GENOMIC INSTABILITY, DEREGULATION OF APOPTOSIS AND TREATMENT RESPONSIVENESS IN SPORADIC COLORECTAL CANCER

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DECLARATION

I declare that this thesis was composed entirely by myself and that the work presented is my own unless otherwise stated.

Wael M. Abdel-Rahman

November 2000

Dedicated to Professor

Andrew Wyllie

ABSTRACT

Different patterns of genomic instability have been described in sporadic colorectal cancers. In a minor subset, deficient DNA mismatch repair causes a '*r*eplication *err*or positive' (RER+) phenotype, while in the major (RER-) subset, a chromosomal instability (CIN) phenotype with large-scale changes to chromosome number and structure has been described. Chromosomal instability has previously been associated with alterations to the *p53* gene. Both patterns of genomic instability lead to further mutations. Data from animal models indicate that both chromosomal double strand breaks and errors of nucleotide mismatch repair engender apoptosis in normal cells. The question thus arises whether cancer cells bearing such lesions show particular phenotypes, reflecting deficiency of specific apoptotic pathways in their origin. Conversely, genes activating apoptosis may be selected as targets for mutations in these genomically unstable cancer cells, as a wider blockade of apoptosis may confer growth advantage and resistance to treatment. Therefore, by examining RER+ and RER- sporadic colorectal cancers, this thesis (1) seeks to delineate distinct chromosomal instability phenotypes associated with RER status, (2) searches for mutations in three apoptosis genes (*Fas, Bik, Bax*) and one growth control gene (*TGFβRII*), and (3) examines sensitivity of these tumours, *in vitro* and *in vivo* to therapies that deliver different types of DNA injury.

Chromosomal instability was studied in 17 colorectal cancer cell lines using Spectral Karyotyping (SKY). RER- lines usually displayed extensive numerical and structural variations with marked heterogeneity. Most RER- lines showed a tendency to approach a near-triploid karyotype, and invariably had structural chromosomal changes including unbalanced translocations. Marked clonal heterogeneity was observed among cells of the same tumour line. Evidence of endoreduplication was much less frequent in these colorectal cancer cells than for other cancers reported in the literature. A minor subgroup of RER- lines retained a near-diploid karyotype, but displayed the same pattern of structural rearrangements and heterogeneity observed in the near-triploid pattern. Chromosomal gains and losses were in broad agreement with previously published data on primary cancers, indicating that the patterns observed in these cell lines are likely to be representative of primary colorectal cancers. In contrast, RER+ lines usually showed marked stability of both chromosome number and structure. *p53* mutation found in some of these RER+ lines was not associated with aneuploid karyotypes. Extensive

chromosomal instability was identified in 3 out of 8 RER+ lines, one of which showed a pattern near triploid as commonly seen in RER- tumours. Two RER+ lines were near-diploid and showed a striking and previously unreported tendency to acquire balanced translocations. Potential mechanisms underlying these different patterns of genomic instability are discussed.

Thirty-three primary (uncultured) colorectal carcinomas were chosen according to their RER status for mutational analysis of apoptosis genes. Twelve of these were RER-, 18 were RER+ showing microsatellite instability at 2 or more microsatellite loci, and 3 tumours were RER+ with only one unstable microsatellite locus. A minimum of 5 microsatellite loci was examined in each case. *Bax* gene mutations were detected in 50% of RER+ tumours, but were not always present at all tumour sites and hence must sometimes have arisen during tumour progression rather than in the founder clone. In contrast, *TGFβRII* mutations were found in 75% of the RER+ tumours, and were present in all the sampled sites: these mutations must have arisen in the founder clone. RER+ and RER- cancers were screened for mutations in the whole coding sequence of *Fas* and in a trinucleotide repeat tract identified in *Bik*, but no mutations were found in either.

Representative colorectal cancer cell lines were investigated for any relationship between mismatch repair and p53 abnormalities and responsiveness to anti-cancer cytotoxic therapy. Cells were treated with cytotoxic agents and examined *in vitro* for clonogenic survival and induction of apoptosis. Tumour cells from primary carcinomas were implanted as xenografts in Severe Combined Immunodeficiency (SCID) syndrome mice, and apoptosis was scored following treatment in *vivo*. Apoptotic response to ionising radiation (IR) was present in tumours with intact *p53*, or functional DNA mismatch repair, whereas response to 5-Fluorouracil (5FU) associated only with intact *p53*. Increased tolerance of RER+ carcinoma cells to a methylating agent (Temozolomide) was also observed. These data may help to inform the design of more effective therapeutic protocols based on the commonly observed genotypic alterations in colorectal cancers.

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ABBREVIATIONS

APS	Ammonium persulphate
bp	Base pairs
BSA	Bovine serum albumin
CCD	Charged coupled device
cdk	Cyclin-dependent kinase
cDNA	Copy deoxyribonucleic acid
CGH	Comparative genomic hybridisation
CIN	Chromosomal instability
COX-1	Cyclo-oxygenase-1
COX-2	Cyclo-oxygenase-2
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DAPI	4,6-diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
DCC	Deleted in Colorectal Cancer
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DSBs	DNA Double strand breaks
dTTP	deoxythymidine triphosphate
dITP	deoxyinosine triphosphate
DMSO	dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
ddNTP	dideoxynucleotide triphosphate
DDW	Distilled deionised water
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
FAP	Familial Adenomatous Polyposis
FISH	Fluorescence *in situ* hybridization
5-FU	5-Fluorouracil
GTBP	G-T binding protein
hMLH1	Human MutL Homologue
hMSH2	Human MutS Homologue
hPMS1	Human post-meiotic segregation homologue 1
hPMS2	Human post-meiotic segregation homologue 2
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IAP	Inhibitor of apoptosis protein
IR	Ionizing radiation
LOH	Loss of heterozygosity
MCC	Mutated in colorectal cancer
MDE	Modified acrylamide (trade name)
MMR	Mismatch repair
MTHFR	Methyltetrahydrofolate reductase
MNNG	N-methyl-N'nitro-N-nitrosoguanine
MNU	N-methyl-N-nitrosourea
MSI	Microsatellite instability
mRNA	Messenger ribonucleic acid
NAT1, NAT2	N-Acetyltransferases
NER	Nucleotide excision repair
NRS	Normal rabbit serum

NSAIDs	Non-steroidal anti-inflammatory drugs
OD	Optical density
PBS	Phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	Polymerase chain reaction
RER+	Replication error positive
RER-	Replication error negative
RNA	Ribonucleic acid
SAP	Shrimp alkaline phosphatase
SCID	Severe combined immunodeficiency syndrome (mouse)
SDS	Sodium dodecyl sulphate
SKY	Spectral Karyotyping
SSC	Salt/sodium citrate buffer
SSCP	Single-stranded conformational polymorphism
SST	Sequence specific transactivation
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	N,N,N',N',-tetramethylethylenediamine
TGFβ	Transforming growth factor β
TGFβ RII	Transforming growth factor β receptor subunit II
TS	Thymidylate synthase
UDDW	Ultrapure distilled deionised water
UV	Ultra violet

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CHAPTER 1

INTRODUCTION

Different forms of genomic instability exist producing characteristic patterns of abnormalities in genomic organization. Consistent patterns of instability found in colorectal cancers reflect and may ultimately reveal the specific genetic defects that underlie them. Depending on the nature of the underlying defect, patterns of genomic instability may be related to the capacity of mutated cells to survive and replicate. Thus, genes activating apoptosis may be selected as targets for mutation in these genomically unstable cancer cells, as a wider blockade of apoptosis may confer growth advantage and resistance to treatment. Hence, it is important to study patterns of genomic instability in cancers in order to identify, more clearly, their causes and effects. Colorectal carcinoma is a good model in this regard as two of the commonly observed genomic instability phenotypes—microsatellite instability or '*r*eplication *err*or positive' (RER+) phenotype and chromosomal instability (CIN)—were first described and also prevalent within colorectal cancers. There are data suggesting that some apoptotic pathways are deregulated in colorectal cancers. Furthermore, these tumours are notoriously resistant to both chemotherapy and radiotherapy and most patients for whom surgery alone is not curative are doomed to die of their disease. The basis of this resistance to therapy is still not completely understood.

To introduce these issues, this chapter gives an overview of current knowledge of the genetic events and environmental influences that play a role in sporadic colorectal tumour development and responses to different treatment modalities. The specific types of genomic instability pertinent to this study will be discussed, namely microsatellite instability, chromosomal instability, and potential causative mechanisms including the role of p53 and relevant apoptotic pathways. Finally, the aims and objectives of this project are presented.

1.1. Clinical aspects and aetiology of colorectal cancer

1.1.1. Incidence and distribution of colorectal cancer

Large bowel cancer is the second most common cancer in western countries. Over 34,000 new cases of bowel cancer are diagnosed each year in the UK. Incidence varies considerably worldwide. Within Britain bowel cancer is more common in Scotland than it is in England and Wales (Cancer Research Campaign, 1999a). Incidence is increasing in low-risk populations, such as Mediterranean countries, Central and South America, Asia and Eastern Europe, as lifestyles become increasingly westernised (Weisburger, 1991). Large bowel cancer accounts for 17,620 deaths per annum in the UK (11% of all cancer deaths) (Cancer Research Campaign, 1999a). The risk of developing colorectal cancer increases with age; the likelihood of developing cancer at age 30-34 is small (2.9/100 000) compared with the risk at age 85 or older (531.6/100 000) (Levin & Dozois, 1991).

1.1.2. Natural history of colorectal cancers: the adenoma to carcinoma sequence

Adenocarcinoma accounts for more than 90% of colorectal cancers. Most sporadic colorectal cancers are thought to develop via an earlier adenoma stage, progressing through a number of histologically distinct steps, for which the term "adenoma to carcinoma sequence" was coined (Muto *et al.*, 1975). Single dysplastic crypts (uni-cryptal adenomas) are thought to be the first manifestation of tumour development (Ilyas *et al.*, 1999). Adenomas can gradually grow in size. They show either tubular or villous architecture or a combination. The cells show a range of nuclear pleomorphism from mild through moderate to severe dysplasia, which may progress to malignant change resulting in local invasion and eventual metastasis to distant sites. Several lines of evidence support this model. The population distribution of adenomas reflects that of carcinomas, and migrants acquiring increased risk of colorectal cancer through moving to areas with high cancer incidence also acquire an increased risk of developing adenomas (Weisburger, 1991). The distribution of adenomas throughout the large bowel is similar to that of carcinoma, with an age of onset approximately 5 years earlier (Muto *et al.*, 1975), consistent with sequential progression. Foci of carcinoma can sometimes be found within adenomas, and islands of residual adenoma may be seen adjacent to carcinomas suggesting common origin (Lane, 1976). Although the progression from adenoma to carcinoma is thought to occur in the vast majority of western colorectal cancers, it is possible that *'de novo'* cancers sometimes arise without pre-existing adenoma. In particular, it has been suggested that cancers with flat morphology may arise *de novo* in the Japanese population (Wada *et al.*,

1996), though this is disputed (Owen, 1996). The process of tumour development probably takes up to 20 years or more and not all adenomas will progress into carcinomas (reviewed by Ilyas *et al.*, 1999).

Recently it has been proposed that aberrant crypt foci 'ACF' (small areas of epithelium with irregular glandular architecture but no evidence of dysplasia) are precursor lesions which give rise to adenomas, however the data regarding mutations found in ACF remains inconclusive and their relationship to adenomas remains unproven (Jen *et al.*, 1994b; Smith *et al.*, 1994a; Yamashita *et al.*, 1995; Nucci *et al.*, 1997).

1.1.3. Treatment and prognosis of colorectal cancer

There are several systems for staging colorectal cancers including Dukes' staging system, TNM staging system and the American Joint Committee on Cancer (AJCC) staging system. The following discussion refers to AJCC stage. Each stage carries specific prognostic and therapeutic implications (Cohen *et al.*, 1997). Overall, prognosis for this disease is relatively poor with an average five-year survival rate of 37% (Cancer Research Campaign, 1999b). The tumour stage remains the main histopathological predictor of survival (Laurent Puig *et al.*, 1992). In addition, certain genetic factors have a significant relationship with prognosis. Notably, relatively poor 5-year survival is associated with alterations to the *p53* gene and certain chromosomal alterations and deletions, whereas several studies indicate improved survival in patients whose cancers have a diploid karyotype and in patients whose tumours demonstrate microsatellite instability (Jass *et al.*, 1994; Bubb *et al.*, 1996; Jass *et al.*, 1998).

Surgery with resection of the primary tumour is recommended for all stages of colon cancer. Nevertheless, a proportion of patients at each stage have residual micro-metastasis, which are the targets of adjuvant therapy. 5-Fluorouracil (5-FU) alone or in combination with levamisole—or less commonly 5-FU, levamisole, and leucovorin—represent the standard adjuvant therapy. This treatment increases the 5-year overall survival by 33% for stage III colon cancers (Moertel *et al.*, 1990; Moertel, 1994; Moertel *et al.*, 1995; O'Connell *et al.*, 1998). Minimal improvement in survival is observed in stage IV with adjuvant therapy (Buroker *et al.*, 1994). For stage II and III rectal adenocarcinoma, pre-operative or post-operative combined radiation and chemotherapy with 5-FU is considered beneficial (O'Connell *et al.*, 1994). Many lines of treatment are followed for metastatic colorectal cancers most of which use 5-FU or one of its new derivatives, however, the main benefit is palliative (Cohen *et al.*, 1997).

1.1.4. Risk factors in the development of colorectal cancers.

1.1.4.1 Environmental and other non-genetic risk factors

It has long been postulated that dietary factors contribute to colorectal cancer formation. Mutagens are present in the stool of many people who eat a western diet (Reddy *et al.*, 1989) and the nature of genetic and cellular damage initiated by dietary components may determine subsequent genetic events, however, the exact role of these dietary mutagens in the aetiology of colon cancer is still an interesting area requiring more research. Principally, an increased risk of cancer is associated with a diet high in saturated fats and meat and low in fibre, though the type of fat and fibre consumed is important. Intake of calcium and certain vitamins may also reduce risk. Tobacco smoking and excessive alcohol are associated with increased risk (Palmer & Bakshi, 1983; Weisburger, 1991; Cancer Research Campaign, 1993; Olsen *et al.*, 1994). Dietary factors also have an impact on the formation of adenomas (Winawer & Shike, 1992).

1.1.4.2 Interaction between environmental influences and molecular genetic pathways

Vegetables: One important constituent of vegetables is folate. Folate is central to methyl group metabolism and may influence both methylation of DNA and the available nucleotide pool available for DNA replication and repair. Folate deficiency produced large numbers of tumours in rats and mice (Yunis & Soreng, 1984; Wagner, 1995; Potter, 1999). Fibre ferments in the large bowel and produces short chain fatty acids, like butyrate, which induce apoptosis in colonic cell lines (Hague & Paraskeva, 1995). More generally, vegetables contain large numbers of anti-oxidants and bio-active compounds with potent anti-carcinogenic properties, which may have protective effects (Wattenberg, 1978).

Meat: Cooked meat contains carcinogens like heterocyclic amines (such as PhIP: 2-Amino-1-methyl-6-phenylimidazo[4,5-b] pyridine) and nitrosamines which have been shown to be carcinogenic in animals (Kakiuchi *et al.*, 1995; Lynch *et al.*, 1998) and probably in humans (Bingham *et al.*, 1996) via direct mutagenic effects on genes such as the *APC* gene.

Bile acids: Secondary bile acids are formed after enzymatic deconjugation and dehydroxylation of primary bile acids in the large bowel by anaerobic bacteria. It has been shown that these compounds can have tumour-promoting capacities in animal experiments. In epidemiological studies, colonic cancer risk is related to the faecal bile acid concentration. Secondary bile acids are toxic to several cell systems at physiological

concentrations. The exact mechanism by which these molecules exert their action is not well understood (Nagengast *et al.*, 1995).

Non-steroidal anti-inflammatory drugs (NSAIDs): NSAIDs inhibit cyclo-oxygenases (COX-1 and COX-2), which are responsible for metabolism of arachidonic acid to prostaglandins. Decreased levels of prostaglandins are associated with protection against tumour formation (Kalgutkar *et al.*, 1998). NSAIDs produce inhibitory effects on epithelial proliferation and promote apoptosis (Barnes *et al.*, 1998) and have effects on angiogenesis (Tsujii *et al.*, 1998). More recently, NSAIDs have been suggested to suppress tumour formation in HNPCC families by selecting for mismatch repair proficient subsets of cells (Ruschoff *et al.*, 1998). Therefore, clinical trials to establish the efficacy of NSAIDs more generally are underway.

Tobacco: Tobacco smoke is a major source of wide variety of carcinogens which may act in a way similar to those derived from meat.

Alcohol: Acetaldehyde may cause DNA adduct formation and inhibit DNA repair. Alcohol may also exert effects through nutrient deficiency, particularly folate (Garro & Lieber, 1990).

Physical activity: Exercise stimulates peristalsis, reduces exposure time, and has favourable effects on the immune system (Potter *et al.*, 1993). Furthermore, physical activity is associated with a metabolic milieu that is less favourable to the growth of cancers (McKeown-Eyssen, 1994).

Many risk factors tend to cluster in the lives of some individuals.

1.1.4.3 Genetic predisposition to colorectal cancers: high prevalence polymorphisms

1.1.4.3.1 N-Acetyltransferases (NAT1, NAT2) and Cytochrome P450 (CYP) enzymes:

Heterocyclic amines are thought to act as colorectal carcinogens and their effectiveness may vary with differences in their metabolism which may be due to genetic variability of at least 3 enzymes (NAT1, NAT2, CYP1A2). Therefore, polymorphisms of these enzymes may influence the risk of colorectal neoplasia. Some studies suggest that combined polymorphisms of NAT1, NAT2, and perhaps, CYP1A2 may bring about rapid acetylation phenotype, which is associated with increased colorectal cancer risk, in the presence of a high intake of meat (Chen *et al.*, 1998; Potter, 1999). However, other studies did not find any association between NAT1 and NAT2 polymorphisms and increased colorectal cancer risk (Hubbard *et al.*, 1997; Hubbard *et al.*, 1998).

1.1.4.3.2 Methyltetrahydrofolate reductase (MTHFR):

As noted above, folate and vitamin B12, a cofactor in the methyl group metabolism pathway, are associated with reduced risk of colorectal neoplasia (Slattery *et al.*, 1997). MTHFR, a polymorphic enzyme, influences that association such that those at highest risk for both adenomas and carcinomas have the variant (TT) genotype and low intake of folates and vitamin B12 (Chen *et al.*, 1996; Ma *et al.*, 1997).

1.1.5. Predisposition to colorectal cancer and inherited syndromes

The high-risk, inherited cancer predisposition syndromes Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC) are described in the following discussion. In addition, several diseases (*e.g.* ulcerative colitis and Crohn's disease), as well as rare polyposis syndromes, confer an increased risk of bowel cancer and are described where relevant (Howe & Guillem, 1997; Howe *et al.*, 1998; Ilyas *et al.*, 1999).

1.2. Genetic pathways to colorectal cancer

Carcinogensis is a multi-step process of accumulating genetic changes resulting in either activation of dominantly acting oncogenes or inactivation of tumour suppressor genes (Foulds, 1958; Fearon & Vogelstein, 1990; Kinzler & Vogelstein, 1996; Bodmer, 1997). Each mutation alters the behavioral responses of cells giving some growth advantage. The mutations, which occur, and the order in which they occur form the genetic pathway. Histological progression in precursor lesions, showing progressive increase in atypia leading to malignancy, may accompany the progressive accumulation of these mutations (Muto *et al.*, 1975; Ilyas *et al.*, 1999). Tumours arising from a particular tissue escape the same growth controls and hence may be expected to develop along similar genetic pathways. However, genetic pathways for a particular tumour arising in a particular tissue may vary according to the individual genetic background, regional differences and the nature of the first mutation may have a strong influence on the mutations to follow (Ilyas *et al.*, 1999). Recent work had strengthened the proposal that a certain degree of genetic instability is required to drive the generation of multiple mutations found in malignant tumours (Lengauer *et al.*, 1997; Cahill *et al.*, 1998; Lengauer *et al.*, 1998; Cahill *et al.*, 1999a). Clonal selection seems to be essential to drive the accumulation of subsequent mutations in the progenitor cell, as waves of clonal expansion give rise to daughter cells that have the growth advantage of cancer (Cahill *et al.*, 1999b; Tomlinson & Bodmer, 1999). A Diagrammatic illustration of the common genetic pathways in colorectal cancer is presented in Figure 1.1.

The remainder of this Chapter describes the putative genetic pathways in colorectal carcinogenesis, with particular stress on those genetic alterations relevant to the work in this thesis.

Figure 1.1

Genetic pathways to colorectal cancers

APC mutations are thought to initiate tumour formation in both sporadic and inherited colorectal cancer; deregulation of apoptosis may be required at this stage. Neoplasia progresses as a result of mutations in *K-ras*, and then one or more tumour suppressor genes located on chromosome 18q, particularly those associated with the TGFβ pathway, and *p53*. Mismatch repair gene mutations may speed up the tumorigenic process by increasing the rate of mutation in these or other genes where 'target' repetitive sequences are present, such as in the *TGFβRII, IGFIIR , Bax* and *E2F4*.

This figure is based on: Fearon & Vogelstein, 1990; Ilyas *et al.*, 1999; and Arends, 2000.

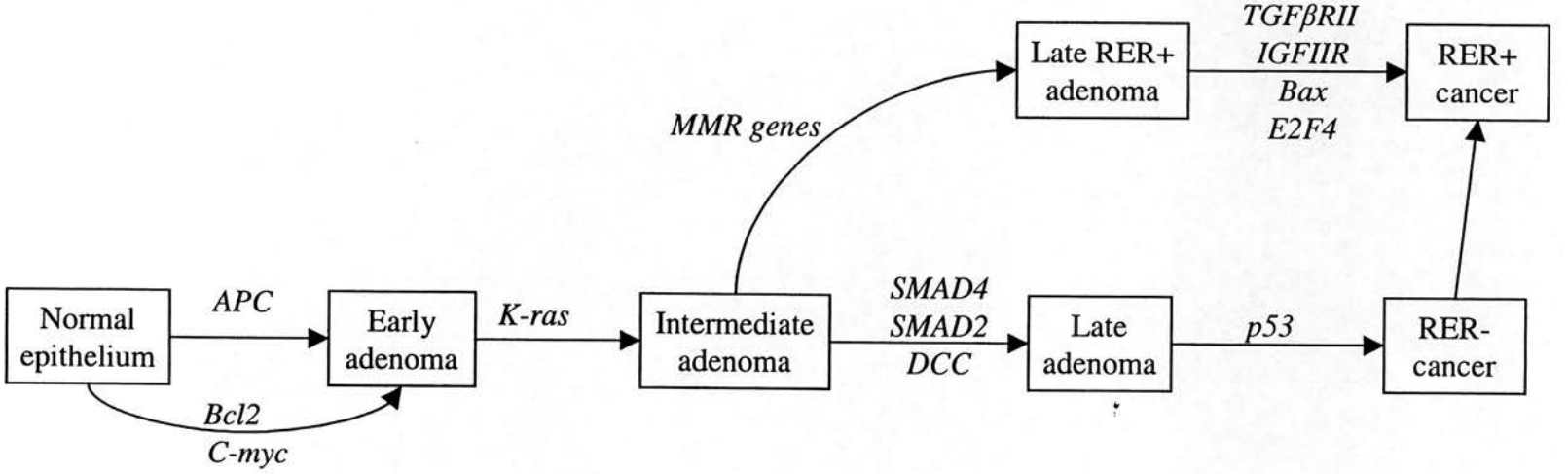

Figure 1.1: Common genetic pathways in colorectal carcinogenesis

1.2.1. The *APC* gene

Mutations of the Adenomatous Polyposis Coli (*APC*) gene, located on chromosome 5q21-5q22, (Bodmer *et al.*, 1987; Leppert *et al.*, 1987; Solomon *et al.*, 1987) are the earliest and commonest gene mutation in sporadic colorectal cancers (Nakamura *et al.*, 1992; Powell *et al.*, 1992). Germ-line *APC* mutations are found in Familial Adenomatous Polyposis (FAP) (Groden *et al.*, 1991) which is an autosomal dominant condition characterized by the development of hundreds to thousands of colorectal polyps as well as other extra-colonic manifestations. Some of the polyps in FAP inevitably progress to cancer, if left without surgical removal, when normal function of the remaining *APC* allele is lost. Thus, APC functions as a classical tumour suppressor gene according to Knudson's hypothesis (Knudson, 1971).

Mutations of the *APC* are almost all truncating mutations resulting in loss of the downstream functional domains. The one important consequence of this is loss of the ability to down-regulate β-catenin, an important molecule in the Wnt signalling pathway, leading to increased nuclear signalling in association with the transcription factor Tcf-4 (Morin *et al.*, 1996). One of the genes inappropriately activated in this pathway is *c-myc*, thus linking *APC* pathway to apoptosis deregulation (He *et al.*, 1998). *APC* mutations probably disrupt pathways mediated through proteins that bind APC, some of which are yet to be elucidated.

1.2.2. The *K-ras* genes

The Ras proteins are part of signal transduction pathways. Up to 50% of colorectal carcinomas and adenomas more than 1 cm in diameter contain *K-ras* gain-of-function missense mutations, predominantly in codons 12 and 13 (Bos *et al.*, 1987; Bos, 1988). Mutant *K-ras*, in conjunction with other oncogenes, is capable of transforming cells in culture (Ruley, 1983). Furthermore, disruption of mutant *K-ras* in colorectal cancer cell lines reduces the tumorigenic potential and aggressive phenotype with concomitant reduction in expression of the *c-myc* oncogene (Shirasawa *et al.*, 1993). Ras activation of PI 3-kinase suppresses apoptosis but Ras also promotes apoptosis through the Raf pathway [Kauffmann Zeh, 1997 #807]. Recently, however, other effects of mutant *ras* have been reported. Active Ras can lead to phosphorylation of pro-caspase-9, thereby inhibiting cytochrome-c-induced apoptosis (Cardone *et al.*, 1998). Whereas, β-catenin activates transcription from the cyclin D_1 promotor, Ras signalling pathways can further increase

cyclin D_1 transcription (Tetsu & McCormick, 1999). Cyclin D_1 binds to cyclin-dependent kinase (cdk) to bring about cell cycle progression through G1 to S phase and is an attractive target in these pathways because of its possible role in allowing cell proliferation under conditions that promotes differentiation and senescence in normal cells (reviewed by McCormick, 1999). However, there have been several reports of *K-ras* mutations in histologically normal colonic mucosa suggesting that dysplasia is seen only when *K-ras* mutations occur in association with other mutations such as *APC* (Jen *et al.*, 1994b).

1.2.3. The *p53* gene

p53, the guardian of the genome, is a key molecule in the maintenance of genomic integrity, and loss of normal p53 activity contributes to a wide variety of human cancers (Hollstein *et al.*, 1991; Lane, 1992; Hansen & Oren, 1997; Albrechtsen *et al.*, 1999). p53 is a nuclear phosphoprotein which has the ability to regulate many cellular processes including cell cycle progression and apoptosis via its sequence-specific transactivation (SST)-dependent or -independent functions (Haupt *et al.*, 1995; Gottlieb & Oren, 1998; Sionov & Haupt, 1999). Figure 1.2 summarizes the roles of p53 in cellular responses to DNA damage. The *p53* gene is located on chromosome 17p13 (Benchimol *et al.*, 1985).

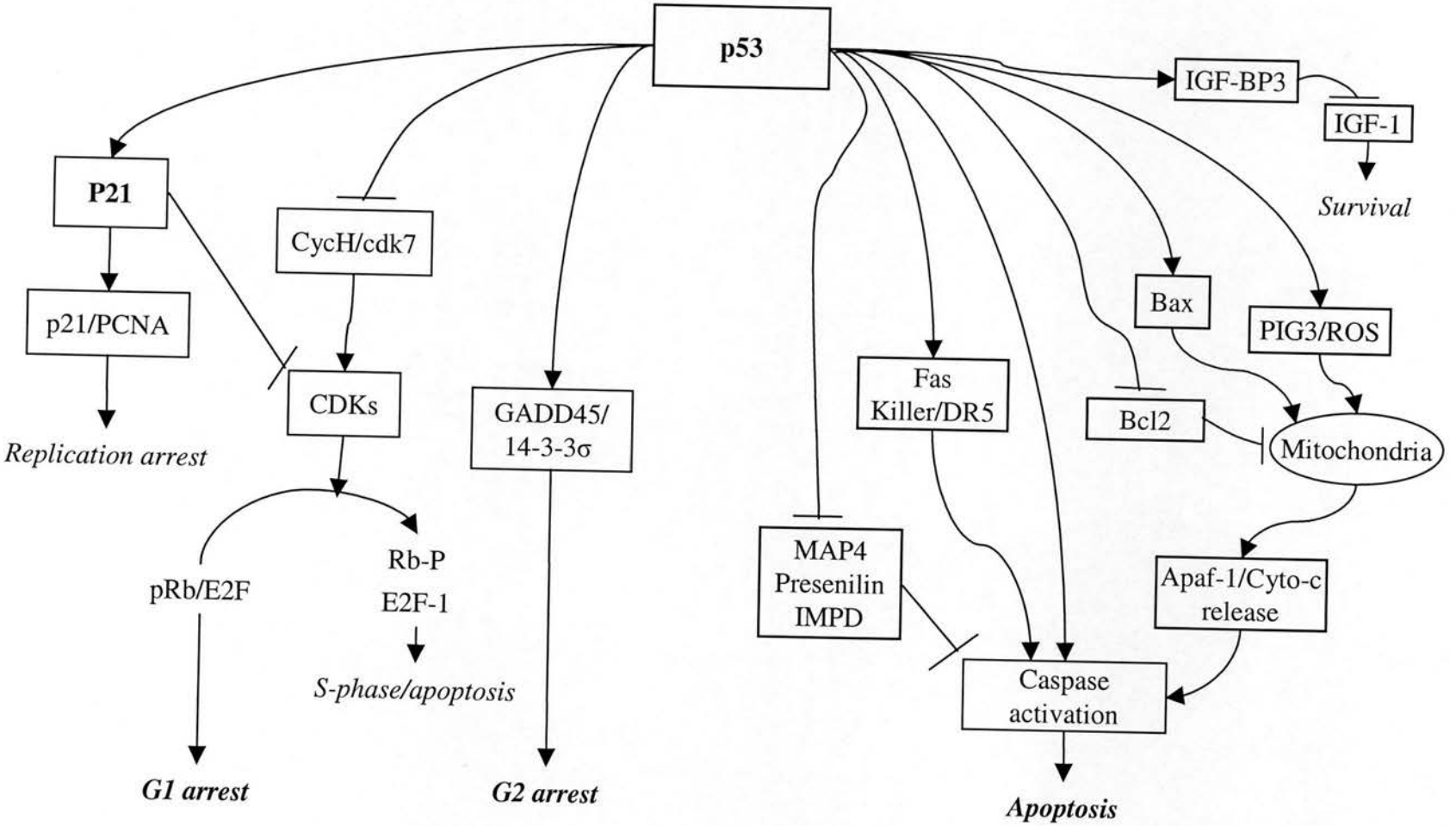

Figure 1.2 Cellular responses to p53 activation

1.2.3.1. The role of p53 in cell growth arrest

p53 regulates vital checkpoints during the G1 and the G2 phases of the cell cycle (Figure 1.2). The induction of $p21^{WAF1/Cip1}$ is responsible for G1 arrest (Waldman *et al.*, 1995), while the induction of *14-3-3σ* gene (Hermeking *et al.*, 1997; Peng *et al.*, 1997; Chan *et al.*, 1999), and to some extent that of the *GADD45* gene (Wang *et al.*, 1999), mediates the G2 arrest. It has been also reported that p21 has a role in G2 arrest (Bunz *et al.*, 1998) and GADD45 can mediate G1 arrest (Kastan *et al.*, 1992; Maity *et al.*, 1994; O'Connor *et al.*, 1997). These checkpoints prevent damaged genomes from undergoing DNA replication or mitosis. p21 mediates p53-dependent G1 arrest by inhibiting the activity of cyclin-dependent kinases (CDKs), which phosphorylate the retinoblastoma (*RB-1*) gene product. In its hypo-phosphorylated form, pRb sequesters the E2F transcription factor, thereby preventing transition from G1 to S phase (Levine, 1997). In addition, pRb recruits histone deacetylase (HDAS1) which blocks transcription by promoting nucleosome compaction (Brehm *et al.*, 1998). p21 also promotes growth arrest by preventing proliferating cell nuclear antigen (PCNA) from activating DNA polymerase-δ essential for DNA replication (Waga *et al.*, 1994). p53 can also trigger growth arrest in a p21-independent manner. By binding to cyclin H and p36Mat1, p53 inhibits the protein kinase complex CDK7/CyclinH1/Mat1 (a CDK activating kinase termed CAK kinase) that activates the CDK2/Cyclin, a kinase required for the G1/S transition (Schneider *et al.*, 1998). p53 can also induce efficient G2 arrest via at least two target genes: *14-3-3σ* and GADD45. The product of *14-3-3σ* gene sequesters the phosphorylated form of cdc25C, a phosphatase of the cyclinB/cdc2 complex that is essential for the G2/M transition (Peng *et al.*, 1997). *GADD45* disrupts cyclinB/cdc2 complex via direct interaction with cdc2 (Wang *et al.*, 1999).

Although G2 arrest can occur in the absence of p21 or p53, both of these proteins are essential for sustaining the G2 arrest after DNA damage (Bunz *et al.*, 1998).

1.2.3.2 The role of p53 in apoptosis

Several p53-induced target genes can promote apoptosis (Figure 1.2), although the expression of each alone may be insufficient to cause significant cell death. Hence, the apoptotic target genes of p53 may need to act in concert to induce significant cell death (Sionov & Haupt, 1999). The apoptotic target genes of p53 may be divided into 2

families: those acting on receptor signalling and those activating downstream effector proteins.

p53-induced proteins acting at the receptor signalling level include the insulin-like growth factor-1-binding protein 3 (IGF-BP3), which blocks the survival signalling by IGF1 (Buckbinder *et al.*, 1995). Combined to this, p53 can suppress the IGF1 receptor via a SST-independent mechanism to ensure an efficient block of this survival pathway (Prisco *et al.*, 1997). p53 also activates an important death receptor, Fas/Apo1/CD95, in response to DNA damage; p53 responsive elements have been identified in the *Fas* gene (Owen-schaub *et al.*, 1995; Mueller *et al.*, 1998; Mueller *et al.*, 1999). In addition p53 may facilitate the transport of Fas from Golgi to the membranes (Bennett *et al.*, 1998). Fas is essential for T-cell killing in response to anti-cancer drugs (Friesen *et al.*, 1996), and for Myc-mediated apoptosis (Hueber *et al.*, 1997). Both responses are p53-dependent, hence linking Fas to the apoptotic responses of p53. Further, p53-induced apoptosis is impaired in *lpr* (Fas-deficient mice) and *gld* (Fas ligand–deficient mice)-derived embryonic fibroblasts (Bennett *et al.*, 1998), but there is controversy regarding this (O'Connor *et al.*, 2000). p53 also induces another death receptor, KILLER/DR5, however, there is no direct evidence for the involvement of this receptor in p53-induced apoptosis (Wu & Levine, 1997).

p53 is linked to the mitochondrial changes associated with early stages of apoptosis via two routes: Bax and PIGs (p53-induced genes-products) that have potential to induce oxidative stress. The Bax protein induces apoptosis via facilitating the release of apoptosis-inducing factor (Apaf-1) and cytochrome c from the mitochondria, which triggers caspase activation (Narita *et al.*, 1998; Susin *et al.*, 1999). Bax is essential for p53-mediated apoptosis in brain tumours (Yin *et al.*, 1997) and fibroblasts (McCurrach *et al.*, 1997), but not in thymocytes (Brady *et al.*, 1996), suggesting that Bax may contribute to p53-mediated cell death only partially. Polyak et al. (1997) isolated several PIGs with the potential to elevate the level of ROS, which in turn signal to the mitochondria to induce apoptosis (Polyak *et al.*, 1997).

Induction of the afore-mentioned genes is dependent on p53-SST function, but, p53 may induce apoptosis via SST-independent function. The mechanisms underlying this SST-independent apoptosis are poorly understood. Possible mechanisms include: (1) loss of pRb function with consequent dergulation of E2F-1 which induces apoptosis (Dyson, 1998; Phillips *et al.*, 1999); (2) suppression of genes essential for cell survival such as those encoding Bcl-2, IGF-1R, microtubule–associated protein-4 (MAP4), inosine-5'-

monophosphate dehydrogenase (IMPD), and presenilin-1 (Prisco *et al.*, 1997; Liu *et al.*, 1998; Roperch *et al.*, 1998; Sionov & Haupt, 1998); (3) direct interaction with apoptosis–inducing proteins like helicases XPB and XPD (Wang *et al.*, 1996); (4) p53 was shown to cause caspase activation in cell free extracts (Ding *et al.*, 1998).

1.2.3.3 The decision between growth arrest or apoptosis

The ability of p53 to promote both growth arrest and apoptosis raises intriguing questions of what determines the choice between these two options. Factors determining this choice include the following:

1.2.3.3.1 The cellular context

The cell type may affect the outcome of DNA damage–induced p53 response. T lymphocytes usually respond to DNA damage by undergoing extensive apoptosis, while fibroblasts enter cell cycle arrest (Bates & Vousden, 1999). The difference may be due to the different physiologic functions as well as levels of expression of apoptotic and repair proteins in each cell (Sionov & Haupt, 1999).

1.2.3.3.2 The efficiency of DNA repair mechanisms

There is evidence suggesting that so long as DNA repair is not completed p53 remains active, while cells defective in DNA repair mechanisms are more prone to p53-induced apoptosis. Repair mechanisms that have shown such an effect include nucleotide excision repair (Dumaz *et al.*, 1997), transcription coupled repair (McKay *et al.*, 1998), and O^6–methylguanine-DNA methyltransferase-mediated repair of DNA adducts (Maze *et al.*, 1996).

1.2.3.3.3 Oncogenic composition of the cell: the pRb-E2F-1 balance

Activation of p53 in normal cells often results in cell cycle arrest whereas p53 activation in transformed cells generally induces apoptosis (Bates & Vousden, 1999). This may be due to abrogation of the p53-pRb signalling pathway in the majority of cancers by a variety of mechanisms. This results in the deregulation of E2F-1 with the consequent induction of apoptosis (Dyson, 1998; Phillips *et al.*, 1999). Introduction of p21 switches the p53 response from apoptosis to growth arrest (Waldman *et al.*, 1997), consistent with the anti-apoptotic function of p21 (Asada *et al.*, 1999).

1.2.3.3.4 Bax-Bcl-2 ratio

As shown in Figure 1.2, activated p53 induces Bax expression and suppresses Bcl-2 expression. Whereas Bax activates, Bcl-2 prevents the release of Apaf-1 and cytochrome c from the mitochondria (Susin *et al.*, 1999). Mitochondrial disruption seems

to be irreversible and prevention of this step appears to be critical in avoiding apoptosis. Bcl-2 can also prevent apoptosis by blocking nuclear import of p53 in E1A-transformed cells (Beham *et al.*, 1997).

1.2.3.3.5 Growth and survival factors

These protect cells from the apoptotic responses to DNA damage by promoting growth arrest (Lin & Benchimol, 1995). This effect may be explained by the cross talk with the apoptosis signalling involving Bcl-2 family members (Dragovich *et al.*, 1998). Survival kinases and 14-3-3 proteins cooperate to inactivate Bad, a pro-apoptotic member of the Bcl-2 family, by BH3 domain phosphorylation (O'Connor *et al.*, 2000).

1.2.3.3.6 The intensity of the DNA damage and p53 expression levels

There are several lines of evidence that extensive DNA damage induces apoptosis via high expression levels of p53 while mild DNA damage tends to trigger growth arrest via inducing low levels of p53 expression (Bissonnette *et al.*, 1997; Reinke & Lozano, 1997; Li & Ho, 1998).

1.2.3.3.7 Factors affecting p53 promotor specificity

There is a tight correlation between the induction of distinct sets of p53 target genes and the ability of cells to arrest in G1 or undergo apoptosis. Data suggest that different p53 mutations or p53-regulatory proteins can change the ability of p53 to transactivate different gene promoters thus predisposing to either arrest or death (Wang & Prives, 1995 ; Barlow *et al.*, 1997; Flaman *et al.*, 1998; Lee *et al.*, 1998).

1.2.3.4. The role of p53 in DNA replication and repair

It is suggested that p53, through its various biochemical activities and via its ability to react with components of the repair and replication machinery, actively participates in various processes of DNA repair and recombination. In particular, the 3'-5' exonuclease activity of p53 is proposed to play a significant role in various aspects of DNA repair (Albrechtsen *et al.*, 1999; Janus *et al.*, 1999). p53-mediated up-regulation of genes involved in DNA repair, such as the two genes *GADD45* (Kastan *et al.*, 1992) and *p48* xeroderma pigmentosum gene (Hwang *et al.*, 1999), might be envisioned as a first step to initiate a repair process. More recently, p53 was shown to induce a ribonucleotide reductase gene, *P53R2,* that is directly involved in DNA repair, thus supplying stronger evidence for p53 involvement in DNA repair (Tanaka *et al.*, 2000). There is controversial evidence suggesting a role for p53 in nucleotide excision repair (NER) (Ford & Hanawalt, 1995; Ford & Hanawalt, 1997). Data showing direct association of p53 with components

of repair pathways provided clues as to how p53 is involved in DNA repair. Examples of these proteins are members of the transcription factor IIH (TFIIH) multi-protein complex that couples transcription with NER (Leveillard *et al.*, 1996), and Cockayne syndrome B protein (CSB) (Troelstra *et al.*, 1990). p53 shows biochemical activities related to DNA repair, it directly binds to double-stranded and single-stranded DNA in a non-sequence specific manner (Steinmeyer & Deppert, 1988), to ends of double strand breaks (Bakalkin *et al.*, 1995), to Holliday junctions (Lee *et al.*, 1997), and to DNA mismatches (Lee *et al.*, 1995). In this last respect p53 shows similarity to MSH2. In fact, p53 can bind to the promoter region of *hMSH2* gene thus suggesting a potential regulatory action of mismatch repair (Scherer *et al.*, 1996).

1.2.3.5 Mutation of p53 in colorectal cancer

Alterations to, or loss of normal p53 function are associated with the transition from benign adenoma to malignant colorectal carcinoma. Inactivation of p53 in sporadic cancer often involves both alleles; allelic deletions occur in more than 75% of carcinomas (Vogelstein *et al.*, 1988; Cunningham *et al.*, 1992), accompanied by mutations of the second allele in 86% of cases with a lost allele (Baker *et al.*, 1990). The key selective advantage of p53 mutations is most probably resistance to apoptosis, as critical apoptotic stress may arise through relaxation of cell-cell and cell-extracellular matrix adhesion at this stage of tumour progression as a result of initiation of tumour cell invasion (Ilyas *et al.*, 1999).

1.2.4. Mismatch repair defects in colorectal cancer

1.2.4.1. HNPCC and microsatellite instability

The human DNA mismatch repair genes were identified largely by their homology to bacterial or yeast genes and their involvement in HNPCC. The HNPCC syndrome is an autosomal dominant disease that accounts for up to 5% of all colorectal cancers (Leach *et al.*, 1993). HNPCC patients may also develop endometrial and ovarian cancer and sometimes cancers of the stomach, small intestine, hepatobiliary system, kidney and ureter. HNPCC kindreds are defined classically by the Amsterdam Criteria, which includes onset of histologically verified colorectal cancer in at least three relatives, one of them a first degree relative of one of the other two; at least two successive generations affected; and cancer onset under 50 years of age in at least one of the affected persons (Vasen, 1994). The syndrome arises as a result of germ-line mutations in one of several DNA mismatch

repair genes including predominantly *hMSH2* and *hMLH1* and to a lesser extent *hPMS1, hPMS2, hMSH3* and *hMSH6 (GTBP).* Mutations in *hMSH2* account for about half and in *hMLH1* for about a quarter to a third of the HNPCC kindreds (Nystromlahti *et al.*, 1994; Beck *et al.*, 1997).

1.2.4.2. The mismatch repair (MMR) system

The mismatch repair system recognizes and repairs nucleotide mismatches or heteroduplex loops that arise during DNA replication particularly to repetitive DNA sequences such as microsatellites. Microsatellites are short tandem repeat (mono-, di-, tri-, or tetra-nucleotide) sequences distributed throughout the genome, but a clear understanding of their function is still lacking. In human cells, insertion or deletion loops at microsatellite sequences can be recognized by hMutSα (a heterodimer of hMSH2 and hMSH6) or hMutSβ (a heterodimer of hMSH2 and hMSH3) (Figure 1.3). Subsequent recruitment of hMutLα (a heterodimer of hMLH1 and hPMS2) to the altered DNA, targets the DNA region for subsequent excision, resynthesis and ligation (reviewed by Boland, 1996). Tumours with MMR deficiency demonstrate marked instability of repetitive DNA tracts such as microsatellites, a phenomenon that has been termed *mi*crosatellite *in*stability (MIN) or *r*eplication *er*ror positive (RER+) phenotype (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Thibodeau *et al.*, 1993). Loss of MMR increases the mutation rate up to 100 fold at repetitive sequences and RER+ tumours are said to have a 'mutator phenotype' (Parsons *et al.*, 1993; Loeb, 1994).

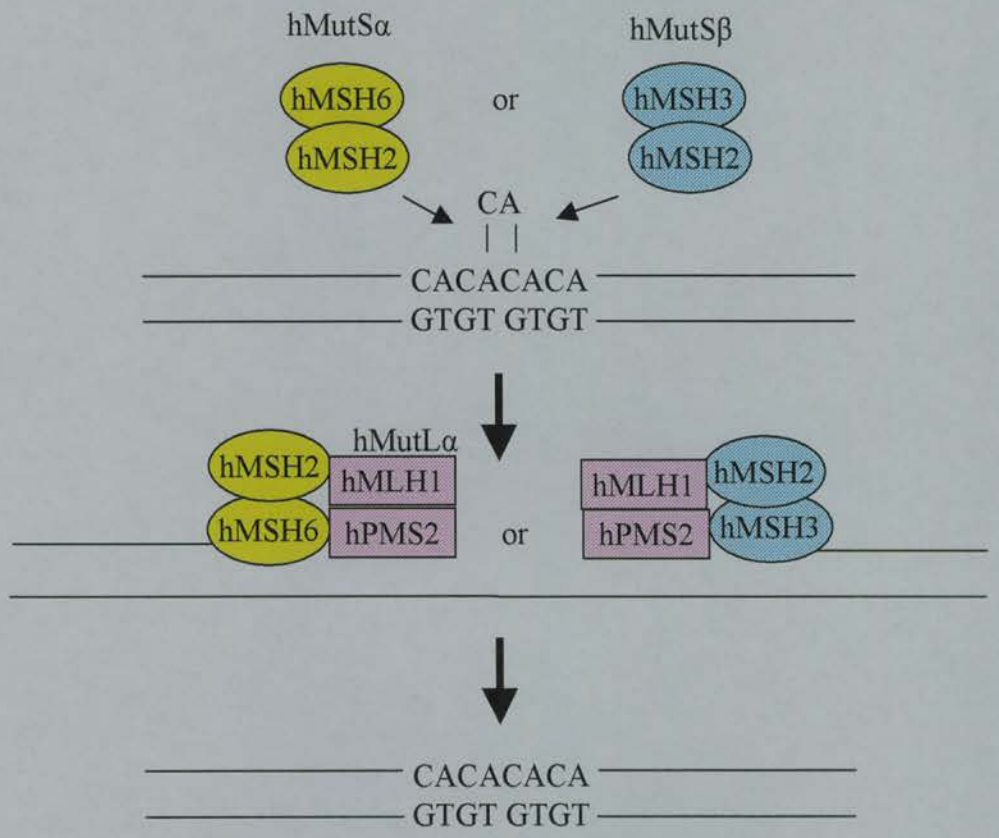

Figure 1.3 The human DNA mismatch repair system

1.2.4.3 MMR in sporadic colorectal cancers

In addition to the tumours in HNPCC patients, approximately 10-15% of sporadic colorectal cancers are found to be RER+ although there is some disagreement as to exact definition of the RER+ phenotype (Liu *et al.*, 1995; Peltomaki, 1995; Bubb *et al.*, 1996; Konishi *et al.*, 1996). RER+ cancers occur at an earlier age and stage of presentation than RER- cancers and are predominantly right-sided. They tend to have either mucinous histology or poor differentiation, with peri- and intra-tumoral lymphocytic infiltrates, they may show more global hypermethylation and better prognosis (Jass *et al.*, 1994; Jass *et al.*, 1998). In addition, in contrast to RER- sporadic colorectal cancers, RER+ cancers are reported to have a near diploid karyotype (Dutrillaux, 1995; Muleris *et al.*, 1995), and, probably as a result, show low frequency of allelic loss (Thibodeau *et al.*, 1993).

1.2.4.4. RER+ carcinogenesis

Several lines of evidence suggest that early development of RER+ tumours follow the usual early carcingenetic steps of mutations in *APC* and *K-ras*, and that loss of mismatch repair function does not give selective advantage in normal epithelium or early adenomas (reviewed by Ilyas *et al.*, 1999). However, one study suggests that loss of MMR may have occurred before APC mutation in HNPCC cancers (Huang *et al.*, 1996), but a study of sporadic cancers did not show similar bias (Homfray *et al.*, 1998). Irrespective of when exactly the loss of MMR occurs, the phenotypic and genotypic evidence suggests that tumours with loss of MMR probably develop in the later stages of carcinogenesis along a genetic pathway different to RER- tumours. Loss of function mutations have been found in four genes with exonic nucleotide repeat sequences in RER+, but not RER-, cancers. These include *TGFβRII* (Myeroff *et al.*, 1995; Parsons *et al.*, 1995), *IGFIIR* (Souza *et al.*, 1996), *Bax* (Rampino *et al.*, 1997) and the cell cycle regulator *E2F4* (Souza *et al.*, 1997). The frequency of mutations in these genes is variable and their relation to each other is not completely known although mutations of *TGFβRII* and *IGFIIR* seem to be mutually exclusive (Souza *et al.*, 1996).

1.2.5. 18q deletion in colorectal cancers, SMAD genes and the *TGFβ* pathway

1.2.5.1 candidate genes on 18q

More than 70% of colorectal cancers demonstrate deletions involving chromosome 18q (Vogelstein *et al.*, 1988). Loss of this region characterises the progression from early to late adenoma (Ookawa *et al.*, 1993). Chromosome 18q status has strong prognostic value in Dukes' stage B cancers, with loss predicting poor survival (Jen *et al.*, 1994a).

Originally, the key oncosuppressor gene important in 18q deletion was thought to be *DCC* (*D*eleted in *C*olorectal *C*ancer) (Vogelstein *et al.*, 1988), a cell adhesion-like molecule. Later research revealed that this gene encodes a netrin-1 receptor, and is probably involved in mediating axon guidance (Keino Masu *et al.*, 1996). The relevance of this gene product to colorectal tumorigenesis is not clear. Other tumour suppressor genes were subsequently discovered on 18q, one of which was originally dubbed *DPC4* (Deleted in Pancreatic Cancer) (Hahn *et al.*, 1996), but was later identified as one of the components of the TGFβ signalling pathway and is now designated *SMAD4*. Data about the frequency of SMAD4 mutations in colorectal cancers in the literature vary from 6 to 30% (Moskaluk & Kern, 1996; Thiagalingam *et al.*, 1996). The SMAD2 gene, coding for another component of the TGFβ signalling pathway, is located close to SMAD4 (Eppert *et al.*, 1996) and it was shown that SMAD2 could be induced by Ras (de Caestecker *et al.*, 1998). Since TGFβ signalling normally results in cell-cycle inhibition and cellular differentiation (Markowitz *et al.*, 1995), it thus appears that defects in the TGFβ pathway, which may occur at different levels in colorectal cancers due to alterations to SMAD4 or SMAD2 or both, may play an important role in carcinogenesis (Myeroff *et al.*, 1995; Grady *et al.*, 1998; Grady *et al.*, 1999). It is worth noting that two further candidate genes are located on chromosome 18q: thymidylate synthase (*TS*) and *Bcl-2*. The roles of these will be discussed later.

1.2.5.2 Interactions with other molecules during colorectal carcinogenesis

As mentioned above, *c-Myc* transactivation was shown to be an important end result of aberrant Wnt signalling. Furthermore, both activated *c-Myc* and *Ras* can co-operate in cellular transformation *in vitro*. Upregulation of both molecules is necessary for the induction of cyclin E-cdk2 activity (Facchini & Penn, 1998). c-Myc drives progression around the cell cycle by activation of cyclin E-cdk2 complex through the release of $p27^{Kip1}$ from this complex. Interestingly, the release of $p27^{Kip1}$ from cyclin E-cdk2 is inhibited by TGFβ (reviewed by Arends, 2000). It therefore appears that the Wnt- and TGFβ-signalling pathways converge on the $p27^{Kip1}$ and deregulation of both might have a synergistic effect on the cell cycle, which might be crucial in colorectal carcinogenesis (reviewed by Arends, 2000). This is further supported by the finding that Ras accelerates $p27^{Kip1}$ degradation through activation of the MAP kinase pathway (Kawada *et al.*, 1997). Increased degradation of $p27^{Kip1}$ has also been reported in aggressive colorectal cancers (Loda *et al.*, 1997).

1.3 Apoptosis deregulation in colorectal cancers

Apoptosis (programmed cell death) is a mode of cell death in which single cells are deleted showing characteristic morphological changes in the midst of living tissue (Kerr *et al.*, 1972). Apoptosis plays a critical role in many physiological and pathological processes. Recent data suggest that loss of particular apoptotic pathways plays a major role in carcinogenesis through permitting survival of genetically unstable cells. Clear identification of these pathways in human tumours becomes an important aim, as it would help define the molecular basis not only of carcinogenesis but also of tumour resistance to various therapeutic measures.

1.3.1 Morphology of apoptosis

In vivo, an important difference between apoptosis and necrosis is that apoptotic cells do not release their contents into the extra-cellular milieu and so do not induce an inflammatory reaction, whereas necrotic cells do. Apoptotic cells first partially destroy their genetic contents by DNA cleavage before being engulfed by phagocytes. This may prove important when inducing death of virus-infected cells by activated cytotoxic T lymphocytes (CTLs). Apoptotic cells rapidly shrink in volume, lose contact with their neighbours and lose specialised surface elements such as microvilli and cell-cell junctions. There is extraordinary cell surface convolution and blebbing (zeiosis) followed by fragmentation of the cell into a series of membrane-bounded, condensed apoptotic bodies (Arends & Wyllie, 1991). The endoplasmic reticulum dilates and fuses with the cell surface. In the nucleus, chromatin condenses into dense caps under the nuclear membrane and is cleaved by the action of endogenous endonucleases into oligo-nucleosomal fragments that can be demonstrated by the characteristic 'ladder' on agarose gel electrophoresis of extracted DNA (Wyllie, 1980). The nucleolus dissociates into a shower of osmiophilic particles with retention of the fibrillar centre. Apoptotic cells are targets for immediate phagocytosis either by phagocytes or adjacent viable cells (Arends & Wyllie, 1991). These characteristic morphological features of apoptosis persist for only a few hours in the tissue (Wyllie *et al.*, 1981).

1.3.2 Signalling cascades and mechanisms of apoptosis

The signal transduction cascades leading to apoptosis in a variety of cells seem to ultimately converge to activate a common apoptotic programme centred on activation of proteases currently known as 'caspases: cysteine-rich, aspartate-cleaving proteases' (Alnemri *et al.*, 1996). Proteins and molecules involved in apoptosis could be schematically classified into signal sensors, transducers, and adaptors/regulator molecules.

Signal sensors are devoid of catalytic activity but recruit catalytic enzymes (caspases), which execute the death signal. Members of the NGF-TNF (nerve growth factor-tumour necrosis factor) receptor super-family like Fas (Apo-1, CD95) are typical examples of sensors, while the Bcl-2 family are the key regulators of apoptosis. The discussion that follows concentrates on the role of these particular molecules (Fas and Bcl-2 family) in colorectal carcinogenesis.

1.3.3 Fas (Apo-1, CD95)/Fas Ligand (FasL) pathway

Fas receptor is a key molecule in the induction of apoptosis (Itoh *et al.*, 1991; Oehm *et al.*, 1992). It is a trans-membrane receptor belonging to the TNF receptor family (Smith *et al.*, 1994b). Binding of its ligand (FasL) (Suda & Nagata, 1994; Takahashi *et al.*, 1994) or cognate antibodies (Alderson *et al.*, 1995; Ju *et al.*, 1995) triggers apoptosis in many cell types. Active Fas receptor also appears to be necessary for apoptosis initiated by other stimuli inducing c-Myc protein expression (Hueber *et al.*, 1997) and exposure to DNA-damaging chemotherapeutic agents (Muller *et al.*, 1997). Fas signal transduction starts with trimerization of the receptor, induced by FasL binding. This activates a protein-protein interactive domain (the so called death domain) to bind to an adaptor protein, FADD/MORT1, which then recruits pro-caspase-8, thus forming the so-called death-inducing signalling complex (DISC). The DISC then leads to cleavage of pro-caspase-8 to caspase-8. Therefore, by a non-transcriptional mechanism, Fas triggers activation of the caspase cascade and hence the terminal effector events of apoptosis (reviewed by Wallach, 1997; Yuan, 1997). Recent evidence, however, suggests that the Fas signalling pathway is significantly more complex and involves at least two major pathways termed type I and type II. In type I cells, caspase-8 is recruited in sufficient amounts at the level of the DISC to directly activate caspase-3, a central executioner caspase (Scaffidi *et al.*, 1998), whereas, if caspase-8 in the DISC is present at lower levels, active caspase-8 then cleaves Bid (Luo *et al.*, 1998), a Bcl-2 family member that causes release of cytochrome-c (Kluck *et al.*, 1997). On release from mitochondria, cytochrome-c interacts with APAF-1 and pro-caspase-9 to form a complex known as the apoptosome that cleaves pro-caspase-9 to caspase-9, which in turn activates caspase-3. This pathway is termed type II (reviewed by Pinkoski *et al.*, 2000). The existence of these two pathways helps explain why in some circumstances, Bcl-2 is capable of preventing Fas-mediated death, such as in type II cells but not type I cells (Scaffidi *et al.*, 1998).

Not all Fas-bearing cells are susceptible to Fas-mediated killing. Expression of apoptosis inhibitors such as the inhibitor of apoptosis protein (IAP) (Deveraux & Reed,

1999) and FLICE inhibitory protein (FLIP) (Irmler *et al.*, 1997), as well as , expression of decoy receptors, DcR1 and DcR2 that bind to FasL but do not transmit death signals (Ashkenazi & Dixit, 1999), or secretion of soluble FasL that binds Fas and similarly does not transmit death signals (Nagao *et al.*, 1999), are all possible mechanisms of resistance to Fas-mediated killing. In addition, A variety of tumour cells including colorectal cancer cells have been shown to express FasL and may thus escape immune surveillance by inducing apoptotic cell death in tumour infiltrating CTLs (reviewed by Walker *et al.*, 1998).

Fas protein is constitutively expressed in normal colorectal epithelium and most colorectal adenomas, but is expressed in only a minority of carcinomas (Moller *et al.*, 1994). During colorectal malignant transformation, it appears that the constitutive Fas-positive phenotype can change to an inducible INF-γ/TNF-α-sensitive Fas-positive phenotype or to stable, clonal deficiency in Fas expression (Moller *et al.*, 1994). The mechanism responsible for these changes has not been established, however.

1.3.4. Bcl-2 family

The Bcl-2 family of proteins controls an important checkpoint prior to activation of the caspase family of proteases in apoptosis. Bcl-2 is capable of inhibiting cell death induced by various stimuli including chemotherapeutic agents. A large number of Bcl-2–related proteins have been isolated and divided into three categories: (1) anti-apoptotic members such as Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1 (Bfl-1), and Boo, all of which exert anti-apoptotic activity and share sequence homology within four Bcl-2 homology (BH) regions 1-4; (2) pro-apoptotic members such as Bax, Bak, Bad, Mtd (Bok), Diva, which share BH1-3; and (3) the pro-apoptotic BH3-only proteins Bik, Bid, Bim, Hrk, Blk, Bnip3 and Bnip3L. Bcl-2 family members have the ability to homodimerise or heterodimerise, thus potentiating or inhibiting their activity (Reviewed by White, 1996; Brown, 1997; Kroemer, 1997).

There is accumulating evidence that Bcl-2 family members exert their effect mainly through controlling the apoptotic mitochondrial changes, mainly the release of cytochrome c and Apaf-1, which trigger caspase activation (Narita *et al.*, 1998; Susin *et al.*, 1999). It has been suggested that the Bcl-2 family might constitute a convergence point for various apoptotic regulating signals. As discussed before, Bid is implicated in the Fas mediated apoptosis and Bax in DNA damage-induced apoptosis.

In the normal colonic crypt, Bcl-2 expression is restricted to the lower half, suggesting a role in protecting the proliferating cells against apoptosis (Hockenbery *et al.*, 1991). Bcl-2 expression is retained in around half of all sporadic colorectal cancers, and this is associated with poor prognosis (Ofner *et al.*, 1995).

One prominent, widely expressed member of this family is Bax, a 21 KDa protein with the capacity to homodimerise, to form a potent death inducer, or to heterodimerise with other members of the Bcl-2 family (Oltvai *et al.*, 1993). Bax is important for tumour suppression *in vivo* (Yin *et al.*, 1997), and as mentioned above, p53 can transactivate *Bax* (Miyashita & Reed, 1995), whilst down-regulating *Bcl-2* (Miyashita *et al.*, 1994). Recently, clonally expanded, inactivating mutations in *Bax* have been observed in a proportion of colorectal cancers, together with evidence for underexpression of the Bax protein in these tumours (Rampino *et al.*, 1997; Ouyang *et al.*, 1998; Yagi *et al.*, 1998; Yamamoto *et al.*, 1998).

Bik is a potent death-inducing protein that interacts with the endogenous survival promoting proteins, Bcl-2 and Bcl-x_L, and their functional viral homologues Epstein Barr virus-BHRF1 and Adenovirus E1B-19kDa. In baby rat kidney cells Bik has been shown to be sufficient for induction of apoptosis in the presence of mutant p53 and low levels of Bax, suggesting that it functions independently of both to induce apoptosis (Boyd *et al.*, 1995; Han *et al.*, 1996). The significance of Bik expression in colorectal carcinogenesis has not been explored hitherto.

1.3.5 c-myc

The *c-myc* gene encodes a transcription factor and exerts diverse overall effects including induction of apoptosis (Evan *et al.*, 1992) and activation of the cell cycle. As mentioned above, c-myc expression is an important end result of Wnt signalling. The c-myc protein also binds to other transcription factors including p53 and pRb. As such, c-myc is thought to be one of the crucial elements regulating the balance between cellular proliferation and apoptosis, however, our understanding of the complex functions of c-myc is still far from complete (reviewed by Arends, 2000).

Elevated levels of c-myc product are not seen in tumours with distant metastases, or in the metastases themselves, indicating its involvement at an earlier stage of development. Expression of the oncogene c-myc is frequently increased in sporadic

colorectal cancer, sometimes due to amplification of the gene, However, it is not correlated to clinical prognostic parameters (Sikora *et al.*, 1987; Erisman *et al.*, 1988a; Erisman *et al.*, 1988b).

1.4. Chromosomal instability in colorectal cancer

Most human cancer cells are aneuploid, and the appearance of aneuploidy in early stage tumours suggests that the altered DNA content, and its underlying causes, play an important role in the development and progression of human cancers. Non-random patterns of acquired chromosome changes have been detected in all tumour types that have been investigated in any great detail (Mitelman *et al.*, 1997). Hematolymphoid malignancies demonstrate a recurrent theme of balanced structural aberrations leading to deregulation of oncogenes or to the creation of hybrid genes (Rabbitts, 1994). In contrast, available data indicate that the development of solid tumours is more dependent on combination of deletions and amplifications of multiple chromosome segments (Mertens *et al.*, 1997). It should be noted that much less cytogenetic information has been gathered for carcinomas partly due to technical difficulties in obtaining good metaphases for analysis from neoplastic epithelial cells and partly due to the karyotypic complexity (Mertens *et al.*, 1997).

1.4.1 Chromosomal changes in primary colorectal cancers

Colorectal cancers display a wide range of chromosomal changes both structural and numerical. Cytogenetic analysis of short-term cultures from primary tumours and cell lines (Reichmann *et al.*, 1981; Muleris *et al.*, 1988; Muleris *et al.*, 1990; Yaseen *et al.*, 1990; Konstantinova *et al.*, 1991; Bardi *et al.*, 1993a; Bardi *et al.*, 1995; Mertens *et al.*, 1997; Eshleman *et al.*, 1998) and, recently, comparative genomic hybridisation (CGH) performed on DNA directly extracted from tumour tissue (Ried *et al.*, 1996; DeAngelis *et al.*, 1999; Georgiades *et al.*, 1999), have identified the most common clonal changes in colorectal cancers to be loss of chromosomes 17p and 18. Other common chromosome losses include 1p, 4, 5q, 6q, 8p, 10q, 14, 15, 21, 22 and Y. Gain of chromosomes 7, 8q, and 20 are widely reported followed in incidence by gain of 1q, 5p, 9, 12q, 13q, and 16. Structural abnormalities in the form of deletions, amplifications, insertions, inversions and translocations are common, although no specific structural abnormality was detected by classical cytogenetic analysis in colon cancer and balanced rearrangements are reported to be rare (Bardi *et al.*, 1995; Dutrillaux, 1995). There is a striking progressive increase in chromosomal number and structural rearrangement in the transition from proximal to distal large bowel (Reichmann *et al.*, 1982; Muleris *et al.*, 1990; Bardi *et al.*, 1995). There have been some attempts in the literature to subclassify colorectal cancers according to their cytogenetic profiles. This is discussed later (see 1.4.6).

1.4.2 Chromosomal abnormalities in colorectal adenomas

Clonal cytogenetic changes have been recorded in up to 65% of all colorectal adenomas. Some of these show only numerical changes, most commonly gain of chromosome 7. Loss of 14 and 18 and to a lesser extent gain of 20 and 13 were also recorded. Other adenomas show both numerical and structural rearrangements, the most often involved chromosome is chromosome 1, with loss of 1p with a breakpoint at 1p32-36. Loss of 8p, gain of 8q and 13q and loss of 17p are also reported. Adenomas with structural chromosomal abnormalities are generally larger and had a higher degree of dysplasia (Bomme *et al.*, 1994; Dutrillaux, 1995; Bomme *et al.*, 1996; Ried *et al.*, 1996). Loss of genetic information from 1p36 is suggested to be an early, seemingly primary, premalignant event in intestinal tumorigenesis that may occur at induction of the hyperproliferation stage (Bardi *et al.*, 1993b; Bomme *et al.*, 1998), although this is debated (Reichmann *et al.*, 1984).

1.4.3. Chromosomal and other genetic lesions and metastasis

Cytogenetic similarities between primary and secondary cancers were found in almost all cases studied, indicating that many of the chromosomal aberrations presumably occurred before tumour spread took place. Compared with the primaries, the metastases appeared to exhibit decreased clonal heterogeneity but, concurrently, an increase in the karyotypic complexity of individual clones. Among the aberrations recurrently found in metastatic lesions, in order of frequency, are del(1)(p34), i(17)(q10), -18, -Y, -21, +7 and +20, all of which have been seen repeatedly in series of primary colorectal carcinomas, and del(10)(q22) and add(16)(p13), which so far have not been associated with primary tumours and which may play a particular pathogenetic role in the metastatic process (Bardi *et al.*, 1997). Loss of the p53 and DCC on 17p and 18q appear to be essential to metastasis. Loss of regions of 13q (including the *Rb-1* locus) and 14q occur more frequently in liver metastases when compared to the corresponding primary tumours. These changes are also more frequent in late stage primary carcinomas compared to earlier stages (Ookawa *et al.*, 1993). Loss of heterozygosity of the gene *nm-23*, located on chromosome 17q21, supposed to be associated with metastatic progression is not found in all studies (Ookawa *et al.*, 1993). The gene encoding motility-related protein (MRP/CD9), implicated in cancer cell motility and metastasis, is highly expressed in primary cancers, compared with the low levels of expression in metastases (Cajot *et al.*, 1997).

1.4.4. Common chromosomal losses suggesting tumour suppressor genes in colorectal cancer

1.4.4.1 1p loss

As mentioned above partial deletion of chromosome 1p occurs in up to half of all colorectal cancers and is associated with poor prognosis. Some studies suggested that it is a primary change appearing very early at the hyperplastic stage (Bomme *et al.*, 1996),while other studies found 1p loss to be a later event (Reichmann *et al.*, 1984; Leister *et al.*, 1990). Detailed deletion mapping, using loss of heterozygosity (LOH) studies, suggests that at least three separate loci may be involved (Praml *et al.*, 1995a; Praml *et al.*, 1995b), though no tumour suppressor genes have yet been unequivocally identified. Introduction of 1p36 into colon cancer cells suppresses tumorigenicity (Tanaka *et al.*, 1993), and this region contains a candidate tumour suppressor, p73, which encodes a protein with homology to p53 that can activate p53 target genes and interact with p53 (Kaghad *et al.*, 1997).

1.4.4.2 8p loss

Loss of material on chromosome 8p also occurs in about half of all sporadic colorectal carcinomas (Cunningham *et al.*, 1993) and may be associated with the change from adenoma to carcinoma (Cunningham & Dunlop, 1994; Cunningham *et al.*, 1994). There is strong evidence to suggest that this region of the genome harbours at least two colorectal tumour suppressor genes (Cunningham *et al.*, 1993), though no such gene has yet been convincingly identified. A recently identified candidate gene is KILLER/DR5, a tumour necrosis factor (TNF)-receptor related protein which may be involved in p53-dependent apoptosis (see above). Partial gene screening of the remaining allele in colorectal cancers has not yet identified mutations (Wu *et al.*, 1997).

1.4.4.3 5q loss and MCC

Loss of heterozygosity studies which led to the eventual identification of the tumour suppressor gene *APC*, initially suggested another candidate gene, *MCC* (mutated in colorectal cancer), located within 100kb of *APC*, as being frequently lost in colorectal cancer as part of the 5q deletion event (Solomon *et al.*, 1987; Vogelstein *et al.*, 1988). The gene product of *MCC* shows sequence homology to a murine G protein receptor important in signal transduction. Mutations in the gene were detected in several sporadic cancers, leading to speculation that it may play an important role in colorectal cancer development (Kinzler *et al.*, 1991a; Kinzler *et al.*, 1991b). However, no further mutations have been

reported and *MCC* seems to have no role in colorectal carcinogenesis (Curtis *et al.*, 1994; Cripps *et al.*, 1995).

1.4.5 Gene amplification

In mammalian cells, the ability to amplify genes is restricted to genetically unstable transformed or tumorigenic cells (Otto *et al.*, 1989; Tlsty, 1990) and is a recessive trait (Tlsty *et al.*, 1992). Cytologically, amplified sequences may be present intra-chromosomally, as homogenously-staining regions (HSRs) or extra-chromosomally, in the form of double minutes (DMs) (Tlsty & Adams, 1990). Both DMs and HSRs are rare in primary bowel cancers, although presence of DMs has been demonstrated *in vitro* in one study (Bruderlein *et al.*, 1990) and metastases of colorectal cancers have been reported to contain DMs (Reichmann *et al.*, 1981).

In mammalian cells, sequences that contain amplified genes are not simple repeats and often appear at new chromosomal locations, suggesting that their generation involves several steps (Stark *et al.*, 1989; Stark, 1993). Several models for gene amplification have been proposed. The unequal exchange model and the re-replication model (Stark & Wahl, 1984)—with multiple initiations of replication—have not been observed in mammalian cells (Windle & Wahl, 1992). A third model suggested that DNA breakage can induce the breakage-fusion-bridge cycle, leading to chromosomal instability and gene amplification (Stark, 1993). Hypoxia, which occurs when tumours outgrow their vascular supply, has been demonstrated to facilitate gene amplification (Rice *et al.*, 1986), and this has been postulated to be due to the induction of endonuclease activity, which may create fragile sites and potentiate break-related genomic instability (Stoler *et al.*, 1992; Coquelle *et al.*, 1997; Coquelle *et al.*, 1998).

1.4.6 Cytogenetic classifications of colorectal cancers

1.4.6.1 Dutrillaux's model

This model suggests two distinct types of abnormal karyotype, possibly indicating the presence of separate underlying mechanisms of tumour development (Reichmann *et al.*, 1982; Muleris *et al.*, 1988). These groups have been termed 'Monosomic type' (MT) and 'Trisomic type' (TT) (Muleris *et al.*, 1990; Dutrillaux, 1995). Based on data from cytogenetic analysis of short term-cultured colorectal cancers, MT was reported to be found in 70% of colorectal cancers, and is characterised by loss of chromosome 18 and 17p together with progressive structural changes with loss of chromosomal fragments thus

creating a hypo-diploid karyotype. Endoreduplications are then suggested to occur leading to the formation of a hypo-tetraploid clone, which is seen mixed with the hypodiploid one in 40% of MT tumours, as shown by the presence of many duplicated markers. Structural changes continue, reducing the chromosome number and creating a pseudo-triploid pattern, however, there are still many duplicated markers. Examples of MT were identified in breast cancers (including some in which endoreduplicative event was apparent) and similar patterns were identified in colorectal cancers, particularly in the distal colon and rectum. Later studies linked this group to the RER- cancers (Remvikos *et al.*, 1995) and a high frequency of abnormal p53 function (Remvikos *et al.*, 1997).

In contrast, the TT was found in 20-25% of colorectal cancers, and shows few structural rearrangements and whole chromosome losses, but rather tends to gain chromosomes (+7, +13, +20, +12, +X). Polyploid clones are exceptional indicating that endoreduplication is a rare event. TT tumours occur commonly in the rectum (Lasserre *et al.*, 1994). Additionally, a small proportion (around 7%) of cancers have apparently normal karyotypes (termed NT), and are associated with significantly lower age of diagnosis than those with abnormal karyotypes, proximal location in the bowel, microsatellite instability and improved patient survival (Remvikos *et al.*, 1995).

The mechanisms driving each type of karyotypic evolution are not understood, but may reflect differences in local environment such as carcinogen content throughout the colon, or, since endoreduplication can occur as a consequence of hypoxic conditions, degree of angiogenesis (Loffler, 1987). Alternatively, fundamental molecular changes associated with each karyotypic group may drive the acquisition of different chromosomal abnormalities.

1.4.6.2 Chromosomal instability (CIN) versus microsatellite instability

RER- tumours demonstrate a high degree of karyotypic instability affecting both chromosome number and structure. In contrast, RER+ tumours demonstrate a marked degree of chromosomal stability with a near diploid karyotype and few structural changes (Eshleman *et al.*, 1998). Further studies by Lengauer *et al.* (1997) discovered a striking defect in chromosome segregation in RER- cells resulting in chromosome gains or losses in excess of 10^{-2} per chromosome per generation (equivalent to a gain or loss every 5 cell generations), as a dominant trait (Lengauer *et al.*, 1997). This form of chromosomal instability (CIN) is sufficient to drive carcinogenesis in RER- colorectal cancers (Lengauer *et al.*, 1997). CIN is shown to be consistently associated with the loss of function of a mitotic checkpoint, and in two cell lines CIN was associated with mutations of the mitotic

checkpoint gene *hBUB1* which is responsible for normal mitotic delay upon spindle disruption (Cahill *et al.*, 1998). The cellular conditions that favour or permit CIN are not yet fully understood, and it does not appear to correlate absolutely with p53 status (Lengauer *et al.*, 1997; Eshleman *et al.*, 1998).

1.4.7 Mechanisms underlying chromosomal changes in cancers

1.4.7.1.The mitotic machinery as a source of numerical chromosomal instability

In human cells, an elaborate macromolecular machine, the mitotic spindle, accomplishes faithful segregation of chromosomes during cell devision. The events of spindle assembly and function in most human cells include depolymerization of interphase microtubules, centrosome-mediated nucleation of new microtubules, centrosome separation, chromosome condensation, nuclear envelope breakdown, chromosome congression, chromosome cohesion and movement, and finally, cytokinesis. It is not difficult to envision how defects in components of this complex machinery, such as the molecules that control its organization and function, and the regulators that temporally couple spindle operation to other cell cycle events, could lead to chromosome mis-segregation (reviewed by Pihan & Doxsey, 1999). The following discussion will concentrate on some of these components that might contribute to the generation of aneuploidy in tumours.

1.4.7.1.1 Centrosomes

Recent studies indicate that centrosome defects may contribute to spindle abnormalities, aneuploidy and tumour development and progression (Doxsey, 1998). Using antibodies to pericentrin and γ-tubulin, it has been shown that the vast majority of malignant tumours including colorectal carcinomas exhibit abnormal centrosomes (Pihan *et al.*, 1998). Centrosome abnormalities included: supernumerary centrosomes, a-centriolar centrosomes, and centrosomes of aberrant size and shape (Uren *et al.*, 1996). All abnormal centrosomes were able to participate in the formation of structurally and functionally aberrant mitotic spindles. Cells with abnormal centrosomes mis-segregated chromosomes at a high rate producing aneuploid cells with dramatically different chromosome numbers (i.e. CIN) (Uren *et al.*, 1996). Malfunction of three cellular processes—centrosome assembly, centrosome duplication and cytokinesis—either singly or in combination, could produce an abnormal centrosome phenotype (Pihan *et al.*, 1998; Pihan & Doxsey, 1999). Centrosome–associated regulatory molecules implicated in centrosome malfunction include the human homologue of Polo (Plk), the human

homologue of *Drosophila aurora* (aurora2/Stk15), NEK2 (Doxsey, 1998), and cdk2-cyclin E complex (Hinchcliffe *et al.*, 1999). Supernumerary centrosomes are observed in $p53^{-/-}$ cells (Fukasawa *et al.*, 1996) suggesting a role for p53 in regulating centrosome duplication, but, centrosome abnormalities can also occur in cells with wild-type p53 (reviewed by Pihan & Doxsey, 1999).

1.4.7.1.2 Chromosomes

Cellular structures and processes that remodel chromosomes and facilitate chromosome movement could have profound effects on the fidelity of chromosome partitioning during mitosis. These include kinetochore structure and function and chromosome condensation and cohesion. Centromere DNA lacking kinetochore proteins have been observed in tumour cells (Vig & Rattner, 1989; Vig & Sternes, 1991). Defects in chromosome condensation may induce chromosome mis-segregation (reviewed by Pihan & Doxsey, 1999). Inhibitors of topoisomerase II can induce both numerical and structural chromosomal abnormalities by interfering with chromosome condensation/decatenation (Cimini *et al.*, 1997).

1.4.7.1.3 Cytokinesis

Failure of cytokinesis—the physical division of one cell into two daughter cells—has been observed in tumours: in cells with abrogated p53 and/or p21, cells escape G2 arrest, enter mitosis, segregate chromosomes but fail to undergo cytokinesis leading to a doubling of the DNA complement (Bunz *et al.*, 1998). Recent data showed that p21 restored cyclin E/Cdk2 regulation and prevented endoreduplication induced by defects in microtubule dynamics (Stewart *et al.*, 1999). Cytokinesis failure has also been implicated in polyploidization (Shackney *et al.*, 1989). However, cytokinesis failure on its own—in the absence of other cell cycle or spindle anomalies—would be expected to produce cells with two diploid nuclei (not a single tetraploid nucleus). Cytokinesis can be affected through its regulatory components. In *S. cerevisiae CUT* (cell ultimately torn) mutants undergo premature cleavage and cut DNA randomly at any time during the cell cycle giving rise to both structural and numerical chromosomal changes (Yanagida, 1998). Mutations in other genes regulating cytokinesis can produce polyploid cells. However, these molecules have not yet been linked to tumorigenesis and thus represent an important area for future investigation (Field *et al.*, 1999).

1.4.7.1.4 Regulatory molecules and mitotic check points

Entry into and exit from mitosis are controlled by multiple cell cycle regulatory pathways including the p34cdc2/cyclin B kinase. A human fusion protein from Ewing's

sarcoma (EWS/FLI1) up-regulates a ubiquitin-conjugating enzyme involved in cyclin B destruction (Arvand *et al.*, 1998). Moreover, the human CDC23, a component of the anaphase-promoting complex, is a candidate tumour suppressor gene on chromosome 5q31, an area commonly deleted in haematological malignancies (Zhao *et al.*, 1998). The role of mitotic check point *hBUB1* gene has been discussed previously. More recently, another mitotic checkpoint gene termed *Chfr* was shown to delay entry into metaphase in response to mitotic stress such as that induced by microtubule depolymerizing agent nocodazole (Scolnick & Halazonetis, 2000). *Chfr* was found to be inactive in 50% of tumour cell lines examined owing to either lack of expression or by mutations (Scolnick & Halazonetis, 2000). At least in yeast, the *BIM1/EB1* gene participates in a checkpoint that delays the exit from mitosis in response to an abnormally orientated spindle (Muhua *et al.*, 1998).

1.4.7.1.5 Apoptosis in mitosis

Recent studies indicate that there is a default apoptotic pathway in mitosis that needs to be overcome for the successful completion of mitosis. This pathway appears to involve survivin, an inhibitor of apoptosis (IAP) protein expressed in G2/M and associated with microtubules (Li *et al.*, 1998). Recent data showed that interference with the expression or function of survivin causes caspase-dependent cell death in the G2/M phase of the cell cycle, and a cell-division defect characterized by centrosome dysregulation, multipolar mitotic spindles and multinucleated, polyploid cells (Li *et al.*, 1999). Survivin disruption also results in caspase-dependent cleavage of p21, suggesting that control of apoptosis and preservation of p21 integrity within centrosomes by survivin are required for normal mitotic progression. Other data suggest roles for survivin in controlling the later stages of cytokinesis (Chen *et al.*, 2000), and also in resistance to Fas-mediated cell death (Suzuki *et al.*, 2000). Survivin is over-expressed in many cancers and may constitute an important mechanism whereby cancer cells progress through aberrant mitosis and fail to undergo apoptosis (Altieri & Marchisio, 1999; Tamm *et al.*, 2000).

High doses of anti-microtubule agents induce p53-independent apoptosis during mitosis (Sorger *et al.*, 1997), which is reduced after expression of mutant *BUB1* (Taylor & McKeon, 1997). Bcl-2 appears to be involved in this pathway as disruption of microtubules leads to Bcl-2 phosphorylation and inactivation initiating apoptosis (Haldar *et al.*, 1997). Recent data show that Fas promotes cell cycle progression via a link to the MEK1-ERK (*m*itogen-activated protein kinase-*E*RK *k*inase 1/*e*xtracellular signal-*r*egulated *k*inase) pathway, but the implication of this Fas mediated cell cycle progression is not clear

(Shinohara *et al.*, 2000). These data suggest that apoptosis during mitosis is a safeguard mechanism much like those enacted in other phases of the cell cycle.

1.4.7.2 Mechanisms underlying structural chromosome rearrangements

1.4.7.2.1 DNA Double strand breaks (DSBs)

DSBs are generated by endogenously produced radicals as well as exogenous agents such as ionizing radiation (IR), which is often used in anti-cancer therapy. Repair of DSBs is of cardinal importance to prevent structural chromosomal rearrangements. The deleterious effects of DSBs have triggered the evolution of multiple pathways for their repair. Homologous recombination (HR) repair is an error-free pathway that requires extensive regions of DNA homology. On the other hand, DNA non-homologous end joining (NHEJ) uses extremely limited sequence homology to rejoin the juxtaposed DNA ends. The outcomes of HR depends on the template used for repair: if it occurs between equivalently positioned sister chromatids, it restores the original sequence, while recombination between sequences other than equivalently-positioned sister chromatids results in genome rearrangements including deletions, expansions and translocations, as well as loss of heterozygosity (LOH) with subsequent expression of recessive alleles. NHEJ often results in deletions or small insertions (reviewed by Kanaar & Hoeijmakers, 1997; Kanaar *et al.*, 1998; Dasika *et al.*, 1999; Jasin, 2000). Figure 1.4 illustrates both HR and NHEJ and molecules involved in both pathways. Some components of the DSB-repair pathways have been shown to play a role in suppressing chromosomal aberrations and malignant transformation. Recent data show that two DSBs, each on different chromosome, are sufficient to induce frequent recombinations resulting in balanced translocations and genome instability in mouse ES cells (Ricardson & Jasin, 2000).

Figure 1.4

DNA double strand break repair

In recombinational repair, a DSB is initially processed by Rad50/Mre11/NBS1 nuclease complex yielding a 3' single strand overhangs. Rad52 protects DNA ends and also facilitates the formation of heteroduplex DNA which requires Rad51 and its associate proteins. Intact DNA with sequence homology (blue) is used as template to replace the lost genetic information at the DSB ends. Following the nucleolytic process, Holliday junction formation, branch migration, extension, nuclease resolution of the junction, and then ligation of the DNA complete the recombinational repair.

In Non Homologous end-joining, the Ku heterodimer binds to the DNA ends and recruits DNA-PKcs. The Rad50/Mre11/NBS1 complexes has enzymatic and/or structural roles on the DNA. Xrcc/LigIV joins the DNA ends. In mammalian cells, loss or addition of nucleotides are frequently seen at the broken ends, however, the factors and mechanisms underlying this last step are not yet identified.

The figure and information included are based on: Kanaar & Hoeijmakers, 1997; Kanaar *et al.*, 1998; Dasika *et al.*, 1999; and Jasin, 2000.

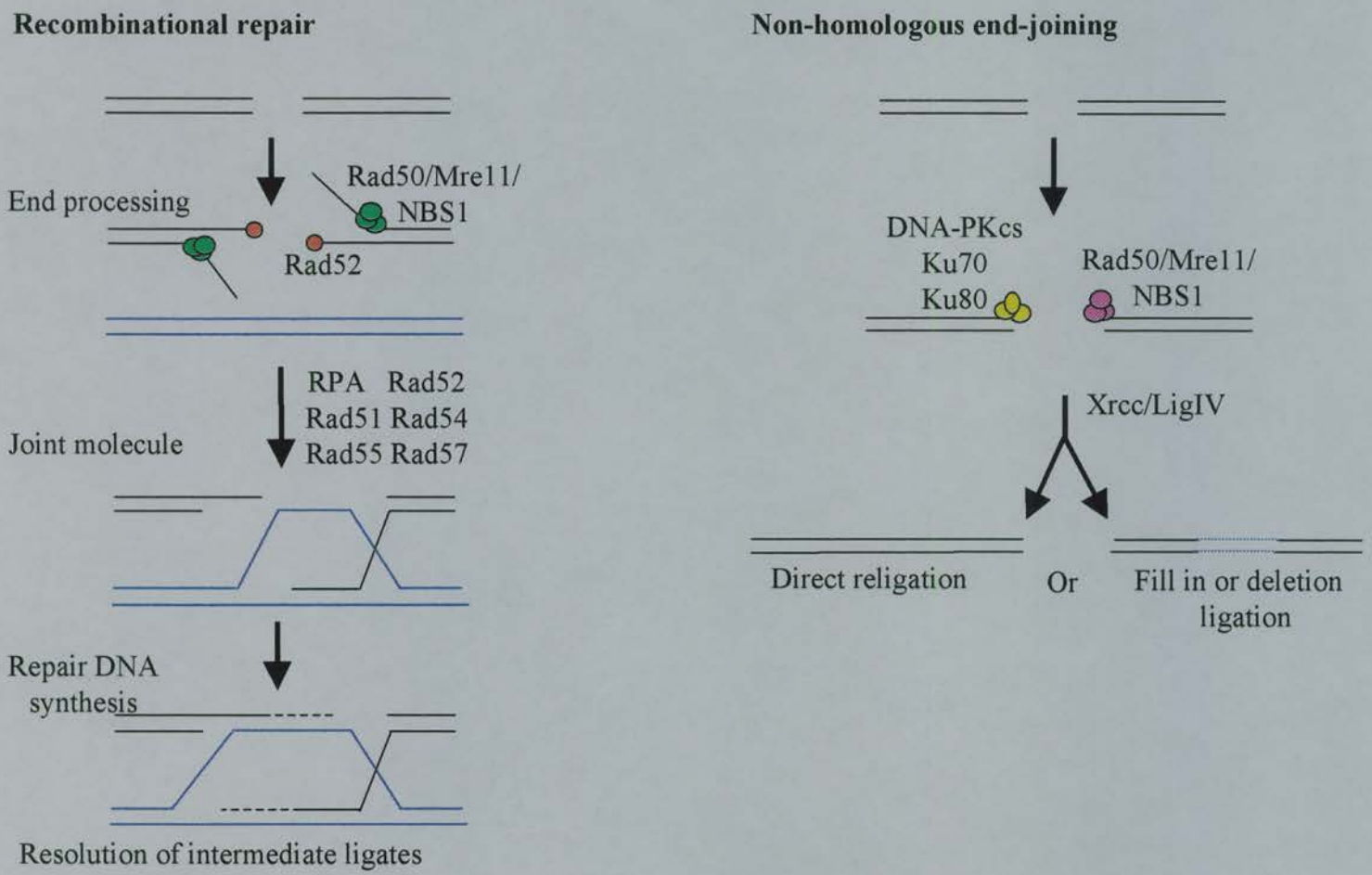

Figure 1.4 DNA double strand break repair

1.4.7.2.2 Chromosomal breakage syndromes and chromosomal fragile sites

Chromosome fragility is a hallmark of two human diseases, ataxia telangiectasia and Nijmegen breakage syndrome. The protein products, ATM and Nibrin, of the genes mutated in these diseases, *ATM* and *NBS1* respectively, play an important role in DNA repair and cell cycle arrest in response to genomic insult. DSBs induce activation of ATM resulting in G1/S arrest through the p53/p21 mechanism, as well as G2/M arrest through the Chk2/Cdc25 pathway. Both ATM and Nibrin are also involved in S-phase arrest in response to DNA damage, however the identities of the downstream effectors in this pathway are not yet identified (Carney *et al.*, 1998; Carney, 1999).

Chromosomal fragile sites are loci that are especially prone to forming gaps or breaks on metaphase chromosomes when cells are cultured under conditions that inhibit DNA replication or repair. They have been shown to display a number of characteristics of unstable and highly recombinogenic DNA in vitro, including chromosome rearrangements, sister chromatid exchanges and, more recently, intra-chromosomal gene amplification. The most frequently observed common fragile sites are FRA3B at 3p14.2, FRA16D at 16q23, FRA6E at 6q26, FRA7H at 7q32, FRAXB at Xp22 (reviewed by Hecht *et al.*, 1988; Vernole *et al.*, 1988; Sutherland *et al.*, 1998). Apart from FRA3B at 3p14.2, which is frequently deleted in several solid tumours (Glover, 1998), no significant association is found between the common fragile sites and cancer breakpoints. These data, along with other chromosome mapping evidence that rare fragile sites are not at the breakpoints in some cancer chromosome rearrangements, cast serious doubt on any role for fragile sites in oncogenesis (Sutherland & Simmers, 1988). However, recent reports by Coquelle et al (1998) showed that hypoxia-induced fragile sites are associated with gene amplifications in tumour cells (Coquelle *et al.*, 1997; Coquelle *et al.*, 1998).

1.4.7.2.3 Telomeric sequences

There is evidence suggesting that telomeres - tracts of hexanucleotide repeats at chromosomal ends that protect against degradation, rearrangement, and chromosomal fusion events – as well as intra-chromosomal telomere-like sequences (Day *et al.*, 1998) are hotspots for recombination, breakage and chromosome fusion (telomeric association) events that may initiate chromosomal instability (Morgan *et al.*, 1998). In germ cells, telomere length is maintained by the enzyme telomerase, but in almost all other somatic tissues, the gene encoding this enzyme is not expressed, resulting in progressive reduction

in telomere length with age (Hastie *et al.*, 1990). Telomere length is substantially reduced by about the same extent in both colorectal adenomas and colorectal carcinomas when compared to normal colonic and blood tissue (Hastie *et al.*, 1990; Bouffler *et al.*, 1996; Slijepcevic & Bryant, 1998; Sprung *et al.*, 1999; Urquidi *et al.*, 2000). However, maintenance of telomere length to some degree could be a critical event in the progression to colorectal malignancy, allowing cells to avoid the acquisition of a senescence signal associated with substantially shortened telomeres. Expression of telomerase, though not present in early colorectal adenomas, is detectable in colorectal carcinomas (Chadeneau *et al.*, 1995). Recent data from telomerase deficient, p53-heterozygous mice suggest that telomerase deficiency in a p53 deficient background favours the development of epithelial tumours, including large intestinal ones, that show evidence of chromosomal instability in the form of frequent non-balanced translocations (Artandi *et al.*, 2000; Hanahan, 2000).

1.4.7.2.4 DNA Methylation

DNA is methylated by a covalent modification of cytosine residues by DNA methyl transferase. Strand breaks are associated with hypomethylation, probably because a reduction in methylation affects chromatin structure, and protein binding ability allowing increased access of changed regions to DNA damaging agents such as nucleases (Lewis & Bird, 1991). Loss of chromosome condensation in this way may also lead to chromosome non-disjunction (Schmid *et al.*, 1984). Hypomethylation, thus may facilitate both structural and numerical chromosomal changes, however, there is not enough data to support this proposal in human carcinogenesis.

Extensive hypomethylation is evident within the genomes of colonic neoplasms, occurring even in very small benign adenomas (Feinberg & Vogelstein, 1987). Hypomethylation does not appear to be a feature of hyperplasic epithelium, and is therefore thought to be an early event in tumour development, preceding malignancy. In colorectal cancer, reduction in methylation occurs selectively, with some genes - consistently hypomethylated and others variable. Thus, whilst hypomethylation would allow increased expression of proto-oncogenes, specific areas of hypermethylation can occur and expression of tumour suppressors could be reduced in this way (reviewed by Feinberg & Vogelstein, 1983; Robertson & Jones, 2000). Transcriptional repression through hypermethylation of the promotor region is indeed a common mechanism of down regulating mismatch repair genes—in particular *hMLH1*—in colon cancers (Herman *et al.*, 1998; Wheeler *et al.*, 1999).

1.5 Summary

This chapter has discussed current knowledge of the development and progression of colorectal cancers. These tumours develop through a complex series of interactions between environmental and genetic factors. Several lines of evidence suggest that sporadic colorectal cancers evolve along different pathways according to the type of underlying genomic instability they possess. Two distinct pathways of genetic evolution of these tumours have been described and both appear to share early steps of *APC* mutation or loss and *K-ras* deregulation. RER+ tumours are characterized by microsatellite instability which targets a group of genes with intra-exonic nucleotide repeats, one of which, *Bax*, codes for a pro-apoptotic gene. RER+ tumours are also characterized by marked karyotypic stability possibly related to the low frequency of p53 abnormalities in these tumours. The RER- group, on the other hand, show a marked degree of structural and numerical chromosomal instability (CIN) the basis of which is not fully understood. Previous studies have linked the chromosomal instability to abnormal p53 function, which is frequently observed in RER- tumours but this correlation is far from consistent. The nature and extent of karyotypic abnormalities in colorectal cancers has not been described in detail yet, possibly because of the marked complexity observed. Apoptosis pathways appear to be linked to these pathways generating different types of genomic instability.

1.6. Aims of this study

The aims of this project were to characterize more precisely: (1) the patterns of genomic instability associated with sporadic colorectal cancers; (2) the mechanisms underlying deregulation of key apoptotic pathways; and (3) the impact of different tumour genotypes on treatment responsiveness in colorectal cancer cells.

(1) Genomic instability was studied in RER+ and RER- colorectal cancer cell lines, with or without mutant *p53*, using Spectral Karyotyping (SKY), a recently developed FISH based technique that has the power to identify complex chromosomal rearrangements, in order to determine the patterns of numerical and structural chromosomal changes associated with RER and p53 status.

(2) Deregulation of key apoptotic pathways was studied by analysis of three apoptosis genes (*Fas, Bik* and *Bax*) and one growth control gene (*TGFβRII*) for mutations in both RER+ and RER- tumours in order to establish the frequency and significance of such genetic changes as causes of deregulation of apoptosis in colorectal cancers.

(3) Colorectal cancer cell lines with either mismatch repair or p53 abnormalities were studied for their responsiveness to treatment in terms of clonogenic survival and induction of apoptosis after anti-cancer cytotoxic therapy such as 5-FU, IR and the methylating agent temozolomide. Similarly, xenografts of uncultured primary tumours with different combinations of p53 and RER abnormalities were studied *in vivo* for their responsiveness in terms of induction of apoptosis by cytotoxic therapies in order to determine whether there is a relationship between tumour genotype and treatment responsiveness.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell lines

2.1.1. Source and origin

All cell lines used in this study were supplied by Sir Walter F. Bodmer (Cancer and Immunogenetics Laboratory, Oxford, United Kingdom). These were obtained originally from public sources, kind gifts or from tumours cultured at ICRF laboratories, UK. C70 was established locally at the Cancer and Immunogenetics Laboratory, Oxford, UK. Available information on the origin, clinical history, and molecular genetic analysis of these lines are assembled in Table 3.1.

2.1.2. General maintenance of the cell lines

The colorectal cell lines were cultured in Glasgow Minimal Essential Medium (GMEM) with L-Glutamine, 10 - 15% (v/v) Foetal Calf Serum, and 1% (v/v) Penicillin-Streptomycin (stock solution contains 5000 U Penicillin and 5000 ug Streptomycin per 1 ml, GibcoBRL, UK). All cells were grown at 37°C in a humidified atmosphere of 5% CO2. The VACO cell lines were cultured in Iscove's Modified Dulbecco's Medium* (GibcoBRL, UK). Cell lines were split no more than 1:4 at passage. When confluent they were rinsed briefly with buffered EDTA saline (Versene, GibcoBRL, UK) before being incubated for approximately 5 minutes at 37°C with Trypsin (0.5 mg ml^{-1} in buffered EDTA saline). The enzyme was inactivated by adding fresh growth medium with serum and the cells were pelleted, re-suspended in growth medium and seeded into tissue culture flasks, to the required density.

For frozen stocks, pellets of cells were re-suspended in freezing mix (10% v/v DMSO, 90% cell culture medium), and placed in Nunc cryotubes. After slow freezing in a -70°C freezer at least overnight, cells were transferred to liquid nitrogen for long-term storage. To restart from frozen samples of cells, the cells were thawed rapidly in a 37°C water bath, diluted in 10 ml pre-warmed growth medium (added slowly, drop by drop), then pelleted and re-suspended in fresh medium before being seeded into tissue culture flasks.

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* Contains 9 more amino acids in addition to the GMEM components as specified in the GibcoBRL catalogue.

2.2 Fluorescence *in situ* hybridization (FISH) techniques

2.2.1 Preparation of cell suspensions for making metaphase spreads

Cell lines were passaged into two 25 cm^2 flasks and cultured for 18-24 hours. One of the flasks was incubated in Colcemid (0.05 $\mu g\ ml^{-1}$) for 1-2 hours. Due to the observed chromosomal shrinkage and distortion after adding Colcemid to some lines like C70, the other flask was used for metaphase spread preparation directly, without Colcemid, and the better metaphase spreads were used for hybridisation. Cultures were harvested by trypsinization as usual but all the supernatants were added to the cells, transferred into 10 ml centrifuge tubes and spun at 500 x g for 5 minutes to pellet cells. The supernatant was removed and the pellet re-suspended in 10 ml pre-warmed (37°C) 0.075M KCl hypotonic solution and incubated in a 37°C water bath for 10 minutes. 0.5 ml fixative solution (3 parts methanol:1 part glacial acetic acid) was added and the tubes were spun again at the same speed, the supernatant removed and the pellet tapped to loosen it. Cells were fixed dropwise with gentle vortexing to a final volume of 10 ml with fresh ice-cold fixative solution and ice-cooled for 1 hour. Tubes were spun again at the same speed, the fixative solution was replaced by fresh fixative solution and ice-cooled for 10 min. Fixing and spinning was repeated once again to obtain a white pellet, cells were resuspended in 1 ml of fixative solution and stored at –30°C for up to one year.

2.2.2 Preparation of metaphase spreads for FISH analysis

2.2.2.1 Metaphase spread preparation by dropping

Pre-washed microscope slides were soaked in 100% ethanol at least overnight. Stored cell suspensions (prepared in 2.2.1 above) were spun at 500 x g for 5 minutes to pellet cells, the supernatant discarded and the cell pellet re-suspended in a few drops of fixative solution to form a milky cell suspension. Slides were polished with a soft lint-free cloth just before use and one drop of fixed milky cell suspension was dropped directly on to the slide to make metaphase spreads. The position of the drop on the slide was marked with a diamond pencil. Metaphase spread quality was checked on a phase contrast microscope at x400 magnification. If necessary, spreading of chromosomes was facilitated by re-dropping the cell suspension and adding a drop of adjusted fixative solution (1 part methanol:1 part glacial acetic acid) just before drying of the metaphase spread. If necessary slides were pepsin-treated for 3-10 min with a pepsin solution (10 μl of 10% pepsin in 50 ml 0.01 M HCl) at 37°C. Slides were washed rapidly in DDW (de-ionised

distilled water) and then in 1x SSC four times for 2 min each. Slides were soaked in 2xSSC pH 7.0 for 30 min at 37°C. Slides were dehydrated by incubation in ascending grades of ethanol (70%, 90% and 100%) and air-dried.

20xSSC: 88.2 g sodium citrate, 175.3gm NaCl, pH adjusted to 7.0 using HCl (and different concentrations were prepared from this stock).

2.2.2.2 Metaphase spread denaturation

Slides were then placed into denaturing solution (see below) at 68°C in a fume cabinet for 90 seconds, using a thermometer to keep a constant check on the formamide temperature. This was always carried out in Coplin jars, adding no more than 2 slides at a time to each jar. Slides were then plunged immediately into ice-cold 70% ethanol and left for 5 minutes before dehydrating through the ethanol series as before, air dried, and then kept at 37°C ready for hybridization. The required probe solution was placed onto the middle of the chromosome spread and the coverslip was placed and sealed with rubber solution, care was taken during this last step not to create air bubbles. Slides were placed in a humidified chamber at 37°C for the required time.

Deionised formamide: 500 ml formamide added to 25g ion-exchange resin beads, stirred for 45 minutes, then filtered through a paper filter. Stored at -20°C. Denaturing solution: 35 ml deionised formamide, 5 ml 20xSSC, 10 ml water, pH adjusted to 7.0 using HCl.

2.2.3 Spectral Karyotyping (SKY)

2.2.3.1 The SKY probe

The SKY™ probe kit (Applied Spectral Imaging, Migdal Ha'Emek, Israel) was used for the 24-colour FISH, according to the manufacturer's instructions. The chromosomes in this probe set were labelled with combinations of up to five fluorochromes or haptens, (Table 2.1a&1b). The fluorochromes used in the SKY™ kit are Rhodamine-dUTP, Texas Red-dUTP, Biotin-dUTP (detected with Avidin-Cy5), FITC-dUTP, and Digoxygenin-dUTP (detected with anti-digoxygenin-Cy5.5). A unique florescent emission spectrum for each chromosome is obtained by the labelling of each chromosome with a different combination of these fluorochromes/haptens (Table 2.1 a & b). This process involves up to 4 DOP-PCR reactions for each chromosome, one reaction for each fluorochrome or hapten incorporated. Once all of the component DOP-PCR

reactions for each chromosome are completed, all of the reaction products are pooled in the to produce the SKY™ probe kit. For example, in the SKY™ kit the spectrum for chromosome 1 is made up of Texas Red, Biotin-Cy5 and FITC, therefore three separate DOP-PCR reactions are performed labelling chromosome 1 specific DNA with each of these fluorochromes/haptens. The products from these reactions are then combined, along with those for all of the other chromosomes, into the final probe mixture. The DOP-PCR reactions were performed by Applied Spectral Imaging (Migdal Ha'Emek, Israel) on flow cytometric-sorted human chromosomes and the probe was supplied commercially with other components of the SKY™ kit ready for use.

Table 2.1a: Chromosome labelling scheme for the SKY™ kit

Chromosome	Probes[a]	Chromosome	Probes[a]	Chromosome	Probes[a]
1	BCD	9	ADE	17	C
2	E	10	CE	18	ABD
3	ACDE	11	ACD	19	AC
4	CD	12	BE	20	A
5	ABDE	13	AD	21	DE
6	BCDE	14	B	22	ABCE
7	BC	15	ABC	X	AE
8	D	16	BD	Y	CDE

[a] The fluorescent probes are denoted as follows: A, Rodamine-dUTP; B, Texas Red-dUTP; C, Biotin-dUTP (detected with Avidin-Cy5); D, FITC-dUTP; E, Digoxygenin-dUTP (detected with anti-digoxygenin-Cy5.5).

Table 2.1b: Characteristics of the flurochromes

	Rodamine	Texas Red	Cy5	FITC	Cy5.5	DAPI
Excitation λ (nm)	550	596	650	495	675	355
Emission λ (nm	570	620	670	525	694	450

2.2.3.2 SKY probe Hybridization technique

24-colour chromosome painting was performed by the 'Spectral Karyotyping' (SKY) technique, essentially as described (Schrock *et al.*, 1996) and as detailed in the SKY™ product instruction manual. Contents of the SKY paint mixture (vial no.1) were vortexed briefly and 10 µl were taken for each slide and denatured by incubation at 75°C for 8 min then the vial was incubated at 37°C at least for 60 min. 10 µl of SKY probe mixture were added to the denatured chromosome preparation on the slide (as described above in 2.2.2.2) and 18 x 18 mm^2 glass coverslip was placed over the probe mix and the edges were sealed with rubber cement, care was taken during this last step not to create air bubbles. Slides were place in a humidified chamber at 37°C for 2.5-3 days. Slides were removed from the humidified chamber and the rubber was carefully removed and slides were transferred to Coplin jar containing washing solution I (50% formamide in 2xSSC), slides were washed 3 times at 45°C for 5 min each wash in a water bath, then three washes in washing solution II (1xSSC) at 42°C for 5 min each, then slides were washed in washing solution III (4xSSC/ 0.1% Tween 20) at 42°C for 5 min. Slides were tilted to allow fluid to drain and then 80 µl of buffer 1 (vial no. 3) were applied to the slide, covered by plastic coverslip and incubated at 37°C for 45 min. Slides were washed 3 times in washing solution 3 at 42°C for 5 min each wash in a water bath. 80 µl of buffer 2 (vial no 4) were applied to the slide and this was covered by plastic coverslip and incubated at 37°C for 45 min and then slides were washed 3 times in washing solution 3 at 42°C for 5 min each wash in a water bath, air dried and then 20 µl of the DAPI (4,6-diamino-2-phenylindole)/antifade solution (vial no. 5) were applied, coverslips were placed on top and slides kept in the dark ready to be examined.

2.2.3.2 SKY Image analysis

Metaphase spreads hybridized with the SKY probe were assessed by fluorescence microscopy and images were captured and analysed using a Spectracube SD200 mounted on a Nikon E800 microscope and SKY software (Applied Spectral Imaging, Migdal Ha'Emek, Israel). In this system, the 5 fluorochromes were excited simultaneously by a triple band-pass filter (SKY-1; Chroma Technologies, Brattleboro, VT) and the spectra of the emitted light were obtained, pixel-by-pixel, with an interferometer and cooled CCD camera. The resulting spectra were matched to reference spectra to determine, pixel by pixel, the identity of each chromosome or fragment of chromosome present. Data were presented as two false-colour images of the chromosomes: the first was a simulation of the

fluorescent colours observed, translated into the range of wavelengths visible to the human eye (spectral image); whereas the second was a classification (Pseudo-colour) image in which each pixel was assigned a colour representing the chromosome identified to be present at that point. In addition, DAPI fluorescence image was captured separately and after reversing and enhancing the image, it was displayed beside the coloured images to provide DAPI banding information.

2.2.4 Single-dye chromosome painting for confirmation of SKY findings

2.2.4.1 Probes for Single-dye chromosome painting

Probes for conventional single-dye chromosome painting were kind gifts from Professor M.A. Ferguson-Smith and Dr Willem Rens (Department of Clinical Veterinary Medicine, Cambridge, UK.). Experiments were designed to test a maximum of 3 chromosomes—each labelled with a single dye—in a single experiment. For such experiments, probe labelling was as follows: Cy3 (direct fluorescence), biotin was detected by sequential incubation with Cy5-labelled Avidin (Amersham International PLC, UK.), biotinylated anti-Avidin antibody, and Cy5-avidin; FITC (incorporated as dUTP derivative) was directly detected or amplified with rabbit anti-FITC (Dako, U.K.), then FITC-labelled goat anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA). Probes were mixed for each hybridization experiment as, 1ul (100ng) of each probe with 1 µl (1µg) of human Cot-1 DNA, made up to a total of 15 µl with hybridisation buffer and denatured at 70°C for 10 min then pre-annealing by incubation at 37°C for 1 h.

Hybridisation buffer: 50% (v/v) deionised formamide, 10% (w/v) dextran sulphate, 2xSSC, 1x Denhardt's solution [50x Denhardt's solution contains5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone, 5 g of bovine serum albuimin and DDW to 500ml], 21mM sodium dihydrophosphate and 29mM disodium orthophosphate.

2.2.4.2 Single-dye chromosome painting procedures and analysis

Metaphase spread preparation on slides were denatured and dehydrated as described above. Pre-annealed probes were hybridised to preparations on the slides overnight at 37°C. After two 5 min washes in 50% formamide / 2xSSC at 45°C, three 5 min washes in 2xSSC at 42°C, one wash in 4xSSC / 1% Tween 20, slides were incubated with the first antibody detection layer for 30 min, then three 5 min washes in 4xSSC / 1% Tween 20, then slides were incubated with the second antibody layer (if required) for 30

min, then three 5 min washes in 4xSSC / 1% Tween 20, then slides were washed three 5 min washes in 4xSSC / 1% Tween 20. Slides were counterstained with DAPI, and mounted in Prolong Antifade (Molecular Probes, U.K.), viewed using a Zeiss Axioplan fluorescence microscope, and images were captured and analysed using Smart Capture / IPLab Spectrum software (Digital Scientific, Cambridge, U.K.).

2.3 Tissue specimens.

Fresh tumour samples were collected from patients with colorectal carcinoma undergoing surgery in the Royal Infirmary of Edinburgh NHS Trust before November 1997. Age, sex, clinical details and other information on the material used for gene analysis and treatment responsiveness studies are supplied in the relevant chapters. Specimens were immediately removed from the operating theatre and delivered on ice to the Department of Pathology at the University of Edinburgh. Blocks of fresh tissue (approximately 10 x 5 x 5 mm) were taken from each colorectal cancer and one from normal mucosa at a point distant from the lesion. Each block of tissue collected from the tumour was subsequently divided into three separate pieces. One portion was placed in 1.5 ml capacity screw-capped vial, snap frozen in liquid nitrogen and stored at -70°C awaiting DNA extraction. The middle portion was fixed in periodate-lysine-paraformaldehyde-dichromate (PLPD), paraffin-processed and sections were stained with haematoxylin and eosin, following standard methodology, for histological assessment. Sections from paraffin blocks were also used for immunohistochemical detection of stabilised p53 protein. The third portion of each of the tissue blocks from the tumours was first washed in PBS and then either placed in a vial with antibiotic medium (Glasgow Modified Eagle's Medium (GMEM) supplemented with 1% v/v, penicillin/streptomycin, 10% Fetal Calf Serum) and immediately taken for implantation in SCID mice. In cases when the implantation could not be performed immediately, samples were placed in freezing medium [10% dimethyl sulphoxide (DMSO) in GMEM] for slow freezing and stored at -80°C until animals were available for establishing xenografts.

2.3.1 Establishment of colorectal cancer xenografts in SCID mice.

All experiments on animals were carried out under project license number 01624 held by Professor Andrew H. Wyllie at Edinburgh University in 1997-1998. Xenografts were established by implantation of two pieces approximately 2x2x2 mm through a small dorsal incision in mice with severe combined immunodeficiency syndrome (SCID), aged 12-16 weeks. Animals were maintained in sterile conditions and provided with sterile food and water. All experimental procedures were undertaken aseptically inside a laminar flow facility. These mice are deficient in both B and T lymphocyte functions due to an inability to correctly rearrange the immunoglobulin and T-cell receptor genes by site-specific [V(D)J] recombination (Bosma *et al.*, 1983; Custer *et al.*, 1985) and thus do not reject implanted foreign tissue. They do, however retain non-specific macrophage-mediated immune responses, useful for removal of bacterial contamination from implanted tumours.

2.4 Polymerase Chain Reactions (PCR)

2.4.1 Extraction of DNA from frozen tissues

DNA was extracted from frozen tissue according to the method of Goelz *et al.*, (Goelz *et al.*, 1985). Frozen tissue was chopped into small pieces in 0.5 ml TE-9 SDS and 0.5 mg ml^{-1} proteinase K and incubated for 48 hours at 48°C, mixing occasionally. An equal volume of water-saturated phenol was added, mixed and centrifuged at 10000 x g for 2 minutes. The top layer was removed and an equal volume of PC-9 added to it, mixed and spun again. The top layer was removed and an equal volume of 24:1 chloroform: isoamyl alcohol was added, mixed and spun. The top layer was once again removed and 0.5 volume 7.5 M ammonium acetate and 2x volume ethanol added, mixed and left overnight at -20°C. This was centrifuged at 4°C and 10000 x g for 20 minutes, the supernatant drained and the pellet vacuum dried. The DNA was resuspended in 250 µl TE, dissolved at 4°C for 3 hours and an OD reading taken at 260nm. Concentration was adjusted to 1 mg ml^{-1}.

TE-9 SDS: 500 mMTris pH8, 20 mM EDTA, 10 mM NaCl, 1% SDS (sodium dodecyl sulphate)
PC-9: 50 ml water-saturated phenol, 50 ml chloroform.
TE: 10 mM Tris, 1 mM EDTA.

2.4.2 Polymerase Chain Reactions

Reactions were carried out in 0.5 ml thin-walled Eppendorf's tubes. PCR was carried out for 35 cycles, each consisting of denaturation for 30 seconds at 94°C, annealing for 30 seconds at a temperature (Ta) calculated according to the primer composition, extension for 30 seconds at 72°C, followed by a final extension step for 10 minutes at 72°C. Reactions consisted of 50 µl volume containing 100 ng genomic DNA, 10-25 pmols of each primer, 200 µM of each dNTP (Advanced Biotechnologies Ltd), 1-4 mM $MgCl_2$, 1.25 U of thermostable DNA polymerase and a final concentration of 1 x buffer consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 0.05% non ionic detergent (Taq DNA polymerase, Life Technologies, UK). Dimethyl sulphoxide (DMSO) at 10% (5 µl per 50 µl reaction) was added for some PCR assays (details are supplied in Table 4.1 and 4.2). The PCR hot start method was used by mixing up all the PCR components except the Taq DNA polymerase enzyme, then adding the enzyme at an initial stage of heating and

holding the PCR mixes at 94°C, then the thermal cycles were allowed to proceed as required. The products were analysed by electrophoresis on 2% agarose gels for detection of the amplified DNA product.

2.4.3 Agarose gel electrophoresis

Agarose gels were prepared by adding 3 g electrophoresis-grade agarose to 150 ml 1x Tris-borate-EDTA buffer (TBE) and heating to boiling point in a microwave oven, with regular mixing, to dissolve the agarose. The gel was stirred and allowed to cool to approximately 55°C before adding 20 ng ml^{-1} ethidium bromide and pouring into a 12x16cm gel mould with a 20-well comb. For checking PCR products, 8 µl product was added to 2 µl loading dye before loading. Products were run against 500 ng of a suitable molecular weight marker, usually Ladder Mix (Fermentas Ltd) or 1-kb ladder (Life Technologies Ltd) were used. Electrophoresis was carried out at 125 V in 1xTBE buffer for variable lengths of time; for most purposes, migration of the bromophenol blue dye approximately 5 cm through the gel was sufficient. Gels were visualised and photographed using a UV illumination box.

10xTBE: 107.8 g Tris (0.89M), 55 g Boric acid (0.89M), 7.44 g Na_2EDTA (0.02M) in 1000 ml DDW pH adjusted to 8.3.
Loading dye: 30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF.

2.5 Analysis of microsatellite instability

2.5.1 Microsatellite loci

The following microsatellite markers were used for analysis of microsatellite instability: D2S119, D2S123, D3S1293, D8S282, D13S160, D15S132, D17S849, D18S58, TGFβR-II, BAT26, BAT40, and L-myc. At least five loci were examined in each patient. The primers and conditions are described in (Table 2.2).

Table 2.2: Primer sequences for amplification of microsatellites.

Locus	$[Mg^{2+}]$ conc. (Mm)	Annealing temp. (°C)	Approximate allele size (bp)	Primer sequences (5'-3')
D2S119	2	55	214-232	CTTGGGGAACAGAGGTCATT GAGAATCCCTCAATTTCTTTGGA
D2S123	2	58	210-240	AAACAGGATGCCTGCCTTTA GGACTTTCCACCTATGGGAC
D3S1293	2	50	116-144	ACTCACAGAGCCTTCACA CATGGAAATAGAACAGGGT
D8S282	1.5	50	260-274	GGGCACAGGCATGTGT GGCTGCATTCTGAAAGGTTA
D13S160	1.5	55	229-241	CGGGTGATCTAAGGCTTCTA GGCAGAGATATGAGGCAAAA
D15S132	1	55	69-83	CTGATAATAAAACCAGGAAGACAC TATTGGCCTGAAGTGGTG
D17S849	1	50	144-174	CAATTCTGTTCTAAGATTATTTTGG CTCTGGCTGAGGAGGC
D18S58,	1.5	60	~110	CTCTTTCTCTGACTCTGACC GACTTTCTAAGTTCTTGCCAG
TGFβR-II,	3	58	267	CCTCGCTTCCAATGAATCTC TTGGCACAGATCTCAGGTCC
BAT26,	1.5	58	80-100	TGACTACTTTTGACTTCAGCC AACCATTCAACATTTTTAACCC
BAT40,	1.5	58	80-100	ATTAACTTCCTACACCACAAC GTAGAGCAAGACCACCTTG
L-myc	1.5	53	140-209	TGGCGAGACTCCATCAAAG CTTTTTAAGCTGCAACAATTTC

(Bubb *et al.*, 1996; Huang *et al.*, 1996; Dietmaier *et al.*, 1997)

2.5.2 Radio-labelling and denaturation of the PCR products

The PCR products were radioactively labelled through primer end-labelling with [γ-^{33}P]ATP using T4 polynucleotide kinase. The radio-labelled products were electrophoretically separated in denaturing 6% polyacrylamide gel and subjected to autoradiography for detection of band shifts.

Oligonucleotide primer sequences were diluted with sterile distilled water before use, giving a final concentration of 10 ng μl^{-1}. The strand primer in each reaction was 5' end-labelled with [γ-^{33}P]ATP using T4 polynucleotide kinase. Labelling was performed prior to each PCR reaction. 10ng of oligonucleotide (10 ng μl^{-1}) was added to 2µl of 5x buffer, 6.2µl of sterile distilled water, 0.3µl of [γ-^{33}P]ATP (equivalent to 3 µCi) and

placed on ice. 0.53 µl (5 U) of T4 polynucleotide kinase was then added giving a total volume of 10 µl and the reaction placed in a water bath at 37°C for one hour. This amount was sufficient for up to 10 PCR reactions.

Polymerase chain reactions were carried out as usual for any microsatellite locus, except that prior to amplification an additional 1 µl labelled primer was added to each reaction. After amplification, 5 µl stop solution was added to 5 µl PCR product, the mixture boiled for 3 minutes and cooled quickly on ice in preparation for loading onto a gel.

2.5.3 Gel preparation and running conditions

A 6% denaturing polyacrylamide gel was used in vertical sequencing gel apparatus with 0.5xTBE buffer. Gels were prepared in 35 cm x 50 cm vertical DNA sequencing electrophoresis plates. Plates were thoroughly cleaned with 'Decon' detergent, rinsed with water and finally wiped with 70% alcohol. One plate was siliconised by wiping with 3 ml Gel Slick, air drying and removing excess with water. Plates were assembled with 0.4 mm spacers and combs. Sixty microlitres of TEMED and 60µl 25% ammonium persulphate (APS) were added to 60 ml stock acrylamide immediately prior to pouring and the gel was allowed to polymerise for 1 hour. The gel was pre-run for an hour in 0.5xTBE at 70 W to reach 55°C. 5 µl of combined PCR product and stop solution denatured as described above were added to the wells after rinsing with TBE to disperse urea. The gel was then run at 70 W for 3-6 hours or until the xylene cyanol marker dye approached the end of the electrophoresis plate. It was then fixed for 10 minutes in a solution of 10% methanol and 10% acetic acid, transferred to Whatman No 17 paper and dried on a vacuum gel drier. Autoradiography was carried out for 1-3 days and autoradiographs were assessed visually for microsatellite instability.

5X T4 polynucleotide kinase buffer: 350mM Tris-HCl pH 7.6, 50mM $MgCl_2$, 500mM KCl, 5mM 2-mercaptoethanol.

Stop solution: 95% formamide, 10mM EDTA pH8, 0.1% bromophenol blue, 0.1% xylene cyanol FF.

Acrylamide solution: 75ml 19:1 acrylamide:bis acrylamide 'Instagel' (Severn Biotech Ltd), 250g 500ml urea (7M), 50ml 10x TBE, 175ml DDW stored protected from light at 4°C.

2.6 Mutation screening methods

2.6.1 Single strand conformation polymorphism (SSCP) analysis

35x50 cm glass plates were used. To 60 ml 0.5xMDE gel solution, 240 µl 10% ammonium persulphate and 24 µl TEMED were added, the solution mixed and poured immediately. The gel was allowed to set for 1 hour before use. Five microlitres of PCR product were denatured for 5 minutes at 50°C in 1 µl denaturing solution then chilled on ice. Three microlitres of the stop solution (95% formamide, 10 mM EDTA pH 8, 0.01% bromophenol blue, 0.01% xylene cyanol) were added and the samples loaded quickly onto 0.75 mm thick MDE gel (Flowgen, UK) containing 5-10% glycerol, after rinsing the wells with 1xTBE. and DNA strands were electrophoretically separated at 20°C at 6 W constant power overnight or 25 W for 4-6 hours. Running time varied according to fragment size; good fragment separation was achieved when samples were run such that the xylene cyanol marker dye had migrated between 20 and 60 cm through the gel. Electrophoresis buffer was 1xTBE.

0.5xMDE gel solution: 15 ml MDE gel, 3 ml glycerol, 6 ml 10x TBE, 36 ml DDW.
Denaturing solution: 0.5 M sodium hydroxide, 10 mM EDTA.

2.6.2 Heteroduplex analysis

A 1xMDE gel containing 3.12 M urea was prepared by addition of 240 µl 10% ammonium persulphate and 24µl TEMED to 60ml gel mix. The gel was poured and allowed to set for 1 hour before use. PCR products for heteroduplex analysis (White *et al.*, 1992) were heated to 95°C to denature double-stranded DNA and then cooled slowly to 37°C over 30 minutes, allowing reannealing of single strands. 4 µl of samples in 2 µl loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25 xylene cyanol FF) was electrophoresed on the MDE gel and run at 0.71-0.80 kV for 16 hours. This slow running of the gel ensured that it remained cool. 0.5 µg of a molecular weight marker, Marker V (Boehringer Mannheim Ltd), and a control sample known to separate into heteroduplex and homoduplex bands were also loaded. Both glass plates were pre-treated as above, and the resulting bands were visualised using the silver staining method as for SSCP.

1xMDE gel mix: 30 ml MDE, 3.6ml 10xTBE, 9 g urea, DDW to 60ml.

2.6.3 Silver staining of mutation screening gels

Gels were stained according to the methods of Bassam *et al.* (Bassam *et al.*, 1991), as detailed in Promega's DNA Silver Staining System protocol with some modifications to avoid detachment of the gel from the plate during the staining process. 35x50 cm glass plates for vertical gel electrophoresis were used with 0.75 mm spacers and square or shark's tooth combs. Both glass plates were pre-treated: the first was wiped with 3ml Gel Slick, allowed to dry for 5 minutes then wiped with 70% ethanol. The second was treated with 20 µl gamma-methacryloxypropyl-trimethoxysilane in 5ml distilled water (pH 3.5 with acetic acid) in order to attach the gel to the plate for staining *in situ* and dried for ten minutes before wiping four times with 95% ethanol. Silver staining of gels was carried out in a shallow glass dish on a gently shaking platform, carefully pouring off used solutions. Ultrapure water was used throughout.

The glass plates were separated and the gel soaked for 10 minutes in 10% ethanol followed by 10 minutes in 1% nitric acid. It was then rinsed for 5 minutes in UDDW and placed in staining solution for 20 minutes. After rinsing for 10 seconds with UDDW, 1/3 of the pre-cooled developer solution was poured on the gel and left in until a brown precipitate appeared, then this developer was replaced with the remaining 2/3 of the fresh cooled developer. When bands appeared, the reaction was stopped by replacing the developer with 0.1 M citric acid for 10 minutes, the gel rinsed in UDDW for a further 5 minutes and then interpreted and photographed on a light box. For re-use, plates were soaked in 5 M sodium hydroxide for one hour and cleaned with detergent and 70% ethanol. Spent silver nitrate was treated with sodium chloride to precipitate the silver before disposal.

Staining solution: 2.04 g silver nitrate in 1 litre UDDW
Developer solution: 160 g sodium carbonate (decahydrate), 1 ml 37% formaldehyde, in 2 litres UDDW.
Stop solution: 19.2 g citric acid in 1 litre UDDW

2.7 Sequencing reactions

2.7.1 Sequencing using Sequenase Version 2.0 kit

Sequencing of *Fas* and *Bik* gene PCR products was carried out according to the protocol provided with the Sequenase Version 2.0 kit (Amersham International plc).

2.7.1.1 Preparation of PCR products

Double-stranded DNA was denatured by incubating at 37°C for 30 minutes in 0.1 volume denaturing solution. This was neutralised with 0.1 volume 3 M sodium acetate, pH 4.5-5.5, and precipitated with 4x volume ethanol. After 15 minutes at -70°C, the DNA was centrifuged at 4°C for 15 minutes, washed with 4x volume 70% ethanol, centrifuged again and vacuum dried. The pellet was resuspended in 7 µl DDW, and to it was added 50 pg of appropriate primer in 1µl volume and 2 µl Sequenase buffer. This was mixed and heated to 65°C for 2 minutes then allowed to cool to <35°C in a water bath over 15-30 minutes. It was then spun briefly and placed on ice. To it was added 1 µl 0.1 M DTT, 2µl labelling mix (diluted 1/5 in DDW) 3.2 U Sequenase in 2µl dilution buffer and 5 µCi ^{35}SdATP. 2.5 µl of each termination mix was warmed to 37°C and 3.5 µl of the the above DNA solution added and mixed thoroughly. After 5 minutes the reaction was stopped by adding 4 µl stop solution and the reactions stored at -20°C for up to one week before use.

Denaturing solution: 2M NaOH, 2mM EDTA.
Dilution buffer: 10 mM Tris.HCl pH 7.5, 5 mM dithiothreitol, 0.5 mg ml^{-1} BSA.
5 x Sequenase buffer: 200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl.
Stop solution: see 2.14.

2.7.1.2 Sequencing gel electrophoresis and autoradiography

Sequencing was carried out on BRL S2 sequencing apparatus using 0.4 mm spacers and sharks-tooth combs. Plates were cleaned with cream cleaner, washed thoroughly and wiped with 70% ethanol. One plate was treated with gel slick as in section 2.5.3. 60 ml gel stock solution was polymerised by adding 60 µl 25% ammonium persulphate and 60 µl TEMED immediately before pouring. The gel was allowed to polymerise horizontally for 1 hour with inverted combs and then pre-run without combs, after rinsing thoroughly with 1xTBE, at 90W for 30-60 minutes in 1xTBE until it reached 50°C. Combs were then replaced to form wells, which were again rinsed thoroughly. Samples were heated to 75°C for 3 min prior to loading and run at 70 W until the bromophenol blue marker reached the bottom of the gel. The gel was fixed twice in 10% acetic acid 10% methanol for 5 minutes

and transferred onto 3 mm paper. It was dried for 2 hours on vacuum gel dryer and exposed to autoradiography film overnight.

Labelling mix (dGTP): 7.5μM dGTP, 7.5μM dTTP, 7.5μM dCTP.
Termination mix: 50mM NaCl, 80μM each dNTP, 8μM of one of ddGTP, ddATP, ddTTP or ddCTP.
Acrylamide solution: see 2.5.3.

2.7.2 Cycle sequencing of PCR products

Direct sequencing of PCR products was carried out for *Bax* and *TGFβRII* gene PCR products using the ThermoSequenase radio-labelled terminator cycle sequencing kit (Amersham International plc). This is a method of cycle sequencing which utilises radio-labelled ddNTP terminators in conjunction with a thermostable DNA polymerase to produce high quality sequence which is largely free of background.

2.7.2.1 Pretreatment of PCR DNA samples

Before sequencing reactions can be carried out, PCR-generated DNA must be pretreated with exonuclease I and shrimp alkaline phosphatase (SAP) to remove single-stranded oligonucleotides, single-stranded PCR-generated DNA and free dNTPs. To do this, 1 μl each enzyme (10 U exonuclease I and 2 U SAP) was added to 5 μl PCR product and incubated at 37°C for 15 minutes. The enzymes were then inactivated by heating to 80°C for 15 minutes. Additionally, an aliquot of untreated DNA sample was quantitated by agarose gel electrophoresis against a quantitative marker.

2.7.2.2 Sequencing reactions

2 μl dGTP termination master mix was mixed with 0.5μl of [α-^{33}P]ddNTP to produce a termination mix for each ddNTP. A reaction mixture was made for each test DNA sample by mixing 50 ng (25 fmol) treated DNA with 0.5 pmol sequence-specific primer (forward-strand primer) and 2 μl reaction buffer in a final volume of 20 μl. 4.5 μl reaction mixture was transferred to each termination tube, mixed and overlaid with one drop of paraffin oil. The tubes were placed on a thermal cycling block and subjected to 30 cycles of 95°C for 30s, 55°C for 30s and 72°C for 1 minute. 4 μl stop solution was added to each tube and the samples heated to 70°C for 2 minutes before loading 3 μl per lane onto a denaturing polyacrylamide gel.

Reaction buffer: 260 mM Tris HCl, pH9.5, 65 mM $MgCl_2$.

dGTP termination master mix: 7.5 μM dGTP, 7.5 μM dATP, 7.5 μM dTTP, 7.5 μM dCTP.

2.7.2.3 Gel electrophoresis

Gel electrophoresis and autoradiography were carried out as in 2.7.1.2, except that glycerol-tolerant buffer was substituted for TBE buffer.

20x glycerol tolerant buffer: 216 g Tris base, 72 g taurine, 4g $Na_2EDTA\ 2H_2O$, made to 1litre with DDW.

2.7.2.4 Elimination of sequencing band compressions using dITP

Where sequencing band compressions (observed as uneven band spacing) caused difficulties in reading a sequence, reactions were repeated substituting dITP termination master mix (supplied with the ThermoSequenase kit) for dGTP. Reactions were carried out as above, except that the termination (extension) temperature was reduced from 72°C to 60°C and the time at this temperature increased to 5 minutes.

dITP termination master mix: 7.5 μM dITP, 7.5 μM dATP, 7.5 μM dTTP, 7.5 μM dCTP.

2.8 Immunocytochemical detection of stabilised p53

The p53 protein accumulation status of the samples was established by immunohistochemistry using antibody DO-7, which recognises an amino terminal epitope and is capable of reacting with both wild type and mutant p53 (Vojtesek *et al.*, 1992). Sections were prepared in the Department of Pathology, University of Edinburgh, by cutting 3 μm sections from tissue stored in periodate-lysine-paraformaldehyde-dichromate (PLPD) and embedded in paraffin wax. Sections were placed onto poly L-lysine (PLL)-treated microscope slides. Matching normal mucosae were stained with each tumour. Unstained sections from known p53 positive and negative colorectal carcinomas were also included as controls for each staining batch. Samples were deparaffinised by placing in xylene for 10 minutes, absolute ethanol for 1 minute, 90% ethanol for 1 minute and 70% ethanol for 1 minute and then rinsed in water. Endogenous peroxidase activity was blocked by treatment in 3% hydrogen peroxide for 15 minutes. This was followed by washing for 5 minutes in DDW and 5 minutes in phosphate buffered saline (PBS). Slides were then wiped to remove excess fluid and marked around the area of the section with a wax pen to retain solutions. 100 μl normal rabbit serum (NRS) diluted 1/5 in PBS was added and left for 20 minutes. This was then drained and the slides incubated overnight at 4°C in a 1/100 dilution of Do-7 antibody (Vojtesek *et al.*, 1992) in 1/5 NRS/PBS solution.

After overnight incubation, the sections were washed twice for 5 minutes in PBS before applying the secondary antibody. Biotinylated rabbit anti-mouse immunoglobulins (Dako Ltd) were diluted 1/400 in the above NRS/PBS solution and 100 μl added to the sections and incubated for 30 minutes at room temperature. Meanwhile, an avidin/biotinylated horseradish peroxidase (HRP) complex (ABC kit, Dako Ltd) was made up by adding 1 drop of avidin and 1 drop of biotinylated horseradish peroxidase to 5 ml PBS and allowing 30 minutes for the reagents to conjugate. After incubation, the slides were washed twice in PBS for 5 minutes and 100 μl ABC complex was added. Slides were incubated for 30 minutes at room temperature then again given two 5-minute washes in PBS. They were then incubated for 3 minutes in diaminobenzidine (DAB) solution at room temperature to allow formation of brown colouration through oxidation of DAB by HRP, washed for 5 minutes in DDW and counterstained for 6 seconds in haematoxylin. They were washed until the water was clear and dehydrated through 1 minute in 70% ethanol, 1 minute in 90% ethanol, and 1 minute in absolute ethanol and 10 minutes in xylene. Slides were mounted with DPX mountant and examined under 100x total magnification on a light microscope. Cases were scored positive if any nuclei stained

positively and the extent of staining, which varied considerably, was noted. Although strength of staining also varied, this was not taken into account as it could vary between experiments.

PLPD: 0.1 M lysine, 0.1 M periodate, 2% paraformaldehyde made to 50 ml with Sørensen's phosphate buffer (0.05 M, pH 7.4). 5% potassium dichromate in 50 ml DDW added immediately before use

PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 to 1 litre DDW pH7.4.

DAB solution: 5 mg DAB, 4.8 ml DAB buffer, 100 µl hydrogen peroxide solution (1/30, [v/v], 30% hydrogen peroxide stock/DDW).

DAB buffer: 24 ml 0.2 M Tris, 38 ml 0.1N HCl, 0.0681g imidazole, DDW to 100 ml, pH adjusted to 7.6.

2.9 clonogenic survival assay

The plating efficiency for each cell line was determined first by incubating 1.5 x 10^2, 2 x 10^2, 3 x 10^2 and 5 x 10^2 cells in 25-cm^2 flasks in triplicate experiments. Cell concentrations, which gave 100-150 colonies after 12 days, were chosen. These were 1.5 x 10^2 Cells for HT29 and 3 x 10^2 for LoVo. Cells were trypsinised, using standard protocols, and the trypsin neutralised with two volumes of cell culture medium. An aliquot of cell suspension was removed and the cell density counted using a haemocytometer. The suspension was also examined to ensure it contained only single cells and not clumps. Cells were plated out, in triplicate flasks, at the required densities. Cells were allowed to reach the log phase over night.

5-FU was dissolved in PBS and temozolomide was dissolved in 10% DMSO/PBS and then added to fresh media to give the required concentrations of each drug. Cells were then exposed to the required concentrations of 5-FU for one hour at 37°C, or different doses of ionizing radiation and then washed and fed by fresh media. Because of the short half-life of temozolomide, cells were exposed continuously to the drug over 12 days. Media were replaced with fresh media containing temozolomide each 2-3 days to ensure that cells were exposed to active drug. Controls were exposed to the drug vehicles and to the same procedures of washing and replacing the media as the treated flasks. Cells were irradiated using X-ray source (Siemens Stabilipan II 300 kv orthovoltage unit) at 0.80 Gy min^{-1} and controls were exposed to the same procedures of transport but were not irradiated. After 12 days, the medium was removed from the plates and the cells washed in PBS, then methanol:PBS (1:1), then fixed in 100% methanol for 10 minutes. Plates were then stained in filtered 50% Giemsa-50% methanol (v/v) for 30 minutes, washed gently with tap water, dried, and the numbers of colonies counted.

2.10 Acridine Orange staining for counting apoptosis

Acridine orange is a metachromatic fluorochrome that differentially stains double-stranded versus denatured DNA. The dye is an intercalating agent that, on excitation with UVC light, emits green light when bound to double-stranded DNA and red light when bound to denatured DNA or RNA. The nucleus of a stained cell thus appears green under UVC light, whilst the cytoplasm stains red. This staining allows the examination of nuclear morphology and hence the sensitive detection of cells which have undergone apoptosis.

At selected time points (1, 2, 3.5 days) after irradiation or drug treatment, cells were trypsinised and centrifuged at 600 x g for 5 min, together with the medium and PBS from the pre-trypsinisation wash. The supernatant was then discarded, cells resuspended in PBS and two volumes of ice cold fixative (90% ethanol, 10% formaldehyde) added and the cells were left to fix for 1-3 hours before counting. Cell suspensions were counted immediately or stored at 4°C until counting. Before counting, cells were centrifuged at 220 x g for 10 minutes at 4°C, re-suspended in 5 ml PBS, spun again and finally re-suspended in 50 to 1000 µl of PBS (depending on cell density). 10 µl of cell suspension was dropped onto a clean glass slide, and an equal volume of Acridine Orange solution (5 µg ml^{-1}, Sigma) dropped onto a coverslip. The coverslip was carefully lowered onto the slide, and the number of cells scored for apoptosis, on the grounds of nuclear morphology, under a fluorescence microscope (Zeiss, Axiovert S100T.V, UK). Nuclear changes characteristic of apoptosis included chromatin condensation nuclear shrinkage and nuclear fragmentaion. Apoptosis was counted in a minimum of 300 cells, each 100 cells, from a different field on the slide.

CHAPTER 3

GENOMIC INSTABILITY PATTERNS IN SPORADIC COLORECTAL CANCERS

3.1. Introduction:

Genomic instability is a feature of most malignant tumours. It implies either increased rate of genomic changes, or an increased tolerance to genomic changes, or both. This genomic instability appears to take different forms in different individual tumours, and leads to different characteristic patterns of abnormality in the genome. Non-random patterns of acquired chromosomal changes have been detected in all tumour types that have been investigated in any great detail (Mitelman *et al.*,.1997), and there is already some evidence that the pattern of chromosomal change reflects the kind of genomic instability in the tumour (Eshleman *et al.*, 1998). The best understood form of genomic instability - microsatellite instability - generates repeated errors in the fidelity of replication of microsatellite sequences resulting from loss of function of the nucleotide mismatch repair pathway. However, the majority of RER+ tumour cell lines have few if any chromosomal rearrangements or numerical changes (Eshleman *et al.*, 1998). Other, RER-, carcinomas have stable microsatellites, but frequently show chromosomal instability (CIN) (Lengauer *et al.*, 1997; Eshleman *et al.*, 1998). As in RER+ tumours, there is clear evidence that the errors responsible for the CIN+ phenotype are repeated many times, at different locations within the genome, during the development of the tumours (Lengauer *et al.*, 1997). The defects that cause chromosomal instability are not yet well established, but are likely to be various, since a range of defects can lead to chromosomal changes in model systems, including defects in double-strand break repair and in centrosomes (see Chapter 1 1.4.7). Deficiency of p53 appears to be not sufficient, but may be permissive (Carder *et al.*, 1993; Carder *et al.*, 1995). Two RER–, CIN+ colorectal carcinoma cell lines have mutations in *hBUB-1*, a mammalian gene whose yeast homologue is essential for normal chromosome disjunction, but these mutations appear to be uncommon (Cahill *et al.*, 1998).

It should be noted that much less cytogenetic information has been gathered for carcinomas, compared with haemato-lymphoid malignancies for example, partly due to technical difficulties in obtaining good metaphases from neoplastic epithelial cells and partly due to the karyotypic complexity and variability making conventional Giemsa (G) banded karyotypes difficult to interpret (Mertens *et al.*, 1997). It seems likely that different patterns of karyotypic changes will be discernable when karyotypes are analysed

in more detail, reflecting different types of chromosomal instability or tolerance of different types of chromosome damage. In other words, there are probably multiple CIN phenotypes. Indeed, not all RER+ colorectal tumours are near-diploid, and there is a substantial subset of RER- human colorectal cancers in which there is little evidence for instability in chromosome numbers (Georgiades *et al.*, 1999). Clear definition of the different patterns of genomic instability in colorectal tumours may give clues to the nature of these undiscovered defects. It may also prove predictive of response to therapy, since consistent patterns of genomic instability are likely to reflect selective defects in cellular ability to recognise and respond to errors or damage of specific types. In particular, nucleotide mismatch and DNA strand breaks, genomic abnormalities that appear repeatedly in human cancers, each trigger apoptosis when they are induced in normal cells (Clarke *et al.*, 1994; Toft *et al.*, 1999). Such specific defects in apoptosis pathways could explain emerging correlations between patterns of genomic instability and tumour resistance to particular therapeutic agents (Bunz *et al.*, 1999). Perhaps related to this is the observation that RER status is one of the strongest factors predictive of long-term survival for patients with colorectal carcinoma (Bubb *et al.*, 1996).

Here, chromosomal changes in a series of 17, well-characterized, colorectal cancer cell lines were studied comprehensively using Spectral Karyotyping (SKY) (Schrock *et al.*, 1996). SKY is a recently developed fluorescent *in situ* hybridization (FISH)-based technique that has the power to identify complex chromosomal rearrangements (Macville *et al.*, 1997; Rao *et al.*, 1997; Ried *et al.*, 1997; Ried, 1998). Tumour cell lines were selected to represent RER+ and RER- groups and within each of these p53 wild type and mutants were selected. SKY findings were confirmed, in some cases, by conventional single-dye chromosome paint FISH, and cytogenetics.

3.2. Materials and Methods:

The 17 human colorectal carcinoma cell lines (Table 3.1) were selected from a set of over 50,collected from many different sources, to represent different combinations of molecular and genomic abnormalities within sporadic colorectal cancers. SKY was as described (Schrock *et al.*, 1996) (see Chapter 2 for details). Briefly, an electronically inverted DAPI image (Figure 3.1 B & 3.2 A) is used for banding of the metaphase chromosomes. The so-called 'spectral image' is obtained from the same metaphase (Figures 3.1 C and 3.2. B) after hybridisation to the SKY paint and spectral imaging of the fluorescent signals on a fluorescence microscope using a spectrometer (Spectracube, Applied Spectral Imaging). The SKY software analyses the spectrum of each pixel of the image, determines which dyes are present and hence which chromosome is being detected . Each pixel of the image is then presented in a false-coloured way using a 'classification or pseudo-colour' to show which chromosome spectrum best matches the spectrum recorded as in Figure 3.1 D and 3.2 C. At least 10 good metaphases were analysed initially for each cell line, this was increased up to a total of 24 metaphase spreads for those lines displaying marked clonal heterogeneity.

The identities of most translocated fragments were verified by conventional chromosome painting using a single fluorescent dye for each chromosome, because SKY occasionally misidentifies small fragments of chromosomes due to overlap between adjacent fluorescent signals. Such errors are, however, almost invariably predictable. Representative karyotypes (Table 3.2) were assembled as follows: all rearranged chromosomes present in at least two metaphases were listed, and the number of copies of each chromosome was that present in the majority of metaphases, except where noted. (Detailed descriptions of the technical aspects of the methods are included in Chapter 2)

Table 3.1 Clinico-pathological and molecular features of cell lines

RER-[1,2]	Age	Sex	Site	Diff	Du.S.	*p53*	*APC*[2]	*β-cat*[2]
C70	69[2]	F[2]	sig[2]	mod[2]	B[2]	wt	mu	wt
HT29[3]	44	F	col	well-mod		mu[4-6]	mu	wt
LIM1863[7]	74	F	Rt col	poor	B	wt		
SW1417[8]	53	F	col	mod	C	wt	mu	wt
SW403[8]	51	F	col	mod	C	mu	mu	wt
SW480[8]	50	M	col	mod	B	mu[4,6,7]	mu	wt
SW620[8]	50	M	LN met		D	mu[5,6]	mu	wt
SW837[8]	53	M	rectum	poor	C	mu[9]	mu	wt
VACO4A[10]	59	M	rectum	mod	D	wt	mu	wt
RER+								
DLD1[11]		M	col	poor	C	mu[6]	mu	wt
GP2D[12]		F	col	poor	B	wt[12]	mu	wt
HCT116[13]		M	col			wt[4-7]	wt	mu
LS174T[14]	58	F	colon	well	B	wt[4,6]	wt	mu
VACO5[10]	78	F	caecum	poor	C	mu[7]	mu	wt
LoVo[15]	56	M	LN met		D	wt[4,7,16]	mu	wt
HCA7[17]	58	F	Hep flx	mod-muc	B	mu	wt	wt
LS411[18]	32	M	caecum	poor	B	wt	mut	wt

Original references describing cell line establishment are supplied after the name of each line. *p53, APC*, and *β-catenin* data either were obtained from work done by Sir Walter F. Bodmer's group or collaborators some of which are published in references 1 and 2. C70 was established in Sir Walter Bodmer laboratory and there is no publication describing its establishment. Additional references for the *p53* status are supplied. Diff, differentiation; Du.S., Dukes' stage; wt, wild type (used to denote absence of abnormalities at the level of the available information); mu, mutant; sig, sigmoid colon; Rt, right colon; LN met, lymph node metastasis; Hep flex, hepatic flexure; mod, moderately differentiated adenocarcinoma; well-mod, well to moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma; mod-muc, moderately - differentiated adenocarcinoma with mucinous differentiation. Gaps in the table indicates absence of information. RER status determined by BAT26 or BAT25 and further r confirmed by D15S58 microsatellite loci.

1, (Wheeler *et al.*, 1999); 2, (Rowan *et al.*, 2000); 3, (Fogh & Trempe, 1975); 4, (Cottu *et al.*, 1996); 5, (O'Connor *et al.*, 1997); 6, (Yoshikawa *et al.*, 1999); 7, (Whitehead *et al.*, 1987); 8, (Leibovitz *et al.*, 1976); 9, (Eshleman *et al.*, 1998); 10, (McBain *et al.*, 1984); 11, (Tibbetts *et al.*, 1977); 12, (Solic *et al.*, 1995); 13, (Brattain *et al.*, 1981); 14, (Rutzky *et al.*, 1980); 15, (Drewinko *et al.*, 1976); 16, (Soulie *et al.*, 1999); 17, (Kirkland, 1985); 18, (Suardet *et al.*, 1992).

3.3. Results and Discussion

3.3.1. Karyotypes of the cell line

The representative summary karyotypes of the cell lines are described in Table 3.2. Representative metaphases analysed by SKY are shown in Figure 3.1 – 3.18. Detailed karyotypes of each of the constituent metaphases of each line are described in Appendix 2.

Some of the initial SKY classifications were incorrect in detail, due to overlap of adjacent fluorescent colours, as illustrated in Fig 3.1 C70-E, F, G, and Fig 3.2 HT29-E, F but their correct composition, was determined by conventional FISH , using single fluorescent dyes for each chromosome is shown in Table 3.2. Single-dye chromosome painting was used to confirm the identity of all translocated fragments in C70, HT29, SW403, SW480, DLD1, HCT116, VACO5, and all potentially misidentified translocations in the other lines. These confirmation experiments are described in detail in the legends for Figure 3.1 – 3.18. The karyotypes ranged from entirely normal to highly aneuploid and multiple chromosomal rearrangements. One line (LS174T) deviated from diploid only in having 3 numerical changes (-X, +7, +15); four others (DLD1, GP2d, HCT116, VACO5) deviated from diploid by having 2-4 rearranged chromosomes and 0-1 numerical changes. LoVo showed 3 trisomies and 3 structural rearrangements. The remaining lines were to varying degrees aneuploid, and/or structurally rearranged with modal chromosome numbers in the range 38 to 127 and with between 8-21 rearranged chromosomes.

Table 3.2

Karyotypes of the colorectal carcinoma cell lines

RER- cell lines

C70: 115~130 (mode 127), XXXXX, 1x4, der(1)t(1;5)(p12;p13)x2, 2x1, del(2)(p21), del(2)(?)* x4, 3x6, 4x2, der(4)del(4)(q31q35)t(4;18)(?p15;?)x2, 5x4, 6x3, 7x9, 8x5, 9x5, 10x4, der(10)t(3;10)(?;q23-24), 11x3, del(11)(?q23), dup(11)(?)x3, 12x6, der(12;22)t(12;22)(?p13;p11)t(12;22)(?q24;q11), 13x6, dup(13)(?)† x2, 14x4, 15x6, 16x3, der(16)t(10;16)(q23-24;q24)x2, 17x2, der(17)t(6;17)(?q23;q24-25)x2, 18x4, 19x4, der(19)t(19;22)(?p13.1;?q11.2)x2, 20x9, 21x2, der(17;21)(q10;q10)x2, 22x3[12]/idem, +der(8)t(5;8)[2]

HT29: 69~73 (mode 70), XX, del(X)(?p21), 1x3, 2x3, der(2)t(1;2)(q32;q11-13), 3x3, 4x2, del(4)(?q31), 5x3, der(5)t(5;6)(p10;?), 6x2, 7x4, 8x2, hsr(8)(p22-23), 9x2, der(6;9)(p10;q10), 10x3, 11x4, 12x3, 13x1, i(13)(q10), 14x2, 15x4, 16x3, 17x2, der(17;19)(q10;?p10), 18x2, del(18)(q12), 19x3, 20x3, del(20), 21x2, 22x3, der(22)t(17;22)(?;q12)[13]/idem, 11x3, der(9;11)[2]/idem, -11, +der(11;13), -13, +der(13)t(7;13)(?;q10), -16, +der(11;16)(q10;?p10)[3]

LIM1863: 66~82 (mode 80), XXXX, 1x3, 2x3, 3x3, der(2;3;8)t(2;3)t(3;8), 4x3, 5x3, 6x2, 7x4, 8x2, del(8)(p?)x3, der(8;17), 9x3, 10x3, 11x3, der(1;11), 12x3, der(12)t(11;12), 13x0, i(13)(q10)x4, der(9;13), 14x3, 15x4, 16x3, 17x0, der(17)t(X;17)x2, 18x3, 19x3, 20x7, 21x2, 22x2[10]/idem, -der(17)t(X;17), +der(17)t(3;17)[2]

SW1417: 66~71 (mode 70), XX, 1x1, del(1)(?)x2, 2x3, 3x4, 4x2, 5x1, del(5)(?)x2, der(5)t(5;17), der(5)t(5;20), 6x2, del(6)(q?), 7x1, dup(7)(q?), der(7)t(1;7)t(1;8)x2, 8x1, der(8)t(1;8), 9x1, del(9)(?)x2, 10x2, 11x4, 12x3, 13x2, 14x3, 15x1, i(15)(q10), 16x3, 17x2, 18x1, del(18)(?), dup(18)(?), 19x1, der(19)t(9;19)x2, 20x2, dup(20)(?)x2, 21x2, 22x4[13]/idem, -2, +del(2)(?), +der(2)t(2;3), +del(10)(?)[2]/idem, der(2)t(2;20), -3, +del(3)(?), +der(5;18), -12, +del(12)(q?), -17, +der(17)t(16;17)[3]

SW403: 60~65 (mode 64), XXX, 1x2, del(1), 2x3, 3x3, 4x2, 5x3, 6x2, 7x4, 8x1, dup(8)(?), i(8)(q10), der(2;8), 9x3, 10x3, 11x3, 12x2, der(12)t(12;15), 13x1, dup(13)(q?)x2, 14x2, 15x2, 16x2, 17x1, del(17)(?), der(17)t(17;22), 18x1, dup(18)(q?), der(18;22), 19x3, 20x4, der(20)t(X;20), 21x3, 22x1, der(22)t(7;22)[9]/66, idem, +del(18)(?)[3]/idem, -22, +der(22)t(18;22) [2]

SW480-clone-1: 52~59(mode 58), XX, Yx0, 1x1, der(1)t(1;9)‡, 2x2, der(2)t(2;12), 3x2, del(3)(?), 4x2, 5x1, der(5)t(5;20)‡, 6x2, 7x2, der(7)t(7;13), der(7)t(7;14), 8x1, der(8;19), 9x1, der(8;9), der(9)t(1;9)‡, 10x1, der(10)t(10;12)(3;12), 11x3, 12x1, del(12)(?), 13x3, 14x2, 15x2, 16x2, 17x2, del(17)(q?), 18x1, del(18)(q?), 19x1, der(5;8;19)t(5;19)t(8;19), 20x2, der(20)t(5;20)‡, 21x3, 22x2[9]

SW480-clone-2: 88~97 (mode 90), XX, Yx0, 1x4, 2x3, del(2)(?), der(2)t(2;12), der(2)t(2;18), 3x3, 4x3, 5x2, der(5;12), der(5)t(5;20)‡x2, 6x3, 7x3, der(7)t(1;7), der(7)t(7;14)x2, 8x4, 9x3, 10x2, der(10;12)x2, der(10)t(10;15), 11x3, der(11)t(11;15)x2, 12x2, del(12)(?), der(12)t(12;14)‡, 13x5, 14x1, der(14)t(12;14)‡x2, 15x3, 16x3, 17x4,

18x1, del(18)(q?)x3, 19x2, der(5;8;19)t(5;19)t(8, 19)x4, 20x4, der(20)t(5;20)‡x3, 21x5, 22x4[13]

SW620: 45~49 (mode 48), XX, Yx0, 1x2, 2x1, der(2)t(2;12), 3x1, del(3), 4x1, del(4), 5x1, der(5)t(5;20)‡, 6x1, der(6)t(6;7)‡, 7x2, del(7), der(7)t(6;7)‡, 8x0, der(8)t(8;13), der(8)t(8;17), 9x2, 10x1, der(10)t(10;13), 11x3, 12x2, 13x1, 14x2, 15x2, 16x1, der(16)dup(16)t(3;16)t(6;16), 17x2, 18x1, der(5;18), 19x2, 20x2, der(20)t(5;20)‡, 21x2, 22x2[11]/46~47, idem, -X, +der(X;18), -der(5;18), +del(5)[3]

SW837: 38~40 (mode 38), der(X)t(X;5), Yx0, 1x0, del(1)(?), der(1)t(1;8)x2, 2x2, 3x1, der(3)t(3;11), 4x2, 5x2, 6x1, del(6)(?), 7x1, der(7)t(7;19), 8x1, der(8)t(8;17), 9x2, 10x2, 11x1, der(11)t(1;11), 12x2, 13x0, der(13;15), 14x2, 15x1, 16x2, 17x1, 18x1, 19x1, 20x2, 21x2, 22x2[7]/idem, +1, -der(1)t(1;8), -2, + der(2)t(2;17), -der(7)t(7;19), +der(7)t(2;7), -16, +der(16)t(16;20)‡, -20, +der(20)t(16;20)‡ [5]

VACO4A: 61~65 (mode 62), XX, Yx0, 1x2, i(1)(q10), 2x3, 3x2, der(3)t(3;10)(q10;q10), 4x2, 5x3, 6x2, 7x4, 8x2, i(8)(q10)x2, der(8;20)(q10;?)x2, 9x3, 10x2, 11x3, 12x3, 13x2, der(13)dup(13)(q?)t(13;15)x2, 14x4, 15x1, 16x2, 17x2, der(10;17), 18x2, 19x2, 20x2, 21x2, 22x2[5]/idem, -der(10;17), +der(4;17)[4]/idem, -X, +der(X)t(X;2)[3]/idem, -9, +del(9)(q?)[2]/idem, -20, +dup(20)(?)[2]

Typical RER+ cell lines

DLD1: 43~46 (mode 46), XY, dup(2)(p13p23), der(6)t(6;11)[14]/ idem, -1, +dup(1)(p?)[4]

GP2d: 45~47 (mode 46), XX, del(3)(?), del(5)(q?), der(6)t(3;6), dup(10)(q?)[12]

HCT116: 43~45 (mode 45), X, Yx0, der(10)dup(10)(q?)t(10;16), der(16)t(8;16), der(18)t(17;18)[18]

LS174T: 46~47 (mode 47), X, +7, +15[14]

VACO5: 43~47 (mode 46), XX[12]/idem, +del(7)(?)[2]/idem, -21, +del(21)(p?)[4]

Atypical RER+ cell lines

LoVo: 48~50 (mode 49), XY, der(2)t(2;12)‡, +5, +7, +der(12)t(2;12)‡, i(15)(q10)[12]

HCA7: 42~43(mode 43), der(X)t(X;4)(p22;q25-26)‡, 1x1, der(1)del(q)t(1;16)(p13;p11.2)‡, 2x1, der(2)t(2;11)(q14-21;q21)‡, 3x0, del(3)(p13p21), der(3)t(1;3)(?p36;q27-29)‡, 4x1, der(4)t(X;4)(p22;q25-26)‡, 5x2, 6x0, der(6)t(6;7)(q21-22;q31)‡, der(6)t(6;18)(q13-15;q11.2)‡, 7x1,
der(7)t(6;7)(q21-22;q31)‡, 8x2, 9x1, der(9)t(9;21)(p12-13;q11.2), 10x2, 11x1, der(11)t(2;11)(q14-21;q21)‡, 12x1, dup(12)(q?), 13x1, 14x1, der(14)t(14;14)(p11.2;q13-21), 15x1, del(15)(?q12q15), 16x1, der(16)t(1;16)(p13;p11.2)t(1;3)(p36;q27-29)‡, 17x2, 18x1,
der(18)t(6;18)(q13-15;q11.2)‡, 19x2, 20x2, 21x1, 22x2[14]/ 40~45, idem, +2, -der(2)t(2;11)(q14-21;q21), -der(3)t(1;3)(?p36;q27-29), +der(3;14), +6, -der(6)t(6;7)(q21-22;q31), +7, -der(7)t(6;7)(q21-22;q31), -10, +der(10)t(3;10), +11,

-der(11)t(2;11)(q14-21;q21), +14, -der(14)t(14;14)(p11.2;q13-21), -der(16)t(1;16)(p13;p11.2)t(1;3)(p36;q27-29), +der(16)t(1;16)(p13;p11.2)‡ [7]/ 48, idem, +der(4)t(X;4)(p22;q25-26)‡, +der(7)t(6;7)(q21-22;q31)‡, -del(15)(?q12q15), +der(15;20), +der(16)t(1;16)(p13;p11.2)‡, +17, +17, -20, +22[3]

LS411: 70~76 (mode 73), X, der(X)dup(X)t(X;5), Yx0, 1x3, del(1), 2x3, 3x3, del(3), 4x3, 5x3, del(5), 6x2, 7x2, dup(7), der(7)dup(7)t(7;12), 8x3, der(8;22), 9x3, del(9), 10x3, 11x3, 12x3, del(12), 13x2, i(13)(q10), 14x1, i(14)(q10), 15x3, 16x3, 17x3, del(17), 18x2, del(18), 19x3, dup(19)(p?), 20x3, 21x1, der(12;21), 22x2, der(6;22)[8]/idem, -del(1), +del(6), +der(10, 17), -dup(19)[3]/idem, -del(1), -der(6;22), +dup(6), +der(6)t(5;6), +del(11)(q?)[3]

Footnote to Table 3.2:

Representative summary karyotypes of the cell lines as obtained by SKY analysis and confirmed by single-dye chromosome painting. For the simple diploid lines, DLD1, GP2d, HCT116, LoVo, LS174T, and VACO5, karyotypes are described according to the standard ISCN (International System for Human Cytogenetic Nomenclature) (Mitelman, 1995), as deviations from diploid. The more complex karyotypes are presented more explicitly as: range of chromosomes per metaphase, (mode), content of normal sex chromosomes, listing of all chromosomes with copy numbers, [number of metaphases with the given composition]. Additional clones are separated by /. Isochromosomes, deletions and duplications are reported where they were evident from size changes; and confirmed by the DAPI image. Breakpoints given for C70, HT29 and HCA7 were judged by cytogenetic banding analysis of the DAPI image following SKY (cytogenetic identification of such breakpoints was according to the advice of an expert cytogenticist [Mrs Patricia Gorman]).

*deletion of 2p at different points with possibility of dup(2)(q?), †tentatively identified as der(13;13)(q10;q10)del(q14q34)t(13;13)(?q14;?q34), ‡apparently balanced translocation.

Figures 3.1 - 3.18: Karyotypes of the cell lines determined by SKY with confirmation by single-dye chromosome painting FISH

Illustration of how karyotypes were produced are supplied with C70 and HT29 (Figure 3.1 B, C, D & 3.2 A, B, C) as explained in the text (see 3.2). SKY classified (pseudo-colour) images of representative metaphases for each of the 17 cell lines are presented in Figure 3.1- 3.18: C70-a, HT29-d, LIM1863, SW1417, SW403, SW480-clone-1, SW480-clone-2, SW620, SW837, VACO4A, DLD1, GP2d, HCT116, LS174T, VACO5, LoVo, HCA7, LS411. These are typical metaphases that may not show all the numerical or structural changes to chromosomes described in Table 3.2. The HCA7 metaphase shown represents the most complex clone. The metaphases for C70, SW1417, and SW837 show rearranged chromosomes unique to that metaphase, t(1;2), der(11)t(X;11), and der(5)t(2;5) respectively, and accordingly these are not recorded in Table 3.2. The chromosomes are shown in classification colours, i.e. each pixel is assigned a colour representing the chromosome that the software has identified from the fluorescence at that position. Satellites at chromosomes 13, 14, 15, 21, 22 as well as pericentromeric heterochromatin (e.g. at chromosome 1) hybridise non-specifically so they are often miscoloured. Illustrative examples of the single-dye chromosome painting FISH are shown. Fig 3.1 C70 E shows a whole C70 metaphase hybridised to chromosome 10 green paint and chromosome 16 red paint showing the rearranged chromosome der(16)t(10;16). Figure 3.2 HT29 F shows use of chromosome 10 green paint and chromosome 22 red paint to prove that the chromosome 10 strip, which appears in the translocation derivative der(22)t(17;22) in Fig 3.1 HT29 D, is just misclassification due to overlap. Fig 3.1 C70 F shows rearranged chromosome der(17)t(6;17), also shown in Fig 3.1 A, in which the right image displays SKY identification of this marker showing that overlap of the fluorescence at the junction between the fragments of chromosomes 6 and 17 creates the fluorescence spectrum of chromosome 10 such that an artefactual thin stripe of chromosome 10 is mistakenly assigned to this region by the software. In contrast, left image shows hybridization with chromosomes 6 paint (red), and 17 paint (green) whereas chromosome 10 paint did not hybridize (not shown). Fig 3.1 C70-G shows C70 rearranged chromosome der(4)t(4;18) also shown in Fig 3.1 A in which the left image hows the translocation as it was originally identified as t(1;4) by the software as the fragment of 18 is very small and its fluorescent signal blends with the adjacent chromosome 4 fluorescence such that the SKY soft ware mistakenly assigned a fragment of chromosome 1 to this region. In contrast, right image shows the correct identification of this very small chromosome fragment using the single-dye chromosome FISH with chromosome 4 green paint and 18 red paint thus establishing it as t(4;18), whereas chromosome 1 paint did not hybridize. The same interpretation applies to Fig 2 HT29-E showing the rearranged chromosome der(2)t(1;2) with the left image showing the overlap at the peri-centromeric region creating two artefactual strips of chromosome 6 and 10 whereas the right image shows correct hybridization with chromosome 1 green paint and chromosome 2 red paint whereas chromosome 6 and 10 paints did not hybridize.

Figure 3.1 C70 A

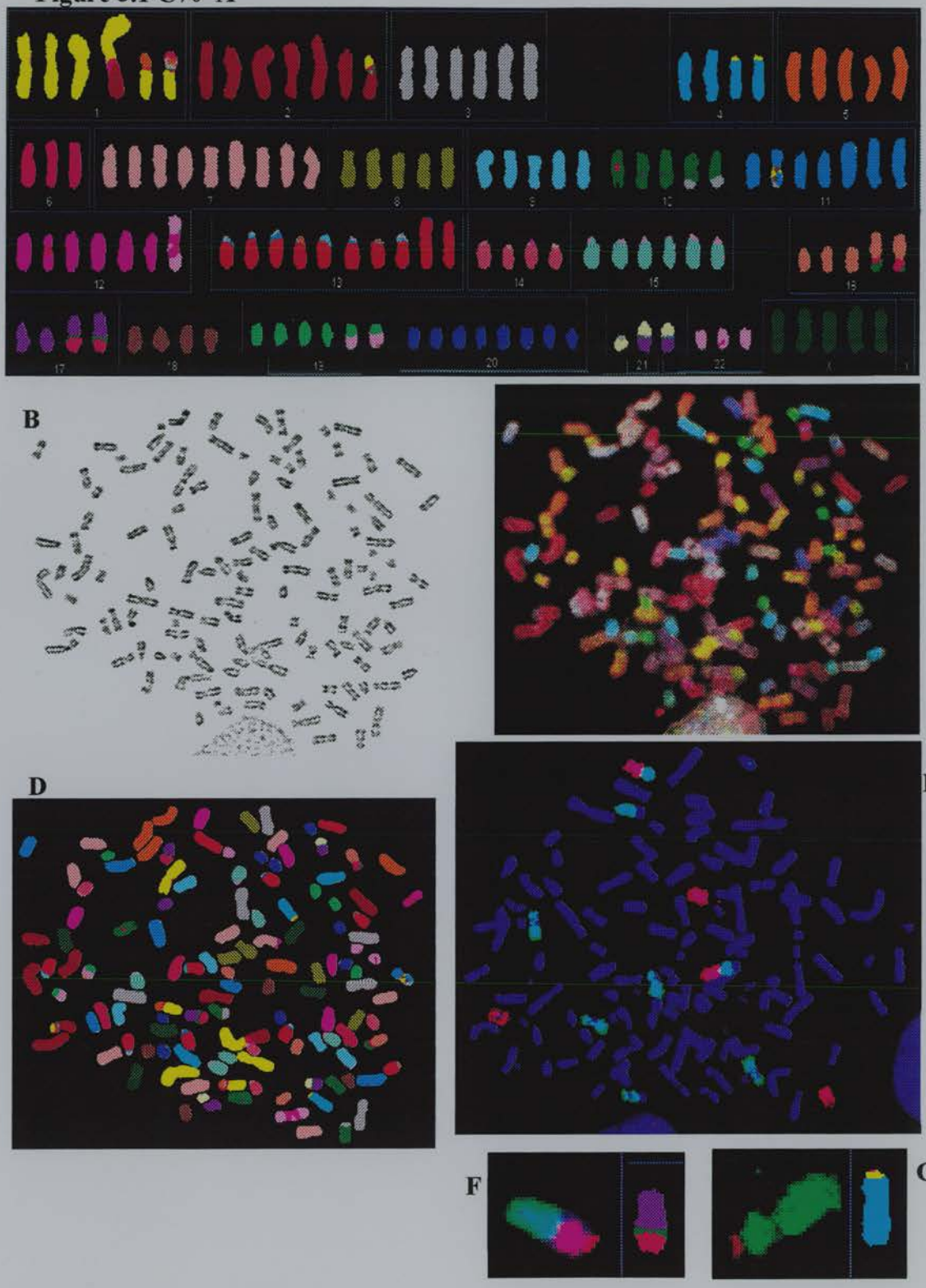
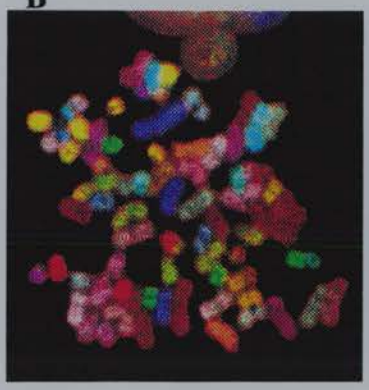


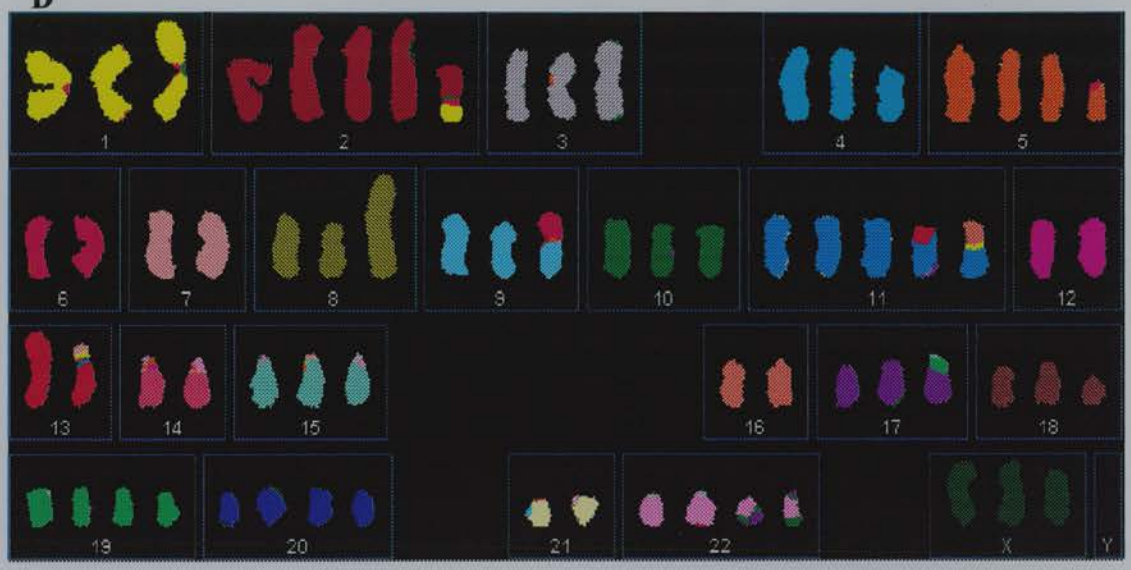
Figure 3.2 HT29 A

B

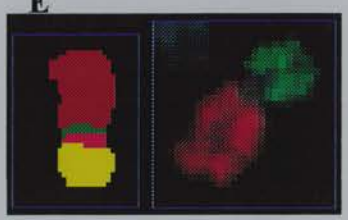


C

D



E



F

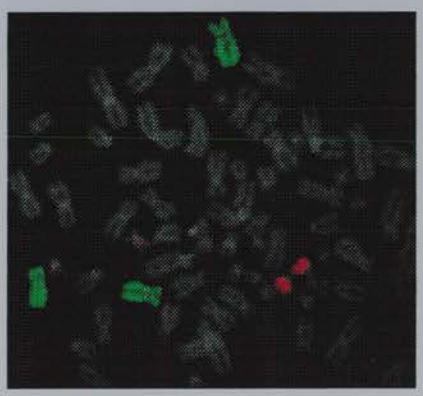


Figure 3.3 LIM1863

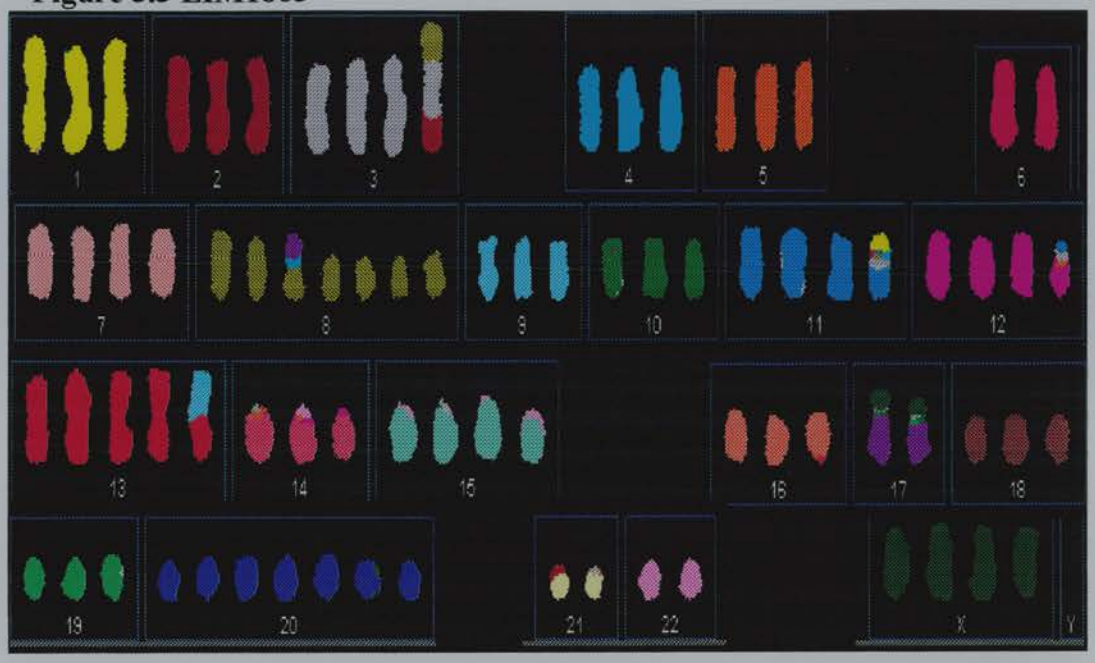


Figure 3.4 SW1417

Figure 3.5 SW403

Figure 3.6 SW480-clone-1

Figure 3.7 SW480-clone-2

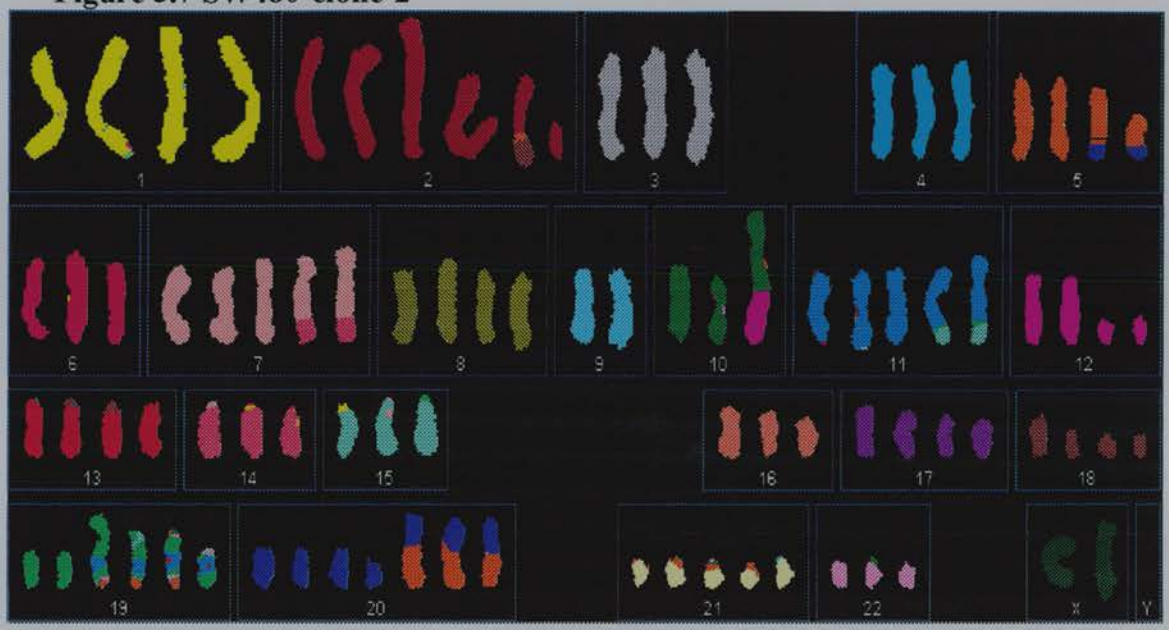


Figure 3.8 SW620

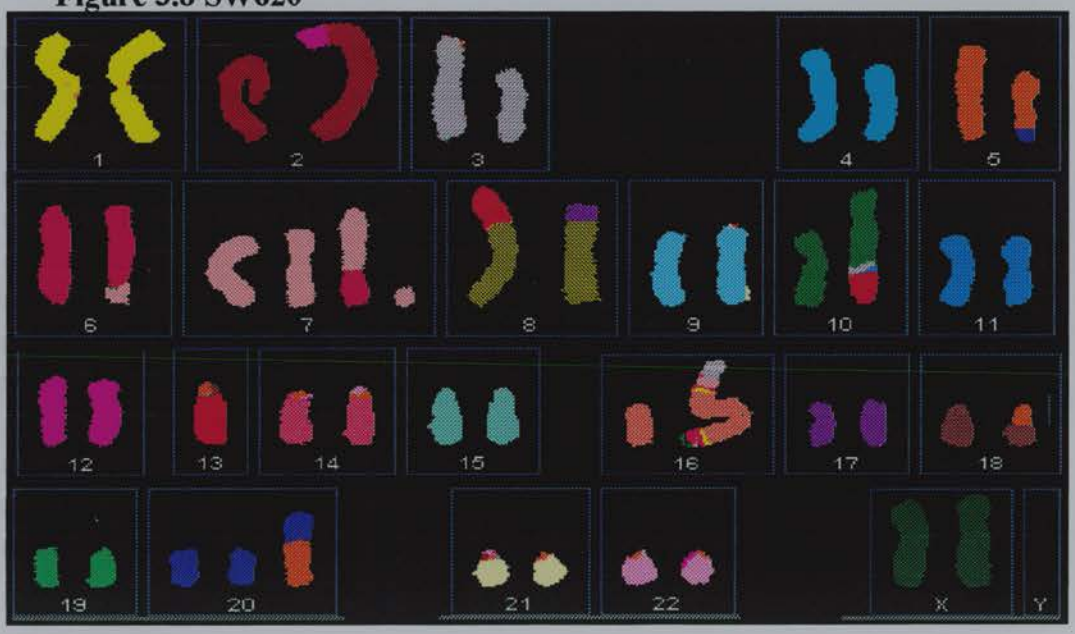


Figure 3.9 SW837

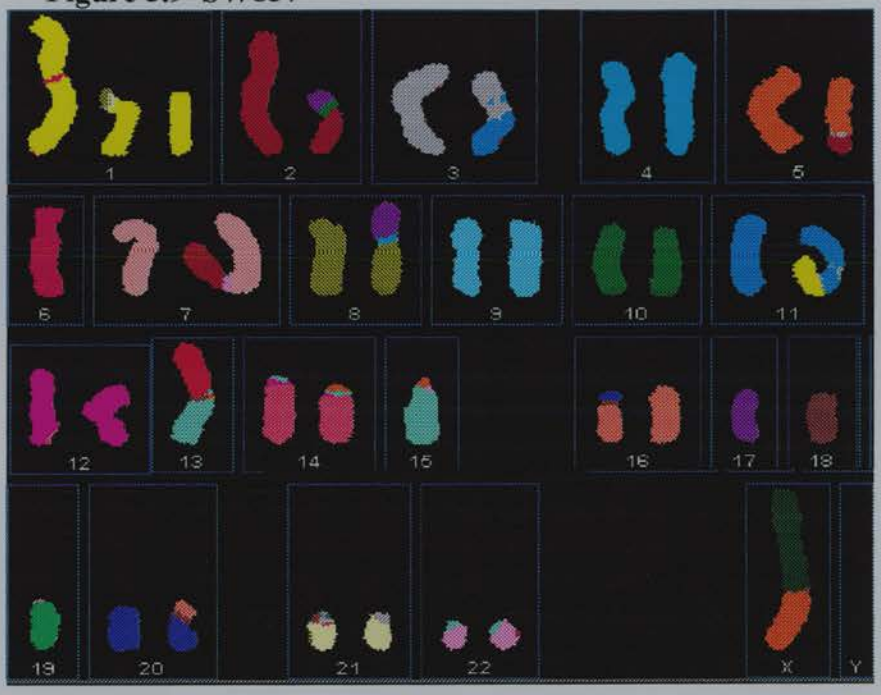


Figure 3.10 VACO4A

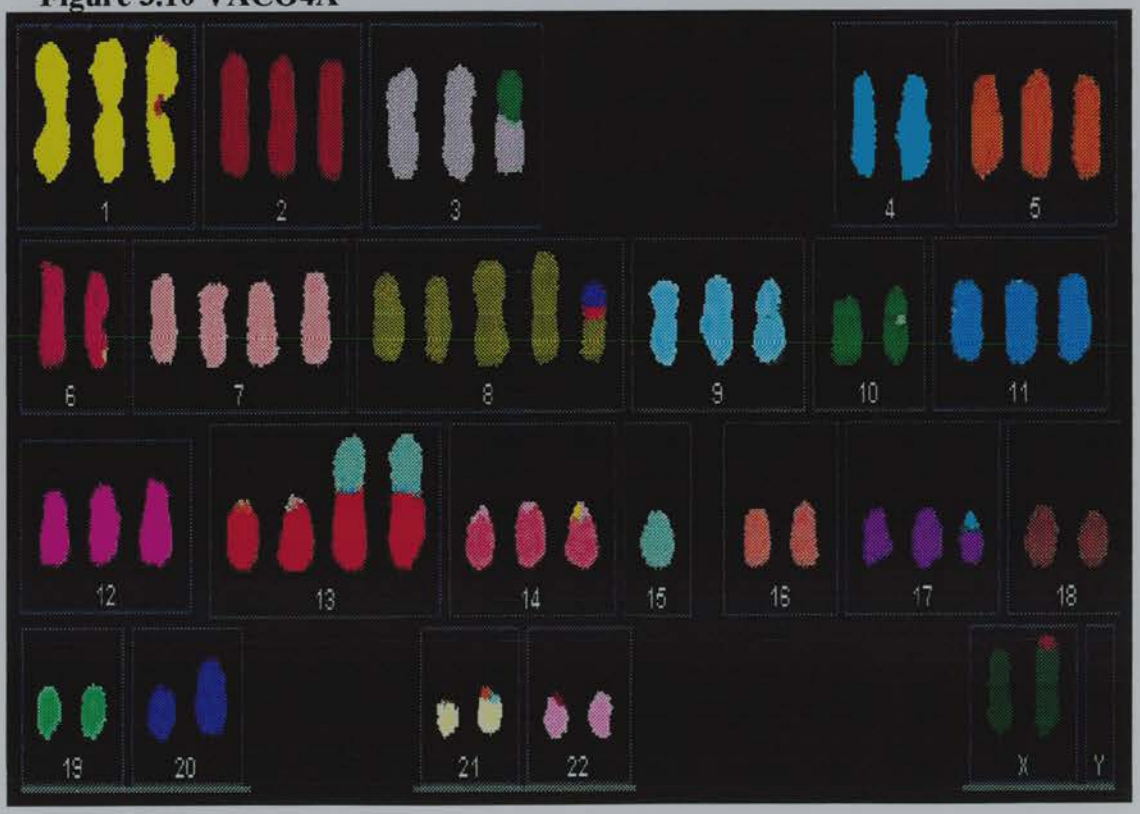


Figure 3.11 DLD1

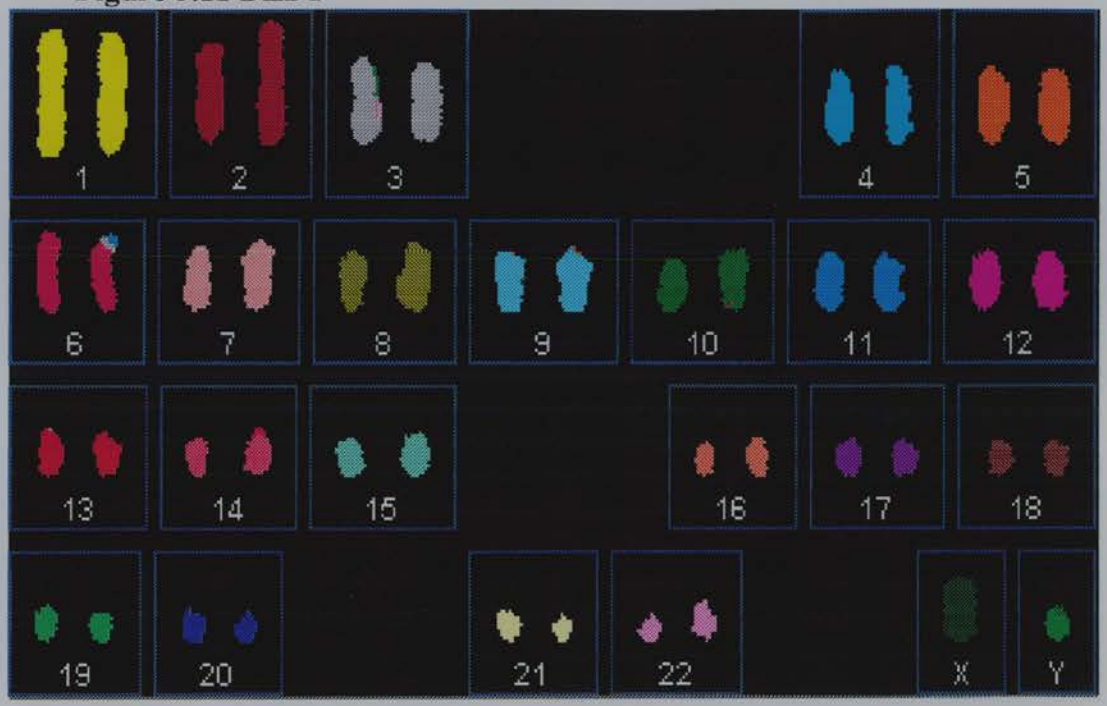


Figure 3.12 GP2D

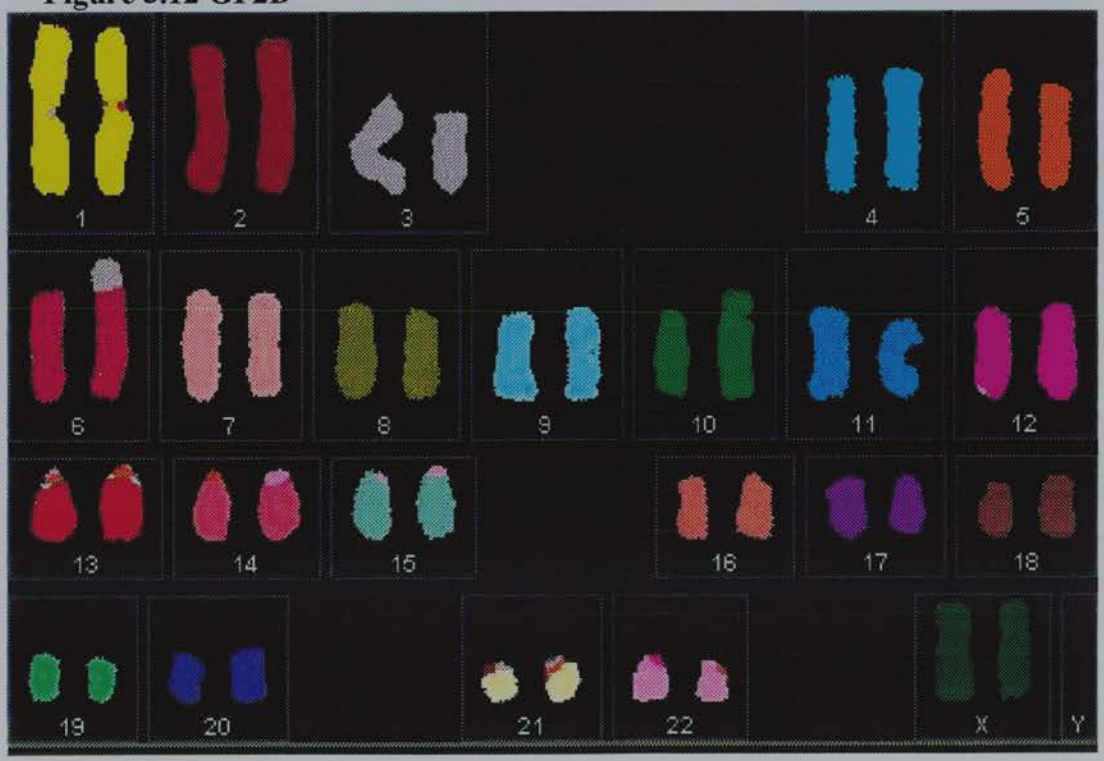


Figure 3.13 HCT116

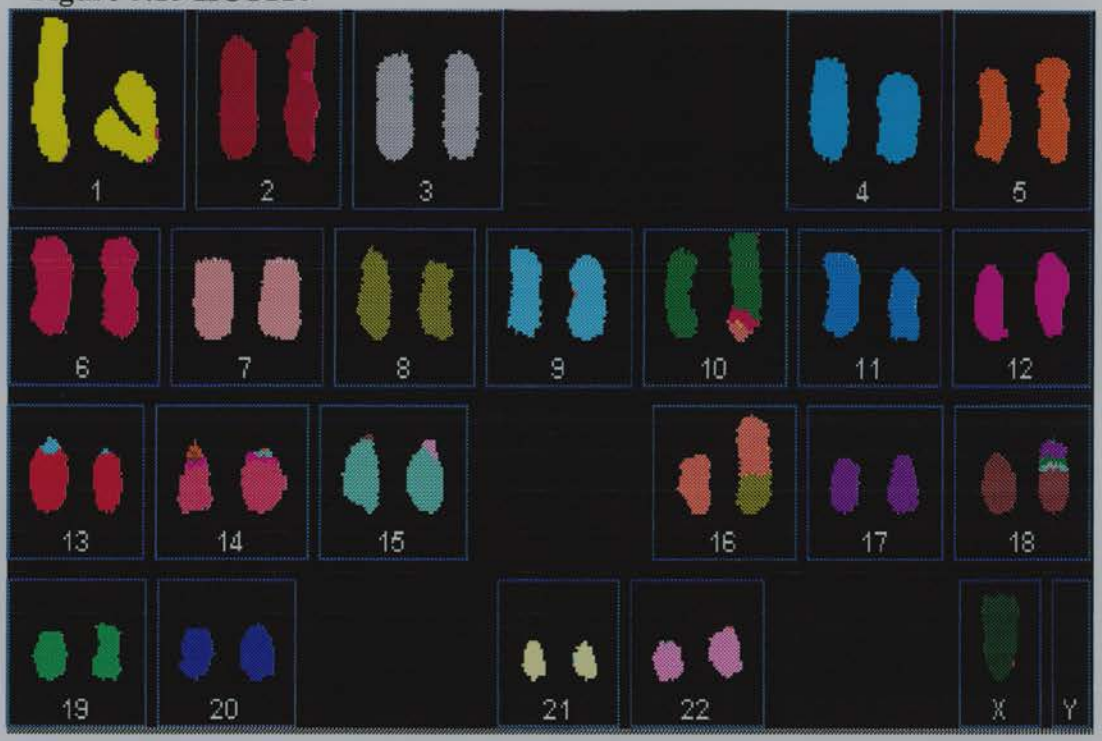


Figure 3.14 LS174T

Figure 3.15 VACO5

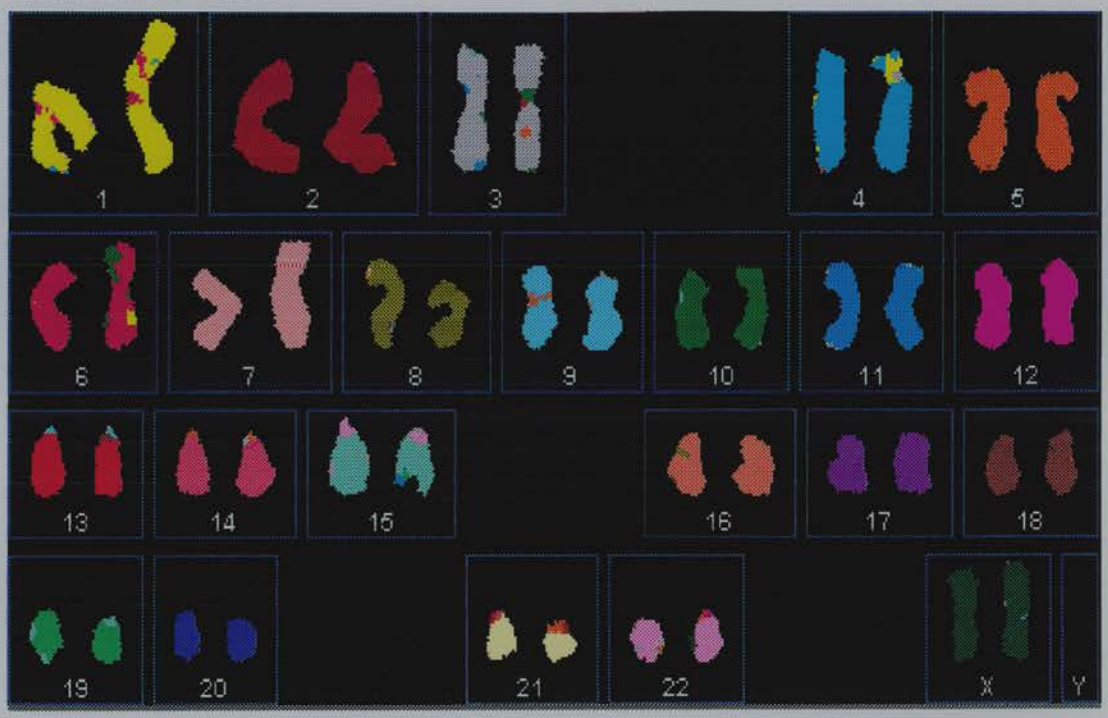


Figure 3.16 LoVo

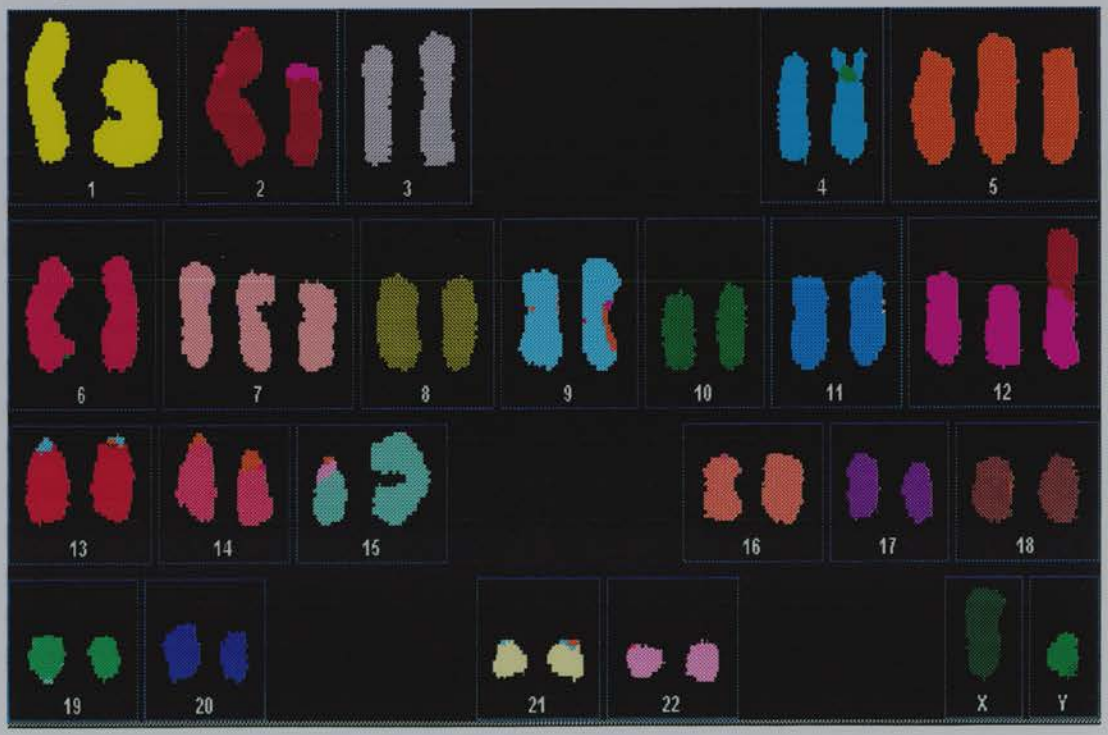


Figure 3.17 HCA7

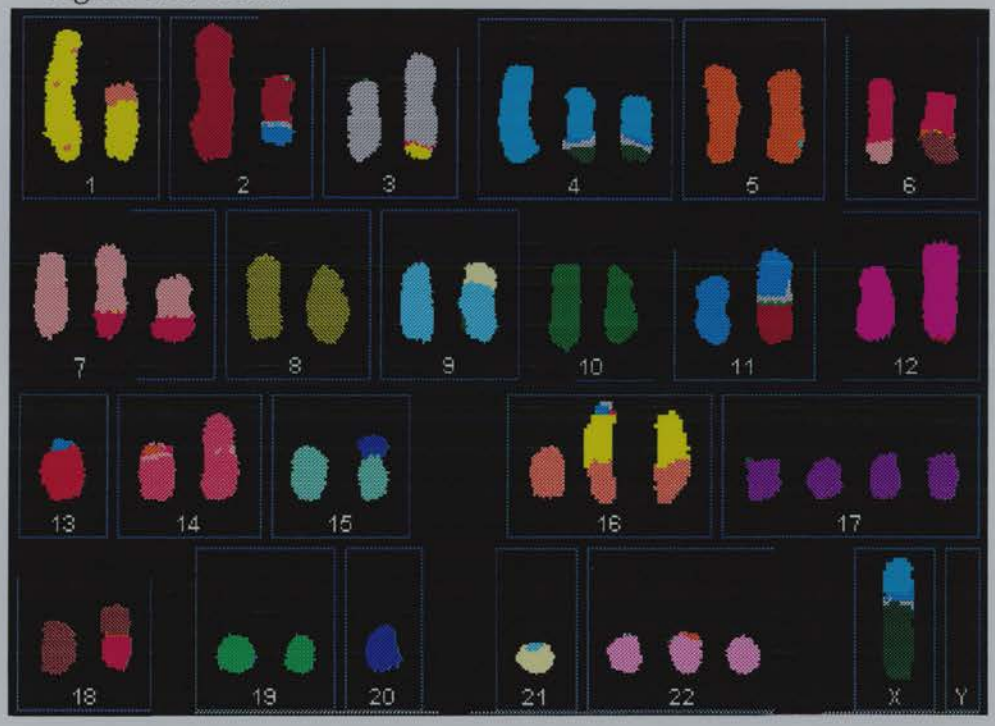
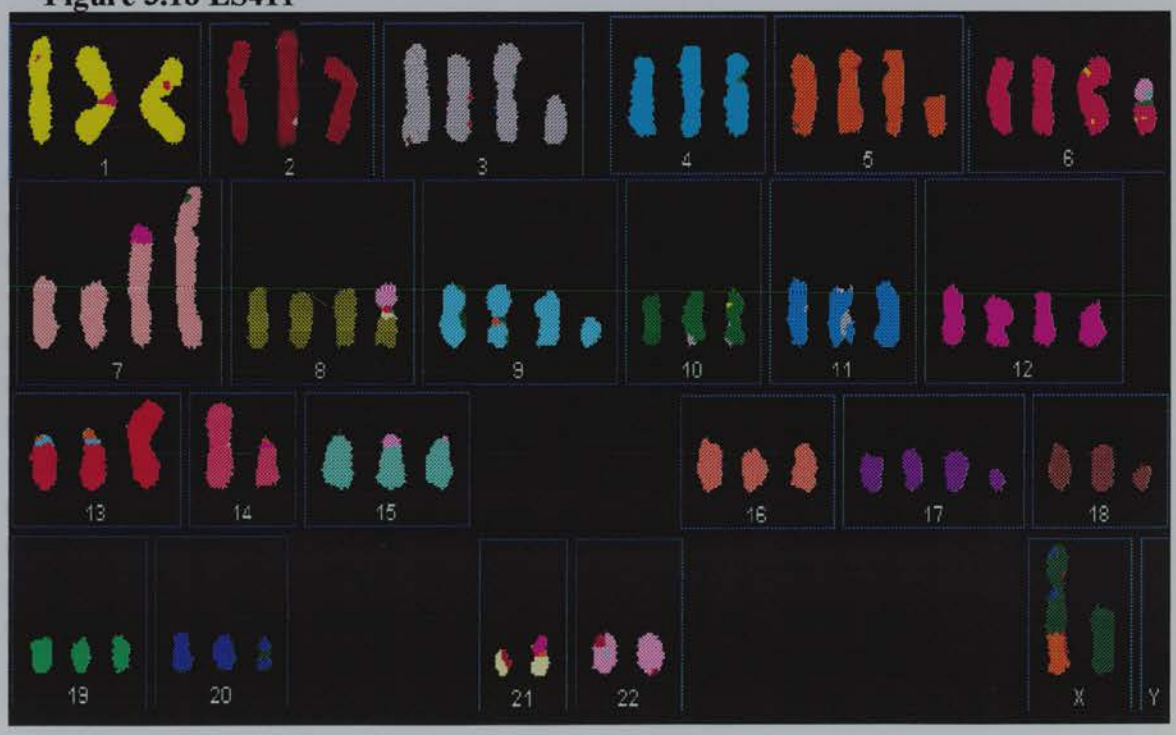


Figure 3.18 LS411



3.3.2. SKY karyotypes are consistent with previously published cytogenetic karyotypes

The karyotypes of thirteen of these cell lines have previously been analysed by cytogenetic banding. The SKY karyotypes obtained here are generally consistent with previously published cytogenetic reports. The matching was high for the simpler karyotypes. In one instance the difference was clearly due to difference acquired through further *in vitro* passaging (LoVo), while in other cases it was due to the SKY revealing the origin of cytogenetically unidentifiable chromosome pieces. In almost all cases of chromosomal rearrangements SKY added more information about the nature of the rearranged chromosomes.

Table 3.3: Comparison of the SKY profiles with the published cytogenetic reports on the same lines

Cell line	Publication(s)	Degree of Matching
HT29	(Yaseen *et al.*, 1990)	B (4y,5n,6ui)
LIM1863	(Whitehead *et al.*, 1987)	A (5y, 0n, 1ui)
SW403	(Chen *et al.*, 1982)	B (4y, 5n, 2ui)
SW480 (both clone 1 and 2)	(Tomita *et al.*, 1992)	A (2 clones identified, 11y,1n,11ui)
	(Yaseen *et al.*, 1990)	B (2 clones not identified, 6y,3n,2ui),
	(Chen *et al.*, 1982)	C (2 clones not identified, 6y,15n,3ui)
SW620	(Gagos *et al.*, 1995); (Gagos *et al.*, 1996)	A (7y,3n,3ui)
SW837	(Chen *et al.*, 1982)	A (7y, 3n, 1ui)
DLD1	(Chen *et al.*, 1995)	B (1y, 0n, 1ui)
GP2D	(Solic *et al.*, 1995)	B (2y, 2ui)
LS174T	(Rutzky *et al.*, 1980)	A (normal karyotype)
VACO5	(McBain *et al.*, 1984)	A (the almost normal karyotype with 2 rare markers described)
LoVo	(Drewinko *et al.*, 1976)	A (2y, 0n, 1ui)
	(Chen *et al.*, 1982)	A (2y, described other rare sets of markers in addition)
	(Soulie *et al.*, 1999)	A (3y, described two other balaned translocations)
HCA7	(Kirkland, 1985), (Kirkland & Bailey, 1986),	Not described in any of them
	(Perry *et al.*, 1999)	Just showed pictures without description of the markers
LS411	(Suardet *et al.*, 1992)	A (6y, 1n, 2ui)

One of three matching bands (A, highly matching; B, moderately matching, C, poorly matching) is assigned for each publication. For some of these cell lines there is more than one cytogenetic report available, with a total of 20 cytogenetic reports on 13 cell lines. 11 fall into band A, 5 fall into band B, and 1 falls into band C. Three cytogenetic attempts to characterise HCA7 by conventional cytogenetics all failed to describe the balanced translocation profile in this line.

y: marker described partially or completely correctly by cytogenetics, n: marker described completely incorrectly by cytogenetics, ui: unidentified markers described by cytogenetics which may match one of the markers fully identified by SKY. The matching bands (A, B, or C) are assigned as follows: band A refers to lines with greater than one third of the total markers being y and no more than one third of the total markers being n; band B indicates lines with less than or equal to one third of the total markers being y and less than or equal to one third of the total markers being n with up to one third being ui; and band C indicates lines with more than one third of the total markers being n.

3.3.3 Patterns of chromosomal changes in the cell lines are similar to those of uncultured tumours as determined by CGH

Samples of all of the 17 colorectal cancer cell lines of similar, or very close, passage number to those used for SKY analysis were supplied to Dr Kanade Katsura, a colleague in the same laboratory, for CGH analysis. The CGH profiles of these lines (kindly supplied by Dr Kanade Katsura) were very similar to those obtained from uncultured primary colorectal tumours (Figure 3.19). CGH data were compared with published surveys of primary uncultured material (Ried *et al.*, 1996; DeAngelis *et al.*, 1998; Meijer *et al.*, 1998; AlMulla *et al.*, 1999; Korn *et al.*, 1999), and RER- tumours and first-pass xenografts of colorectal cancers (Georgiades *et al.*, 1999; Curtis *et al.*, 2000). Common changes were very similar, the lines showing frequencies of gain or loss one to two times that in the primary uncultured material. The only exception was that chromosome 6, particularly 6q, was lost in 8/9 RER- cell lines, but very rarely lost in primary uncultured material, and therefore this may be a change selected in culture. The RER+ lines showed few chromosomal changes detected by CGH the only recurring one being gain of chromosome 7, detected in 3 out of 8 lines. The outstanding feature is the cluster of frequently changed chromosome arms that are common to the lines and primary tumours: 7+ ,8p-, 8q+, 13+, 17p-, 18-, 20+ (Figure 3.19).

Agreement between the SKY-determined karyotype and chromosomal changes detected by CGH was good, as expected (Ghadimi *et al.*, 1999; Macville *et al.*, 1999; Ghadimi *et al.*, 2000). Where chromosomes were unrearranged, the SKY-determined karyotype independently confirmed the CGH estimate of copy number. For many of the translocations, the CGH copy number changes complemented the SKY data by suggesting which parts of the chromosomes were involved in the translocations (Ghadimi *et al.*, 1999; Macville *et al.*, 1999; Ghadimi *et al.*, 2000). For example, the cell lines with relatively simple karyotypes, HCT116 had three translocations involving chromosomes 8, 10,16,17 and 18. CGH (Figure 3.20) showed gains of parts of the long arms of 8, 10, 16 and 17, and no losses identifying these as extra fragments incorporated into the translocations. Similarly, as already reported by (Ghadimi *et al.*, 2000), DLD1 showed an enlarged chromosome 2 and a t(6;11) translocation, and CGH (Figure 3.21) showed regions of increase, at approximately 2p13-21 and 11p15. In some metaphases there was also an enlarged chromosome 1 seen by SKY, and an increase close to 1pter was detected by CGH. In GP2d (Fig. 3.22), CGH showed local loss around the *APC* locus on chromosome 5, corresponding to the short chromosome 5 in the SKY-determined karyotype, while

chromosome 3 shows no CGH changes, suggesting that the del(3) and t(3;6) translocation are balanced. As expected, the balanced translocations in HCA7 was not detected as gains or losses by CGH (Figure 3.23). Some translocation breakpoints deduced this way were in roughly the same position in more than 3 cell lines: 1p10, 6q22, 7p10, 8p10, 8q22, 13q22, 18p10 and 20p12.

Figure 3.19

Comparison of CGH data between cell lines and (**A**) primary uncultured tumours including both RER- and RER+ tumours; and (**B**) selected RER- tumour xenografts. CGH data are expressed as percentage of tumours, xenografts or lines showing either gain or loss at a chromosome arm. Two comparisons have been made. In **A**, pooled data from studies of primary uncultured tumours, unselected for RER status (Ried *et al.*, 1996; DeAngelis *et al.*, 1998; Meijer *et al.*, 1998; AlMulla *et al.*, 1999; Korn *et al.*, 1999), are compared with data from this study, combining the RER+ and RER- cell lines in the ratio 2:8 to mimic unselected primary uncultured tumours. In **B,** data from RER- tumours and first-pass xenografts (Georgiades *et al.*, 1999; Curtis *et al.*, 2000) obtained in one of our laboratories using the same CGH criteria as for the cell lines, are compared with the CGH data from the RER- cell lines. Numbers of changes in RER+ tumours were too small for any meaningful comparison to be made.

Figure 3.19 A: Comparison of CGH data between cell lines and surgical material (primary tumours).

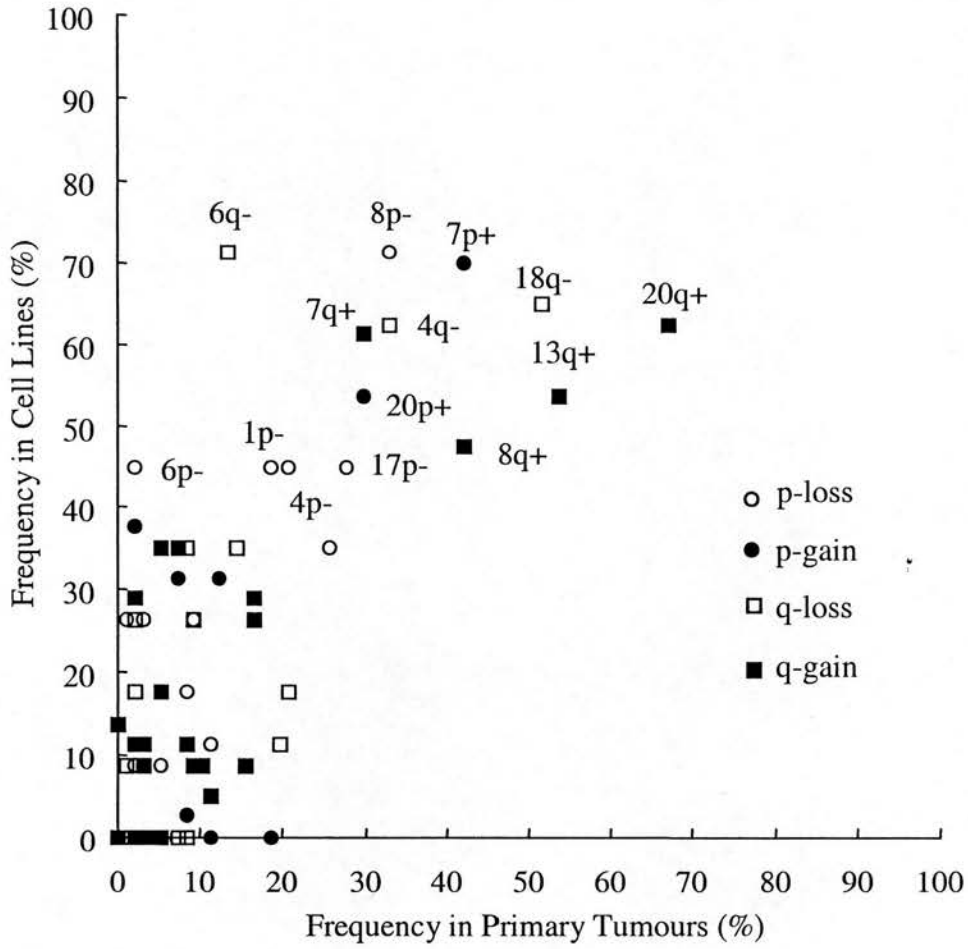
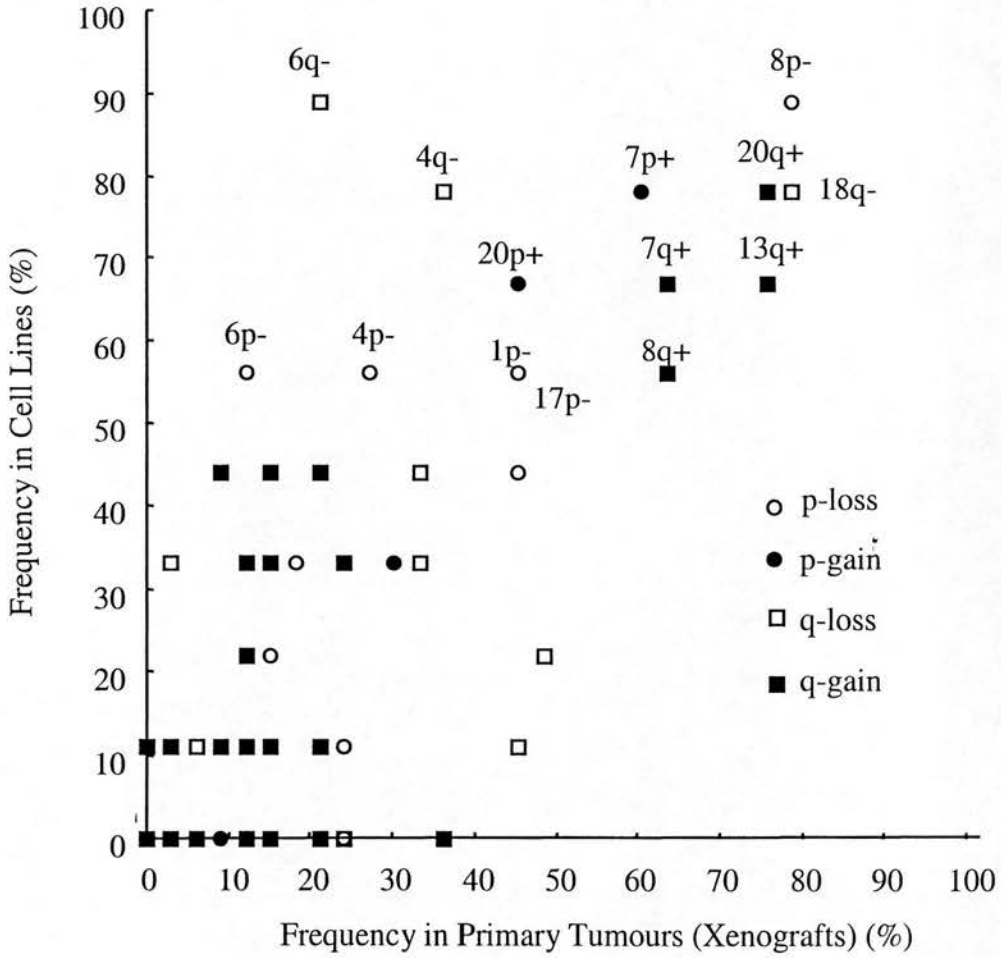


Figure 3.19 B: Comparison of CGH data between cell lines and selected RER- tumour xenografts.



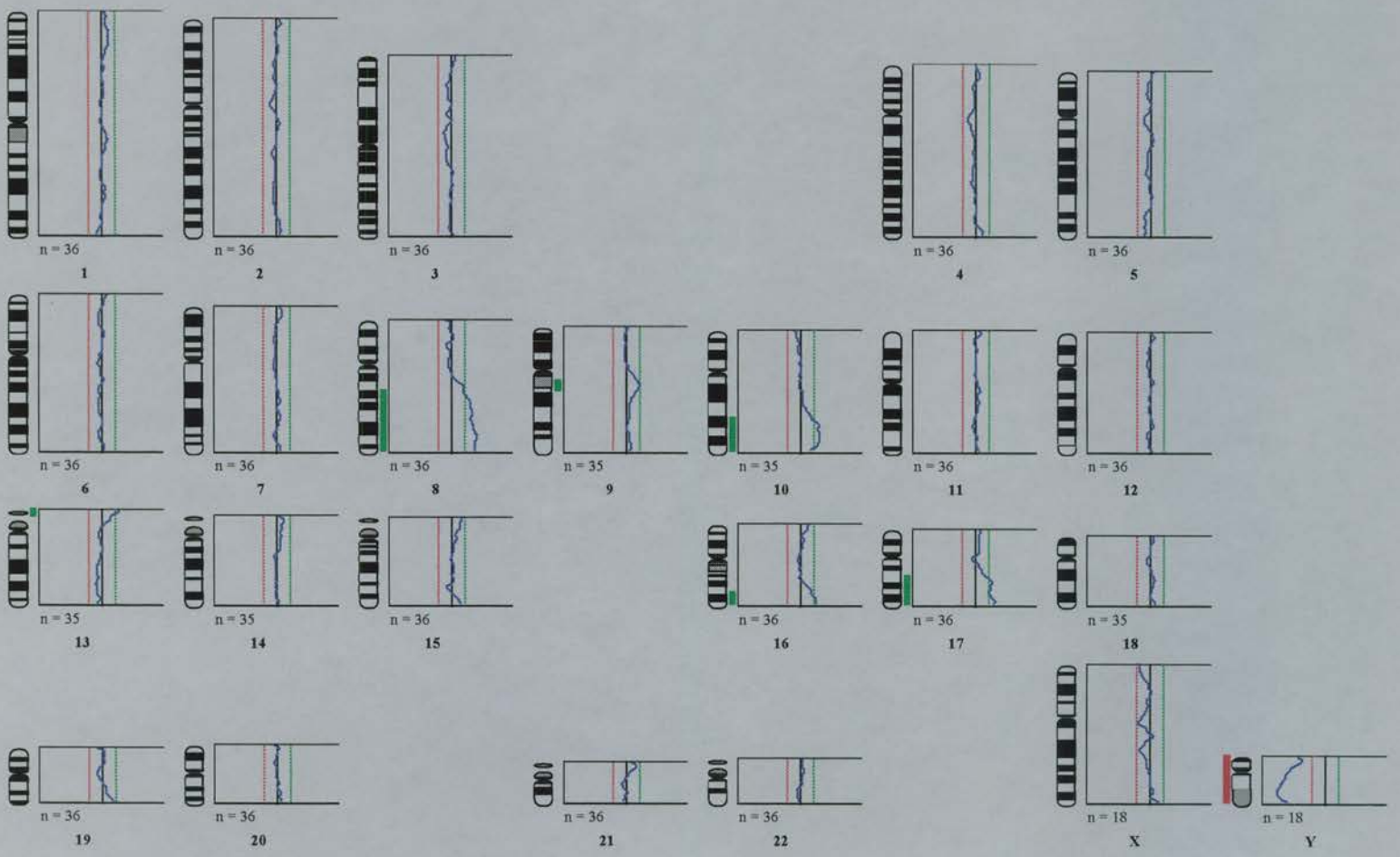


Figure 3.20: CGH ratio profiles of HCT116

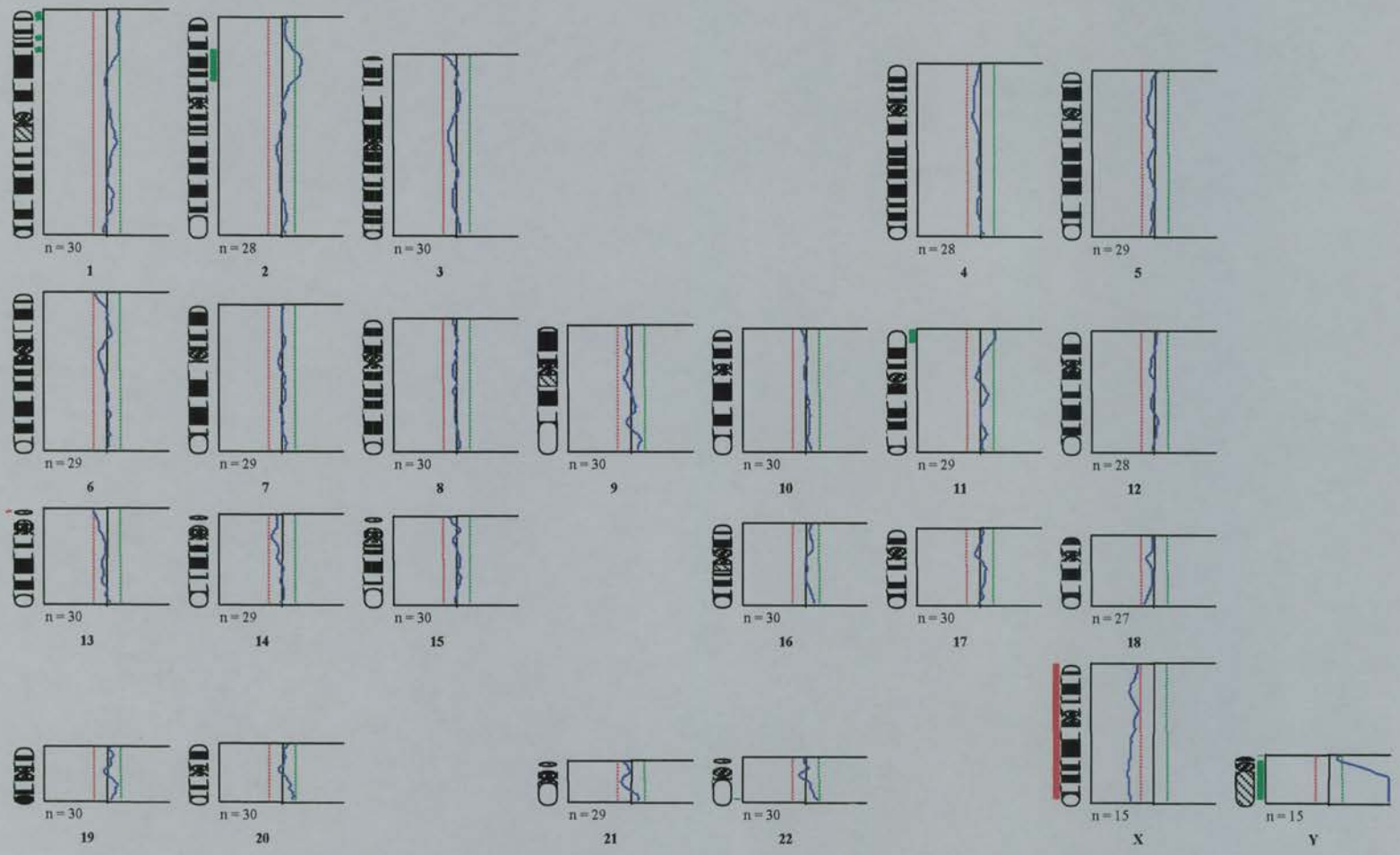


Figure 3.21: CGH ratio profiles of DLD1

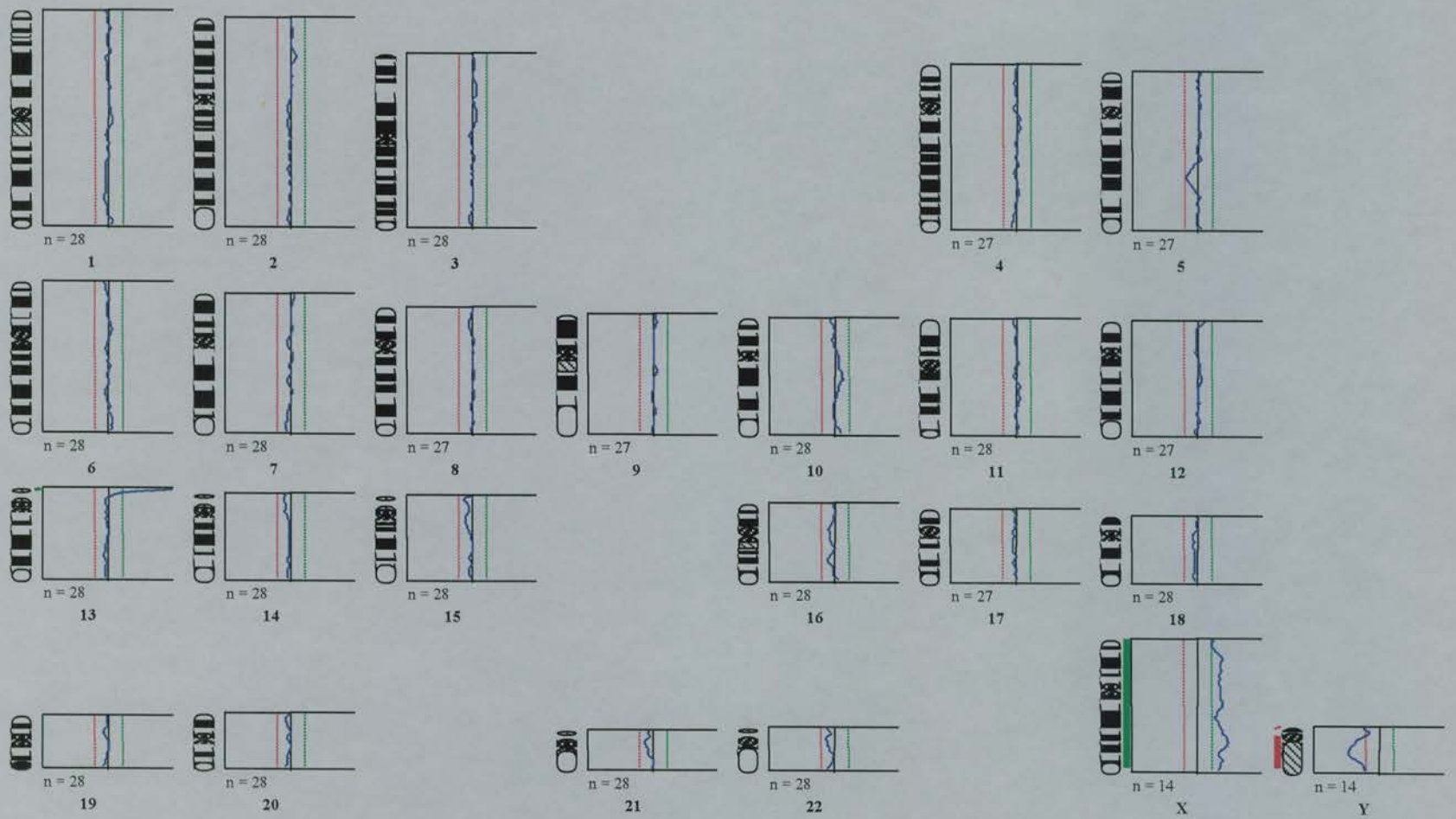


Figure 3.22: CGH ratio profiles of GP2d

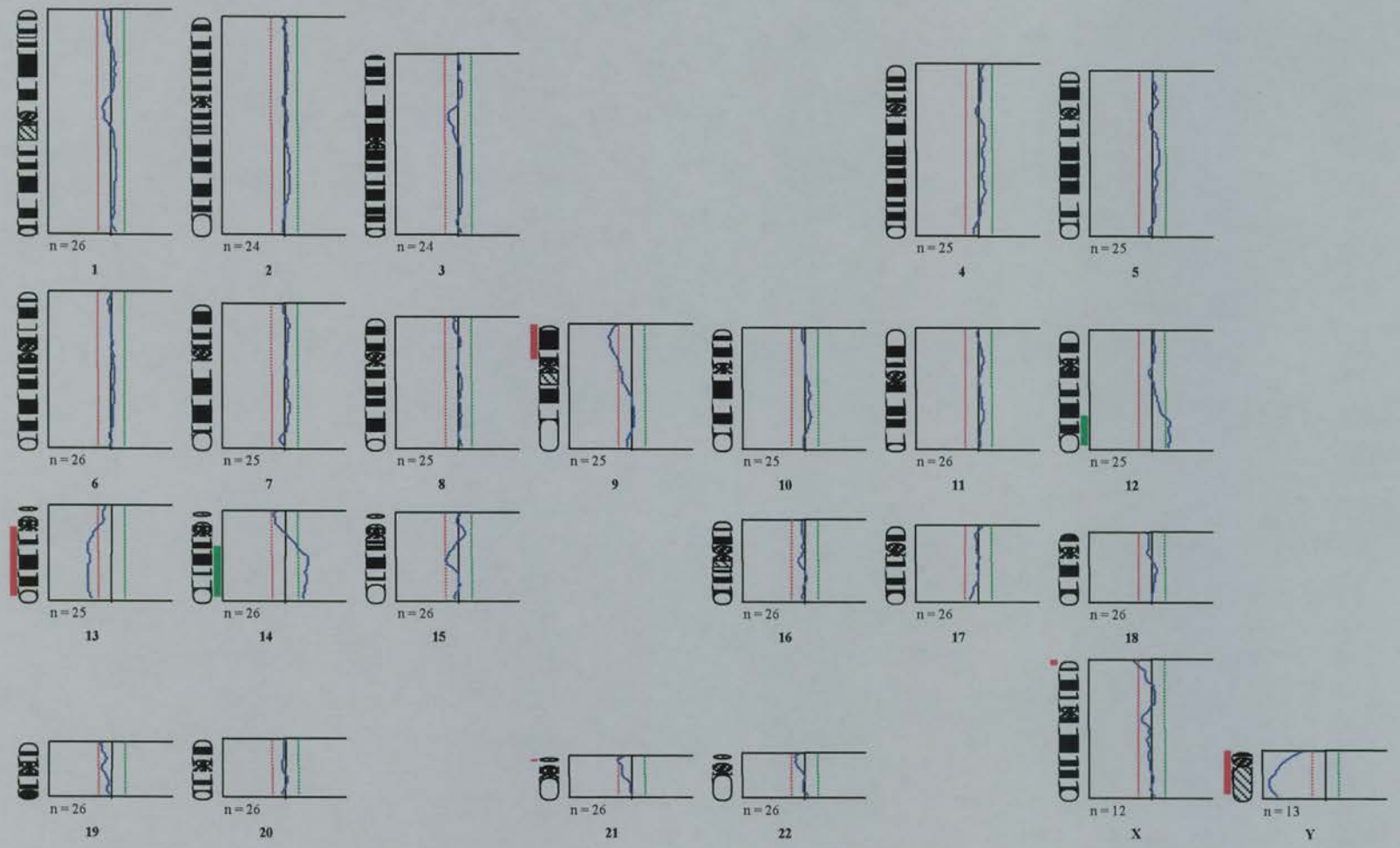


Figure 3.23: CGH ratio profiles of HCA7

3.3.4. Comparison of RER+ with RER- karyotypes

Striking differences between the RER+ and RER- tumour cell line karyotypes were found in terms of both structural and numerical chromosomal changes, as well as metaphase to metaphase heterogeneity.

3.3.4.1 RER- tumour cell lines

The RER- cell lines (Table 3.2) all had very abnormal karyotypes. They were usually near triploid or polyploid, always with evidence of double strand breaks in the form of multiple unbalanced translocations, deletions, duplications, and iso-chromosome formation. All RER- cell lines had numerous (8 to 21 each) structural chromosome rearrangements, suggesting tolerance of breakage or inappropriate recombination. The overwhelming majority of translocations were unbalanced, as noted before for common carcinomas (Dutrillaux, 1995; Davidson *et al.*, 2000) and they were also diverse in site. All chromosomes, except the Y chromosome, were translocated at least occasionally, and they were translocated to a wide range of partners. No particular translocation, however, was present in a substantial proportion of the lines: the most frequent was t(8;17), present in only three of the lines, but even these may have different breakpoints. t(8;17) was also found in some breast carcinoma cell lines; (Davidson *et al.*, 2000).

Almost all the cell lines of RER- tumours had very abnormal modal chromosome numbers. SW837 had a sub-diploid mode of 38; five cell lines (HT29, LIM1863, SW1417, SW403, VACO4A) had modes in the range 62-80; SW480 had two clones with modes of 58 and 90; and C70 was near hexaploid (mode 127). Only SW620 had a near-diploid mode of 48, but it had many structural rearrangements. The chromosomal abnormalities were scored numerically as indices to allow comparison between cell lines (Table 3.4), measuring structural chromosomal changes as a break index (BI) and the numerical chromosomal changes from diploid as a numerical aneuploidy index (NI)—both indices were versions of those described by Eshleman (1998) (Eshleman *et al.*, 1998).

There was marked metaphase-to-metaphase heterogeneity both for normal and rearranged chromosomes, suggesting ongoing instability in most of the RER- tumour cell lines. In this work, it was not possible to distinguish instability from lack of selection against occasional variants because the lines were not cloned. 8/9 RER- lines had more than one cytogenetic clone (*i.e.* two or more identical metaphases) (Table 3.2), and abnormal chromosomes were commonly observed that were unique to one metaphase (according to cytogenetic convention they are not recorded in Table 3.2, but they are described in Appendix 2). The metaphase-to-metaphase heterogeneity was analysed

quantitatively using two further indices: (1) a numerical heterogeneity index (NHI) modified from (Lengauer *et al.*, 1997); and (2) a structural heterogeneity index (SHI) (see Table 3.4 footnote for more details). The ongoing pronounced inter-metaphase heterogeneity strongly suggests that errors in chromosome segregation continue to be repeated during growth, as shown previously (Lengauer *et al.*, 1997). The multiple examples of abnormal chromosomes that were detected in no more than one metaphase suggest that this instability is not restricted to non-disjunction, but includes structural changes that involve chromosome breakage and recombination events. The survival of colorectal cancer cells with this phenotype implies defective coupling between DNA breaks and apoptosis.

Table 3.4: Chromosomal instability indices (NI: numeric index, BI: break index, NHI: numerical heterogeneity index, SHI: structural heterogeneity index)

RER-	Mode	NI	BI	NHI	SHI
C70	127	79	14	33	40
HT29	70	27	15	19	30
LIM1863	80	33	9	11	0
SW1417	70	22	22	13	120
SW403	64	21	14	20	10
SW480	58&90	49	24	43	ND
SW620	48	8	15	10	30
SW837	38	7	13	16	40
VACO4A	62	20	10	14	30
Mean		19.4	15	19.9	40
RER+ (typical)					
DLD1	46	0	3	1	0
GP2D	46	0	4	1	0
HCT116	45	1	3	1	0
LS174T	47	3	0	1	0
VACO5	46	0	2	5	0
Mean		0.8	2.4	1.8	0
RER+(Atypical)					
LoVo	49	3	3	2	0
HCA7	43	2	21	17	50
LS411	73	29	21	15	90

The numerical index (NI) of chromosomal changes (aneuploidy index), was calculated for each line, using a metaphase with modal chromosome number, by assigning one point for each gain of a whole normal chromosome from diploid, and for each loss that was not replaced by a translocation, [modified from (Eshleman *et al.*, 1998)]. The break index (BI) was calculated as the total number of structurally abnormal chromosomes, clonally present (found in 2 or more metaphases), in each line. Duplicate abnormal chromosomes were counted only once. The numerical heterogeneity index (NHI) for a cell line was calculated by calculating the percentage of metaphases whose content of a particular chromosome deviated from the mode for all chromosomes in 12-24 metaphases and averaged. Since each structurally distinct chromosome was examined, the values tend to be higher than those obtained for centromere number (Lengauer *et al.*, 1997). The structural heterogeneity index (SHI) was devised here and was calculated as the total number of structurally abnormal chromosomes found in only one metaphase, divided by the number of metaphases examined and then expressed as a percentage.

N.B. SW480 has two clones, mode 58 and 90 with NI respectively 13 and 49, but the indices were calculated for this line as one line disregarding the constituent clones to allow comparison with other lines. ND: the SHI was not determined for SW480 because the technical complexity involved in distinguishing the two different clones precluded an accurate e assessment of SHI. High metaphase heterogeneity in VACO5 is mainly due to the presence in some metaphases of deleted 7 and 21. The high numerical index for C70 appears to be due to an endoreduplication event, but the index does not indicate this directly.

3.3.4.2. RER+ tumours

The majority of the RER+ lines had striking karyotypic stability; they had fewer abnormal karyotypes than the RER- lines, both by structural and numerical criteria. Five of eight RER+ lines were nearly or exactly (VACO5) diploid, with a maximum of four detectable chromosomal changes (including gains or losses of whole chromosomes, deletions and translocations). A sixth, LoVo, had only three numerical and three structural changes. The translocations, although fewer, appeared as varied in site as in the RER- lines. However, two RER+ lines, HCA7 and LS411, were strikingly different, with multiple abnormalities, much more like the RER- group.

3.3.4.3 The indices show a clear separation of typical RER+ and RER- tumours,

The indices showed a clear separation of typical RER+ and RER- tumour cell lines, with no overlap between the groups, providing the two atypical lines, HCA7 and LS411were exclude from the RER+ group. The break indices ranged respectively from 0 to 4 and 8 to 24; the numerical aneuploidy index ranged from 0 to 3 and 7 to 79; the numerical heterogeneity index ranged from 1 to 5 and 10 to 43, and the structural heterogeneity indices were uniformly zero in the typical RER+ group but ranged from 0 to 120 in the RER- group.

3.3.5. Atypical RER+ lines

LS411 and HCA7 demonstrated two novel patterns of genomic instability. Both stood apart from other RER+ lines as 'atypical RER+' lines.

3.3.5.1. LS411: an RER+ line displaying a pattern similar to the RER- group

LS411 displayed all the chromosomal instability features of the RER- group, as shown by the indices in Table 3.4, while also exhibiting the classic RER+ phenotype, including microsatellite instability and *hMLH1* gene mutation and promotor hypermethylation (Wheeler *et al.*, 1999). This RER+ status of LS411 was reconfirmed on the same stock that has been karyotyped. Furthermore, LS411 was biologically like other RER+ tumours—a poorly differentiated adenocarcinoma in the caecum from a young (32-year-old) patient, who was alive 5 years after surgery (Suardet *et al.*, 1992). The SKY karyotype was recognisably similar to that originally reported using cytogenetics (Suardet *et al.*, 1992), and the cell stock used in this work was the same as that used by Wheeler et al (Wheeler *et al.*, 1999), so it is highly unlikely that an error of sample identification occurred. Possible explanations of the LS411 phenotype would be (a) that there can be selection for both kinds of genomic instability during the development of colorectal

malignancy, or (b) that a tumour with chromosomal instability was treated with a cytotoxic drug such as cisplatin that selects for survival of tumour cells with mismatch repair deficiency (Aebi *et al.*, 1996; Drummond *et al.*, 1996; Fink *et al.*, 1997; Fink *et al.*, 1998b). The latter explanation seems less likely given the available clinical data.

3.3.5.2. Atypical RER+ cell lines predisposed to balanced translocations: HCA7 and LoVo

HCA7 showed a striking, previously undiscribed phenotype: it demonstrated multiple, apparently balanced, reciprocal translocations. This was unexpected in carcinomas, as the great majority of translocations were unbalanced and so result in loss or gain of whole segments of chromosomes, as reported in classical banding studies (Bardi *et al.*, 1995; Dutrillaux, 1995), and confirmed by FISH (Ghadimi *et al.*, 1999; Davidson *et al.*, 2000; Ghadimi *et al.*, 2000). In the present study, the translocations in the other lines were almost all non-reciprocal: out of approximately 160 identified by SKY not more than four contained the same two chromosomes and so could have been reciprocal, although a few reciprocal events could have been missed if the translocated fragment was too small (Elghezal *et al.*, 1999; Fan *et al.*, 2000).

HCA7 is also distinctive in showing a high metaphase-to-metaphase heterogeneity (NHI of 17 and SHI of 50) and break index (21), but limited numerical changes from diploid (NI of 2), that is it appears to be an unstable karyotype that has acquired a large number of structural changes but nevertheless has managed to retain an almost diploid number of chromosomes, though this is in large part merely a reflection, in the numerical score, of the tendency to balanced translocations.

Twenty-one rearranged chromosomes were found in HCA7 that were present in 2 or more metaphases, of which 12 are the partners of 6 balanced translocations. Three related clones were identified in HCA7 in this work. The predominant clone contained all the 6 balanced translocations, and what appeared to be the stem clone contained $\bar{3}$ of them. Interstitial deletions, duplications, or a few unbalanced translocations accounted for 9/21 abnormal chromosomes, but only 3 out of these 9 are common to all three clones. Previous cytogenetic analyses failed to uncover these balanced translocations (Kirkland, 1985; Kirkland & Bailey, 1986; Perry *et al.*, 1999). This cell line has previously been reported to have multiple, biologically and karyotypically different sub-clones (Marsh *et al.*, 1993).

LoVo may also belong in this group that preferentially acquires balanced translocations, although its indices are typical for RER+ lines. It had only one balanced

translocation in the passage we examined, but there is evidence of a tendency to balanced translocations, since cytogenetic banding analysis of another sample by Soulie et al (1999) showed two additional balanced translocations, which must have been acquired on passage, with no unbalanced rearrangements.

3.3.6. SW480, SW620 and clonal heterogeneity

The lines SW480 and SW620 were derived respectively from a primary tumour of the colon and lymph node metastasis from it. SW480 comprises two very different clones, which have different culture behaviour, morphology and *in vivo* aggressiveness as well as different karyotypes (Tomita *et al.*, 1992). The karyotypes of these two lines are displayed as deviations from the normal diploid karyotype in table 3.5.

The three clones illustrate how the parent tumour might have evolved. All three clones are clearly derived from a common clone, containing what is presumably a reciprocal t(5;20), and a non-reciprocal t(2;12) (absent from the particular clone-2 metaphase in Figure 3.7), but have evolved separately almost beyond recognition. SW480-clone-1 and clone-2, but not SW620, have a t(7;14) while SW620 has a reciprocal t(6;7) suggesting that these cells share the break point at chromosome 7q but join up with different chromosomes in the process of translocation. The two clones of SW480, but not SW620, have a complex translocation t(5;8;19) which is not represented at all in SW620 even by the presence of breaks at the same points in these chromosomes.

Comparison of the karyotypes of SW480 (from the primary tumour) with SW620 (the metastasis) does not suggest any candidates for association with the process of metasasis. Although, as noted by others (Gagos *et al.*, 1995; Bardi *et al.*, 1997; Melcher *et al.*, 2000), new breaks and gains involving chromosome 13 and 16 (Table 3.5) wer found. However, another metastatic tumour, LoVo, does not show such changes, but rather shows t(2;12) in common with SW620 and its primary (SW480).

Table 3.5

Deviation from normal diploid karyotype in SW480 and SW620

chr	SW480: clone-1	SW480: clone-2	SW620
1	-1,+der(1)t(1;9)*	+1,+1	
2	+der(2)t(2;12)	+2,+del(2)(?),+der(2)t(2;18) / der(2)t(2;12)	-2,+der(2)t(2;12)
3	+del(3)	+3	-3,+del(3)
4		+4	-4,+del(4)
5	-5,+der(5)t(5;20)*	+der(5)t(5;20)*x2, +der(5;12)t(5;12)	-5,+der(5)t(5;20)*
6		+6	-6,+der(6)t(6;7)*
7	+der(7)t(7;14),+der(7)t(7;13)	+7,+der(7)t(7;14)x2	+del(7),+der(7)t(6;7)*
8	-8,+der(8;19)t(8;19),	+8,+8	-8,-8,+der(8)t(8;13), +der(8)t(8;17)
9	-9,+der(8;9)t(8;9), +der(9)t(1;9)*	+9	
10	-10,+ der(10)t(10;12)(3;12)	+der(10;12)t(10;12)x2,	-10,+der(10)t(10;13)
11	+11	+11,+der(11)t(11;15)x2	+11
12	-12,+del(12)	+del(12)(?),	
13	+13	+13,+13,+13	-13
14		-14,+der(14)t(12;14)x2	
15		+15	
16		+16	-16,+ der(16)dup(16)t(3;16)t(6;16)
17		+17,+17,	-17
18	-18,+del(18)(q?)x2	-18,+del(18)(q?)x3	-18,+der(5;18)t(5;18)
19	-19,+der(19)t(5;19)t(8,19)	+der(19)t(5;19)t(8,19)x4	
20	+ der(20)t(5;20)*x2	+20,+20,+der(20)t(5;20)*x3	+der(20)t(5;20)*
21	+21	+21,+21,+21	
22		+22,+22	
X	+X	+X	
Y	-Y	-Y	-Y
Total	**58**	**90**	**48**

Recorded here are the modal chromosome copy number and structural alterations obtained by the analysis of 12-24 metaphases. Empty spaces indicate that no chromosomal aberrations were found. Markers with a modal number of 0 are not recorded here. *: apparently reciprocal translocations.

3.3.7 Overall patterns of cell lines are like those of fresh surgical materials

These molecular and cytogenetic analyses add to the evidence that cell lines are acceptable models of the genomic abnormalities of authentic colorectal cancers. CGH is equally applicable to cell lines and uncultured cancers, and the cell lines show the typical gains and losses seen in uncultured cancers, as noted by others (Ghadimi *et al.*, 2000). The gains and losses seen in uncultured material were often present at higher frequency in the cell lines. This is likely to be partly due to the masking of changes in surgical material by contaminating normal cells (such as stromal connective tissue, blood vessels and lymphocytes, ect), and may also reflect a relative lack of cell lines representing the subset of RER-tumours that have few imbalances (Georgiades *et al.*, 1999). The SKY-determined karyotypes of these cell lines also broadly showed the abnormalities described in classical cytogenetic studies on uncultured colorectal tumours (Bardi *et al.*, 1995; Dutrillaux, 1995), for example a high frequency of loss or rearrangement of chromosomes 17 and 18; rearrangement of 1 and 8; and gains of 7, 13 and 20. The cell lines appear more rearranged, but this is in part because classical cytogenetic studies sometimes miss or can not interpret correctly certain chromosomal abnormalities (Adeyinka *et al.*, 2000) and primary cultures may select cells that do not fully represent the malignant tumour (Mertens *et al.*, 1992). Thus, the colorectal cancer cell lines appear to carry the genetic changes found in authentic colorectal cancers; are reasonably representative of these tumours; and any additional changes they have acquired *in vitro* are not extensive enough to obscure the changes acquired *in vivo* before culturing. Similar conclusions have been drawn for other tumours, including breast tumour cell lines (Kallioniemi *et al.*, 1994; Davidson *et al.*, 2000).

3.3.8. Multiple patterns of abnormalities of the karyotype

The initial hypothesis under investigation was that there are different forms of chromosomal instability, generated by different kinds of genomic instability present in colorectal cancers. This is well established from comparison of RER+ and RER- colorectal cancers but it is likely, as suggested already (Lengauer *et al.*, 1997), that this may only be an association: tumours that are RER+ are less likely to have all the defects in genomic housekeeping that lead to the more extreme karyotypic changes, but there is no obligatory relationship between RER status and pattern of chromosomal rearrangements. The data obtained in this thesis are consistent with this. While the data confirm that RER- tumour cell lines tend to have very abnormal karyotypes and RER+ lines tend to have

near-normal karyotypes, this appears to be only a negative association between defects in mismatch repair (RER+) and other causes of chromosomal instability. Two RER+ lines had major chromosomal rearrangements: LS411 is derived from a classical RER+ right-sided colorectal carcinoma but shows a range of structural and numerical abnormalities, and metaphase-to-metaphase heterogeneity, that are very similar to those found in members of the RER- group. HCA7 also has a large number of structural rearrangements, but has fewer numerical changes and has many balanced translocations.

Several different patterns of abnormal of karyotype pattern are suggested by the data obtained in this work, which may correspond to different defects in genomic housekeeping. Some of them have been previously described in the cytogenetic literature (Bardi *et al.*, 1995; Dutrillaux, 1995). First, there is the near-normal diploid cell line, VACO5. Second, there are tumour cell lines that show predominantly or exclusively gains of whole chromosomes: LS174T has gained chromosomes 7 and 15, and lost a presumably inactive X. Third, there are three RER+ lines—DLD1, HCT116 and GP2d—that have no net numerical changes (apart from loss of the Y chromosome in HCT116) but have a few structural changes. Several of the structural changes, indeed all in DLD1 and HCT116, lead to the acquisition of additional chromosome fragments. These lines are all RER+, but CGH data on freshly excised colorectal carcinomas suggest that there may be RER- tumours that have similarly near-diploid karyotypes (Georgiades *et al.*, 1999).

The more obviously abnormal karyotypes seem to fall into four patterns. The most striking and novel phenotype is exemplified by HCA7: an apparent strong tendency to acquire balanced reciprocal translocations, in sharp contrast to the overwhelming majority of chromosomal translocations that have been described in solid tumours. Its phenotype is also intermediate between RER+ and RER- tumours in that it shows: many structural chromosomal changes but almost diploid karyotype. LoVo appears to show this same pattern of balanced translocations with near-diploid karyotype.

The majority of the cell lines with very abnormal karyotypes—HT29, LIM1863, SW1417, SW403,VACO4A (all RER-), and LS411 (RER+) are surprisingly similar in overall tendency to acquire large numbers of numerical and structural chromosomal abnormalities, provided the precise chromosomes involved are not considered: they have near-triploid karyotypes with 9-22 rearranged chromosomes, and most chromosomes are present in 3 copies. This pattern of extensive chromosomal instabilities with near-triploid karyotypes (multiple trisomies) seems to be the most common amongst colorectal carcinomas. Two further phenotypes were observed, though whether they are samples of a

continuous spectrum or truly distinct categories remains to be seen. SW837 has many unbalanced structural changes and shows considerable metaphase-to-metaphase variation, yet has retained almost diploid numbers. This suggests that it may not tolerate or generate extensive numerical changes in the way that other lines do. Finally, C70 appears to have an extreme tendency to gain whole chromosomes, with up to 9 copies of certain chromosomes, although this is in part because it appears to have reduplicated its entire complement of chromosomes—which appears to have been similar to the commonly seen near-triploid pattern—at least once.

3.3.9. Evolution of abnormal karyotypes

Most of these colorectal cancer cell lines karyotypes are consistent with evolution by a process of a steady gain of chromosomes, with or without endoreduplication, consistent with the previously described CIN phenotype (Lengauer *et al.*, 1997). This process of steady gain of chromosomes appears to occur with-or without accompanying chromosomal translocation. LS174T has simply gained whole chromosomes 7 and 15. HCT116 and DLD1 clearly show gains of chromosome fragments present as fragments making up the translocated chromosomes, and there are no chromosomal losses.

Surprisingly few, if any, of the lines studied here show the patterns described by Dutrillaux and co-workers (see Chapter 1, Introduction: 1.4.6.1). Only SW837 may show monosomic type (MT) karyotype, and one (C70), or possibly two (SW480-clone-2) show several duplicated markers, as expected for a line that have undergone endoreduplication. However, C70 may have endoreduplicated its entire chromosome complement from a super-diploid rather than sub-diploid state as has been suggested for a typical trisomic type (TT) karyotype. Several of the substantially super-diploid, RER- lines show no sign of endoreduplication, which would usually leave a signature in the form of several duplicated abnormalities, as in C70. Rather they appear to have evolved by accumulation of chromosomes, and C70 resembles such a line that has accumulated chromosomes and then duplicated its entire chromosome complement to become a super-tetraploid. On the other hand, many of the lines studied here, including almost all of the RER- lines, could be considered to show a TT-like pattern: presence of translocations accompanied by gains of chromosomes. Occasional abnormal chromosomes are duplicated, but if the lines gain whole chromosomes this is to be expected. Therefore the overwhelming majority of these lines appear to have karyotypically developed by gradual accumulation of additional chromosomes usually accompanied by some chromosomal translocation events, while

there is an absence of the chromosome loss-followed-by-endoreduplication pattern common in breast cancers.

3.3.10. Mechanisms underlying chromosomal changes

3.3.10.1. p53 status

In contrast to RER status, mutation in *p53* did not appear to correlate with the patterns of karyotypic abnormality in these lines (Table 3.1). It is possible that the *p53* mutation analysis does not detect all p53 abnormalities and conversely, absence of *p53* mutations does not prove that the cells have wild type p53. However, there are data in the published literature on some of these lines that confirm their p53 status using other techniques such as the p53 yeast functional assay (see table 3.1 for references). Two out of five lines in the typical RER+ category (DLD1, VACO5) carried mutations in *p53* but their karyotypes did not show noticeably more numerical or structural chromosomal changes than cell lines with apparently wild-type *p53* within the RER+ group. Similar data were recorded previously (Eshleman *et al.*, 1998). On the other hand there is a group of four out of nine RER- cell lines that have no *p53* mutations (C70, LIM1863, SW1417, VACO4A) all of which show all forms of numerical and structural chromosomal instability. The atypical RER+ line, LS411 is also *p53* non-mutant. This suggests that loss of p53 function by itself does not determine the pattern of chromosomal instability. It appears that loss of p53 is not necessary for generation of chromosomal instability, but the lines with no detectable *p53* mutations may have an unknown equivalent abnormality such as the mdm2 aberrations. It has been shown however that transfecting a mutant *p53* expressing construct into LoVo altered the pattern of chromosomal changes found in clones selected for gene amplification from this line from numerical to structural chromosomal instability. While the original LoVo with its wild type p53 showed gain of whole chromosome 2, transfecting mutant *p53* into this cell line was associated with chromosomal structural rearrangements leading to gain of chromosome 2 material (Soulie *et al.*, 1999). Overall, abnormalities of the p53 by itself appear to be neither necessary nor sufficient for chromosomal instability although they may be permissive.

3.3.10.2. The predisposition to balanced translocation may be a consequence of the RER+ phenotype.

The predisposition to balanced translocation observed in HCA7 and LoVo was found only in RER+ tumour cell lines and therefore may be a consequence of the RER+

phenotype. One possible explanation for a predisposition to balanced translocations would be that when these lines suffer double strand breaks, a *homeologous* (in contrast to homologous) recombination repair process is triggered with a similar but not identical DNA sequence on another chromosome. RER+ cells are defective in mismatch repair, and such cells are known to be more prone to recombination repair between imperfectly matching, *homeologous* sequences (de Wind *et al.*, 1995; Schimenti, 1999). Some translocations would be unbalanced if one product of the translocation does not contain a centromere (Jasin, 2000). An underlying cause generating high levels of chromosomal double strand breaks would be required as well (Ricardson & Jasin, 2000). It may be possible that DNA single strand breaks during DNA replication may generate balanced translocations as well. The high tendency to translocation in RER- cells is likely to occur by a different mechanism such as non-homologous end joining (NHEJ) (see Chapter 1: 1.4.7.2.1).

3.4. Conclusions

In summary, this study has identified, in detail, patterns of chromosomal instability in a group of well-established colorectal cancer cell lines. It has emerged that distinct chromosomal instability patterns characterize these tumours. Whilst the majority, near-triploid (or multiple-trisomic) type is usually RER-, occasional RER+ lines show the same pattern. This is to be expected if the underlying defects are selected for during tumour evolution, since more than one defect of genomic instability may be selected. SKY has also revealed the existence of a new type of instability that has a striking tendency to acquire balanced translocations in contrast to the common patterns previously described in solid tumours. The relationship of chromosomal instability to the mismatch repair deficiency and/or p53 status has been investigated. RER status correlates with, but does not determine, the pattern of chromosomal rearrangements. p53 abnormality itself is neither necessary nor sufficient for chromosomal instability, although it may be permissive. Other possible mechanisms underlying these karyotypic patterns have been discussed. These instabilities appear to give rise to repeated and ongoing genomic alterations, albeit constrained in type, presumably reflecting deficient pathways for recognition and removal of the altered cells by apoptosis. Therefore, it will be of interest to examine the relationships between drug resistance and sensitivity profiles and these different patterns of genomic instability. In addition, the comprehensive karyotypic information provided here on these colorectal cancer cell lines should serve further research in the field.

CHAPTER 4

APOPTOSIS DEREGULATION IN SPORADIC COLORECTAL CANCERS

4.1. Introduction

Many proteins with an established role in carcinogenesis and cancer progression feature in apoptosis pathways. Thus p53, pRb, c-Myc and members of the Bcl-2 and Ras protein families, as well as many viral oncoproteins such as LMP-1 and E1B 19K are all potent regulators of apoptosis (reviewed by Arends *et al.*, 1993; Hale *et al.*, 1996; Wyllie, 1997). The hypothesis has therefore arisen that loss or reduction of susceptibility to apoptosis may be a significant factor in carcinogenesis and tumour progression (Arends & Wyllie, 1991; Evan *et al.*, 1992). Data from genetically manipulated animals show that oncosuppressor deficiency may abrogate the apoptosis of cells that have sustained DNA damage, thus permitting survival and proliferation of cells bearing mutations (Griffiths *et al.*, 1997). Furthermore, there is increasing evidence to support a role of apoptosis in maintaining genomic stability. Recent studies indicate that there is a default apoptotic pathway in mitosis that needs to be overcome for the process to be completed (Li *et al.*, 1998; Altieri & Marchisio, 1999; Li *et al.*, 1999). Apoptosis is widely observed in tumours, however, indicating that changes to the susceptibility to induce apoptosis - if important in carcinogenesis - must be restricted to particular pathways. Clear identification of these pathways in human tumours becomes an important aim, as it would help define the molecular basis not only of carcinogenesis, but also of tumour resistance to various therapeutic measures.

The Fas receptor is a key molecule in the induction of apoptosis (Itoh *et al.*, 1991; Oehm *et al.*, 1992). Active Fas receptor appears to be necessary for apoptosis initiated by wide variety of stimuli including c-Myc protein expression (Hueber *et al.*, 1997) and exposure to DNA-damaging chemotherapeutic agents (Muller *et al.*, 1997). Recent data showed that Survivin confers resistance to Fas-mediated cell death (Suzuki *et al.*, 2000), thus linking resistance to Fas-mediated apoptosis to disturbances in cell cycle and mitotic spindle, possibly by leading to survival and proliferation of genetically unstable cells (Suzuki *et al.*, 2000).

Fas protein is constitutively expressed in normal colorectal epithelium and most colorectal adenomas, but in only a minority of carcinomas (Moller *et al.*, 1994). During

colorectal malignant transformation, it appears that the constitutive Fas-positive phenotype can change to an inducible INF-γ/TNF-α-sensitive Fas-positive phenotype or to stable, clonal deficiency in Fas expression (Moller *et al.*, 1994). The mechanism responsible for these changes has not been established, however, so a series of RER+ and RER- colorectal cancers were analysed for Fas gene mutations.

Recently, clonally expanded, inactivating mutations in *Bax* have been observed in RER+ colorectal cancers, together with evidence for under-expression of the Bax protein in these tumours (Rampino *et al.*, 1997; Ouyang *et al.*, 1998; Yagi *et al.*, 1998; Yamamoto *et al.*, 1998). This provides some circumstantial evidence for the hypothesis that the founder cells of cancers may arise, at least in part, through selective loss of a critical death pathway. Alternative explanations for the *Bax* mutations exist, however. These mutations are restricted to tumours with MMR deficiency, and usually occur in a tract of eight consecutive deoxyguanosines [(G)8] in the third coding exon (Rampino *et al.*, 1997). Since this is a classical target site for nucleotide mismatch, it is possible that mutations within [(G)8] simply reflect the well-recognised effect of MMR deficiency on mutation incidence in tandem repeat microsatellite sequences. The same argument applies as well to mutations of the [(A)10] tract described in *TGFβRII* during colorectal carcinogensis (Myeroff *et al.*, 1995; Parsons *et al.*, 1995), and to Bik, another potent death-inducing protein of the Bcl-2 family (Boyd *et al.*, 1995; Han *et al.*, 1996). The significance of Bik expression in colorectal carcinogenesis has not been explored hitherto.

Therefore, the genes encoding Bik as well as Fas proteins were studied in a series of RER+ and RER- primary colorectal carcinomas, in an attempt to determine the frequency of occurrence of mutations in these apoptosis promoting genes in the process of human colorectal carcinogenesis. Furthermore, *Bax* and *TGFβRII* mutations were studied in a series of RER+ colorectal cancers, each tumour sampled at multiple sites. The basis for such heterogeneity studies is that genetic changes that are critical for carcinogenesis are likely to be shared by all cells in the expanding tumour and should therefore be detectable at all sites throughout the tumour. In contrast, changes that reflect the genomic instability of malignant cells, but are not essential for the transition to malignancy, might be expected to occur in some but not all of the divergent subclones within a given cancer.

4.2.Materials and Methods

4.2.1 *Fas* and *Bik* mutation analysis

All of the samples of DNA extracted from a range of sporadic colorectal cancers were tested for microsatellite instability at a number of loci ranging from 5-11 (D2S119, D2S123, D3S1293, D8S282, D13S160, D15S132, D17S849, D18S58, TGFβR-II, BAT26, BAT40) (see Chapter 2 for details). The BAT26 was always included because it is known to be highly unstable and is claimed to be highly informative for establishment of RER status (Hoang *et al.*, 1997; Stone *et al.*, 2000). A minimum of 5 microsatellite loci was examined in each case and cases were classified as RER+ if they showed instability at one or more microsatellite loci. Following this analysis of RER status 12 RER+ and 12 RER- colorectal cancers were selected for *Fas* and *Bik* mutation analysis. Ten out of the 12 RER+ cases demonstrated shifts at two or more microsatellite loci and 2 out of the 12 RER+ cases demonstrated shifts at only one locus. Primers were constructed from the intronic or non-coding regions surrounding all of the nine exons of the *Fas* gene to allow analysis of exonic and, where possible, splice site sequences (Table 4.1). Splice junction and intronic sequences were retrieved from the GenBank database (accession numbers X81335-X81342). DNA from both tumour and normal tissue samples of each case were used for polymerase chain reaction (PCR) amplification of all the *Fas* gene exons. Exon 9 was amplified in two overlapping fragments (9I & 9II). The PCR products amplified by these primers ranged in size from 108 to 282 base pairs (Table 4.1).

The *Bik* gene was examined less comprehensively, focusing on a potential target site for mismatch repair deficiency mutations: an interrupted trinucleotide repeat tract of 11 CTG [(CTG)2 GCG (CTG)5 GCG (CTG)3 CCG CTG] occupying positions 409-450 included within the coding gene sequence (GenBank database accession number U34584). The PCR primers and amplification conditions are listed in Table 4.2.

The PCR products were tested for mutations using single strand conformation polymorphism (SSCP) analysis. Heteroduplex analysis was performed on the *Fas* exon 5 PCR products for all 24 cases, as these samples showed a banding pattern that proved difficult to resolve by SSCP. Whenever tumours showed aberrantly migrating bands by SSCP or heteroduplex analysis, then their corresponding tumour and normal DNA were directly sequenced using a modified dideoxy chain termination method (sequenase version 2.0).

The p53 protein accumulation status of the samples was established by immunohistochemistry using antibody DO-7 at a dilution 1:100.

Table 4.1: *Fas (Apo-1/CD95)* gene PCR primers and conditions

Exon	Primers (5'-3')	primer conc.	Ta	Mg ion conc	DMSO	P L (bp)
1	TGGGGAAGCTCTTTCACTTCGGAGG	10 pM	62°C	1 mM	5 µl	108
	AGACGGGGTAAGCCTCCACCCGGGC	10 pM				
2	CACGTTGCTTACTTCAGAAATCAAT	25 pM	55°C	1.5 mM	-	260
	TGACTTTCACTGTAATCTCTGGATG	25 pM				
3	CGTGTCCTGTTCAAACACTTGCTCC	25 pM	60°C	1.5 mM	-	220
	TCCAAGCTTGGCCTCTTTCAATTGC	25 pM				
4	TTAGCCGCTATAACTAATAGTTTCC	25 pM	53°C	2 mM	5 µl	184
	TCTAGTGTTTTAATCAGAGAAAGAC	25 pM				
5	TTCTGCCAGGCTTTTGAATTTCTCC	12.5 pM	56°C	1.5 mM	5 µl	144
	GTTGGGGGAAAGGAGAATATAACCG	12.5 pM				
6	TAAAATGTCCAATGTTCCAACCTAC	25 pM	53°C	1 mM	5 µl	118
	CTGCAGTTTGAACAAAGCAAGAACT	25 pM				
7	TTCTACAAGGCTGAGACCTGAGTTG	25 pM	55°C	1.5 mM	-	169
	TCTTTTCAAGGAAAGCTGATACCTA	25 pM				
8	TTTTTATTTGTCTTTCTCTGCTTCC	25 pM	55°C	2 mM	-	115
	TTACTCTAAAGGATGCCATCTCTAT	25 pM				
9-I	CATGGTTTTCACTAATGGGAATTTC	25 pM	55°C	1.5mM	-	232
	CTTTCTGTTCTGCTGTTGTCTTGGAC	25 pM				
9II	CAAAATAGATGAGATCAAGAATGAC	25 pM	55°C	2 mM	-	282
	AACACTAATTGCATATACTCAGAAC	25 pM				

Note: Ta=temperature of annealing, DMSO=dimethyle sulphoxide, P L=product length.

Table 4.2: Primers and conditions for PCR analysis of the *Bik*, *TGβRII* and *Bax* genes

Primers (5'-3')	primer conc.	Ta	Mg ion conc.	DMSO	P L (bp)
***Bik-1(Nbk)* [(CTG)11] tract**					
GGGTCCTGGGTGTCCTGCGAACAGG	12.5 pM	63°C	2 mM	5 µl	121
CGCCTGAGCCGCCGGGGGCTCACTT	12.5 pM				
***TGβRII* [(A)10] tract**					
CCTCGCTTCCAATGAATCTC	12.5 pM	58°C	3 mM	5 µl	267
TTGGCACAGATCTCAGGTCC	12.5 pM				
***Bax* [(G)8] tract**					
ATCCAGGATCGAGCAGGGCG	20 pM	55°C	1.5 mM	5 µl	94
ACTCGCTCAGCTTCTTGGTG	20 pM				

Note: Ta=temperature of annealing, DMSO=dimethyle sulphoxide, P L=product length.

4.2.2 *Bax* and *TGFβRII* mutation analysis

Sixty tumours, sampled at multiple sites, were originally screened for microsatellite instability and only RER+ cases were analysed for *Bax* and *TGFβRII* gene mutations. All the samples were tested at five microsatellite loci including the highly unstable BAT-26 locus. The other 4 loci included TGFβRII, L-myc, D2S123, and D13S160 (see Chapter 2 for details).

Twelve RER+ cases with a total of 45 different tumour sites and the matched normal tissue constituted the material for *Bax* and *TGFβRII* gene mutational analysis. The [(A)10] repeat in exon 3 of TGFβ RII was PCR-amplified using the primers and conditions described in Table 2.2, then the PCR products were analysed for mutations using SSCP. A DNA segment of 94-base pairs encompassing the [(G)8] tract in *Bax* was PCR-amplified using the primers and conditions described in Table 2.2. The PCR products were radioactively labelled through primer end-labelling with [γ-^{33}P]ATP using T4 polynucleotide kinase (see chapter 2 for details). The radio-labelled products were electrophoretically separated in denaturing 6% polyacrylamide gel and subjected to autoradiography for detection of band shifts. At the same time, all the original PCR products were directly sequenced with Thermosequenase radiolabeled terminator cycle sequencing kits (Amersham Life Science).

Tumours were always analysed for mutations along with their matching normal mucosae to supply negative controls. This was useful in excluding polymorphisms observed particularly in the *Fas* gene exon 2. Known mutant sequences of similar nucleotide base pair length were also included as positive controls in mutation screening experiments including SSCP.

4.3. Results

4.3.1.*Fas and Bik* mutation analysis

4.3.1.1.Clinico-pathological data and RER status

The 24 cases selected for this study were classified into 2 groups according to their RER status. The RER+ group included 10 cases that showed microsatellite instability at a number of loci ranging from 2-6 (Figure 4.1, Table 4.3). In this group 9/10 tumours were right sided, 9/10 showed poor or mucinous differentiation, and 4/10 were positive for p53 accumulation determined by immunohistochemistry. In a further 2 tumours (nos. 11 & 12, one left- and one right-sided cancer) only one of the 5 interrogated microsatellite loci exhibited instability. Both were positive for p53 accumulation as determined immunohistochemically, and one included areas of mucinous differentiation. The RER- group (Table 4.4) included 12 cases, which did not show mutations at any of the 5-11 microsatellite loci examined. Only 3/12 were right sided, 3/12 showed mucinous or poor differentiation and 9/12 were p53 positive immunohistochemically.

4.3.1. 2. No mutations in Fas or Bik

On searching the coding sequence of the *Fas* gene for nucleotide repeat tracts several were found. There is a [(T)7] tract in exon 4 occupying positions 397-403, an [(A)6] tract in exon 9 occupying positions 895-900 and a [(T)5] tract in exon 3 occupying positions 288-292 in the coding gene sequence (obtained from the GenBank database accession no. M67454). In addition, there are shorter nucleotide repeat tracts scattered throughout the *Fas* gene. The *Bik* gene contains a potential target site for microsatellite mutator phenotype induced mutations, an interrupted trinucleotide repeat tract of 11 CTG [(CTG)2 GCG (CTG)5 GCG (CTG)3 CCG CTG] occupying positions 409-450 inclusive in the coding sequence (obtained from the GenBank database accession number U34584). PCR-SSCP was selected as a primary screening method because it can detect all types of mutations with a sensitivity of 80%-90% including those associated with the mutator phenotype at microsatellites.

There was no evidence of mutation in any of the 24 cancer or normal mucosa samples examined either in all the *Fas* gene exons, or in the target repeat sequence in the *Bik* gene (representative examples are shown in Figure 4.1).

4.3.2. Heterogeneity studies for *Bax* and *TGFβRII* mutations

4.3.2.1.RER characterisation

Twelve out of 60 patients (20%) demonstrated microsatellite instability (Table 4.3). In some cases (nos. 17, 27, 52, 53 and 55) individual tumour sites from the same carcinoma demonstrated different sets of mutations at the five microsatellite loci tested. In some tumours, biopsies from different sites each exhibited different mutations at the same microsatellite locus (Figure 4.2). However, in no case was microsatellite instability present at one tumour site, and completely absent at all tested loci in others. Hence it was possible to classify all sampled sites of all tumours as RER+, although in 2 tumours (nos. 18 & 22) the instability was evident in only one of the five tested loci.

4.3.2.2.Bax shows two patterns of mutations, but TGFβRII shows a single pattern

Mutation in the *Bax* [(G)8] mononucleotide repeat tract occurred in six out of 12 (50%) RER+ tumours: in five, this involved loss of a repeated nucleotide and in one a gain. Four showed a homogenous pattern, with identical changes in [(G)8] detected in all sites sampled from each carcinoma (Figure 4.2 and Table 4.5). In the other two cases, however, [(G)8] *Bax* mutations were present in some but absent in other sites from the same cancers. Thus, case number 17 showed mutation in tumour sites A and B, but not C and case number 25 showed mutations in A, B and D but not C (Figure 4.2 and Table 4.5). In both these cases, the tumour site in which *Bax* [(G)8] was unchanged showed unequivocal evidence of instability at most of the other tested microsatellite loci. It should be emphasized that all samples from individual cases were taken from single tumours (not from multiple tumours).

In contrast, *TGFβRII* mutations were found in 9/12 cases (75%) and in each of these the mutations were present in all the sampled sites. Two cases showed neither *Bax* nor *TGFβRII* mutation.

Table 4.3

Clinical and pathological data and p53 status of the RER+ cancers for *Fas* and *Bik* mutation analysis

no	age	sex	site in large intestine	Dukes' stage	histology	p53 stain	shifted loci	death genes mutations
1	67	F	sigmoid (Lt)	B	adenocarcinoma	+	2/6	no mut
2	63	F	transverse (Rt)	B	mixed (ad/muc)	-	6/7	no mut
3	69	M	caecum (Rt)	B	mixed (ad/muc)	-	5/5	no mut
4	74	F	ascending (Rt)	C	mixed (ad/muc)	-	5/7	no mut
5	64	F	ascending (Rt)	B	mixed (ad/muc)	-	5/5	no mut
6	66	F	ascending (Rt)	C	mixed (ad/muc)	+	3/5	no mut
7	69	F	ascending (Rt)	B	poorly dif.	+	3/11	no mut
8	49	F	caecum (Rt)	C	mixed (ad/muc)	-	3/6	no mut
9	49	F	ascending (Rt)	B	mixed (ad/muc)	-	5/5	no mut
10	75	F	ascending (Rt)	B	poorly dif.	+	5/5	no mut
11	49	M	rectum (Lt)	B	adenocarcinoma	+	1/5	no mut
12	75	M	caecum (Rt)	B	mixed (ad/muc)	+	1/5	no mut

Note: shifted loci refers to the number of microsatellite loci showing "shifted" or extra bands (as a proportion of the total tested for that case) compared to adjacent normal mucosa.

Histology: ad/muc=mixed adenocarcinoma and mucinous carcinoma pattern, dif=differentiated.

mut=mutation

Table 4.4

Clinical and pathological data and p53 status of the RER- cancers for *Fas* and *Bik* mutation analysis

no	age	sex	site in large intestine	Dukes' stage	histology	p53 stain	shifted loci	death genes mutations
13	57	M	sigmoid (Lt)	C	adenocarcinoma	-	0/8	no mut
14	82	F	sigmoid (Lt)	C	adenocarcinoma	+	0/5	no mut
15	70	F	descending (Lt)	D	adenocarcinoma	+	0/8	no mut
16	73	F	caecum (Rt)	B	adenocarcinoma	+	0/5	no mut
17	59	F	rectum (Lt)	C	adenocarcinoma	+	0/5	no mut
18	40	F	caecum (Rt)	C	adenocarcinoma	+	0/11	no mut
19	67	F	rectum (Lt)	C	poorly dif.	-	0/11	no mut
20	57	M	sigmoid (Lt)	C	adenocarcinoma	+	0/7	no mut
21	72	F	ascending (Rt)	C	adenocarcinoma	+	0/5	no mut
22	75	F	sigmoid (Lt)	A	adenocarcinoma	-	0/5	no mut
23	86	F	ascending (Rt)	C	mixed (ad/muc)	+	0/5	no mut
24	66	M	rectum (Lt)	D	mixed (ad/muc)	+	0/5	no mut

Note: shifted loci refers to the number of microsatellite loci showing "shifted" or extra bands (as a proportion of the total tested for that case) compared to adjacent normal mucosa.

Histology: ad/muc=mixed adenocarcinoma and mucinous carcinoma pattern, dif=differentiated.

mut=mutation

Table 4.5

Mismatch repair deficient carcinomas analysed for RER and *Bax* status

no	age	sex	site	histology	sample site	RER status					*Bax* mutation
						BAT-26	L-Myc	TGFβRII	D13S160	D2S123	
17	81	F	cae	mucoid	A	+	+	+	-	+	+(loss)
					B	+	+	+	-	+	+(loss)
					C	-	+	+	-	+	-
18	67	F	sig	ac md	A	+	-	-	-	-	-
					B	+	-	-	-	-	-
					C	+	-	-	-	-	-
19	70	M	asc	ac pd	A	+	+	+	-	+	+(loss)
					B	+	+	+	-	+	+(loss)
					C	+	+	+	-	+	+(loss)
22	43	F	cae	ac/mu	A	+	-	-	-	-	+(loss)
					B	+	-	-	-	-	+(loss)
25	65	F	cae	ac/mu	A	+	+	+	+	-	+(loss)
					B	+	+	+	+	-	+(loss)
					C	+	+	+	+	-	-
					D	+	+	+	+	-	+(loss)
27	88	F	asc	ac/mu	A	+	+	+	-	+	-
					B	+	+	+	+	+	-
					C	+	+	+	+	+	-
					D	+	+	+	+	+	-
					E	+	+	+	+	+	-
					F	+	+	+	+	+	-
					G	-	+	+	-	+	-
28	75	F	cae	ac/mu	A	+	+	+	+	-	-
					B	+	+	+	+	-	-
38	64	M	asc	ac md	A	+	+	+	-	+	+(loss)
					B	+	+	+	-	+	+(loss)
					C	+	+	+	-	+	+(loss)
					D	+	+	+	-	+	+(loss)
47	60	M	asc	ac pd	A	+	-	+	+	+	-
					B	+	-	+	+	+	-
					C	+	-	+	+	+	-
					D	+	-	+	+	+	-
52	76	M	cae	ac pd	A	+	+	+	+	-	-
					B	+	+	+	+	-	-
					C	+	+	+	+	+	-
					D	+	+	+	+	+	-
53	77	F	asc	ac pd	A	+	+	-	-	-	-
					B	+	+	-	+	-	-
					C	+	+	-	-	+	-
					D	+	+	-	-	-	-
55	70	F	asc	ac pd	A	+	+	+	+	-	+(gain)
					B	+	+	+	-	-	+(gain)
					C	+	+	+	+	-	+(gain)
					D	+	+	+	-	-	+(gain)

cae=caecum, asc=ascending colon, sig=sigmoid colon, mucoid=mucoid carcinoma, ac md=moderately differentiated adenocarcinoma, ac pd=poorly differentiated adenocarcinoma, ac/mu=mixed adenocarcinoma glandular/mucoid pattern, +(loss)=deletion of one G from the [(G)8] tract, +(gain)=insertion of one G in the [(G)8] tract.

Figure 4.1 *Fas* and *Bik-1* mutation analysis

A Denaturing polyacrylamide gel showing RER characterization at the BAT26 microsatellite locus: samples 1 and 2 show band shift in the tumour lane (T) compared to the normal consistent with derivation from RER+ cancers, whereas sample 13 does not show any band shifts and is derived from a RER- cancer.

B SSCP for *Fas* exon 9I for samples 1 and 2 from RER+ tumours , showing no band shifts in the tumour (T) compared to the normal (N) lanes.

C SSCP for *Bik* for samples 1 and 2 from RER+ tumours, showing no band shifts in the tumour (T) compared to the normal (N) lanes.

Figure 4.1 **A**

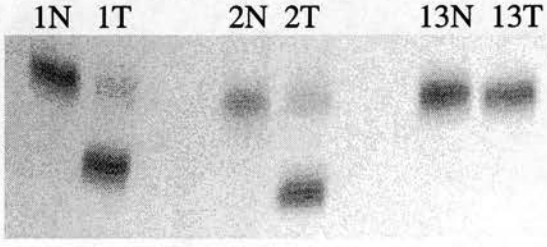


Figure 4.1 **B**

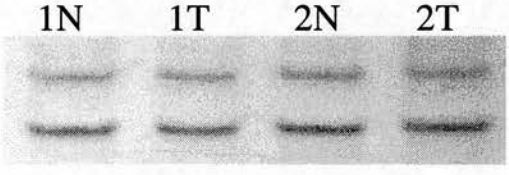


Figure 4.1 **C**

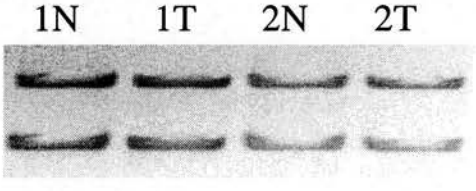


Figure 4.2 *Bax* mutation analysis

A The band shift pattern of case no. 27 at the BAT-26 (left) and L-myc (right) microsatellite loci (N=normal mucosa; A-G=7 different tumour sites from the same carcinoma).

Figure 2

B *Bax* gene analysis of case no. 55 showing the band shift pattern on a polyacrylamide gel demonstrating one nucleotide insertion in the [(G)8] tract from all tumour sites (A-D), compared to normal mucosa (N).

C Sequence analysis of case 17(B&C) and 25(B&C). In both cases, site C does not show the one nucleotide deletion in the [(G)8] tract observed in site B (indicated by arrow).

Figure 4.2 **A**

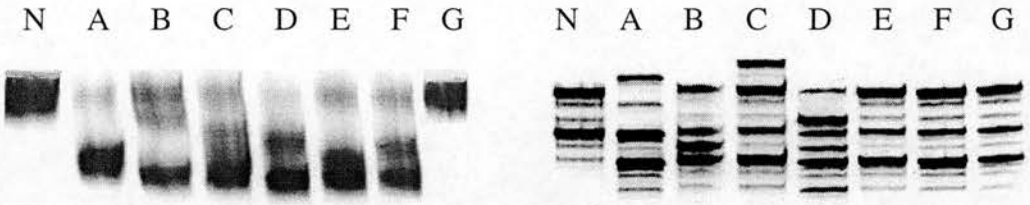


Figure 4.2 **B**

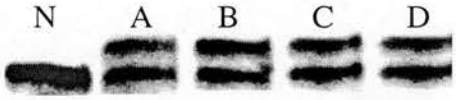
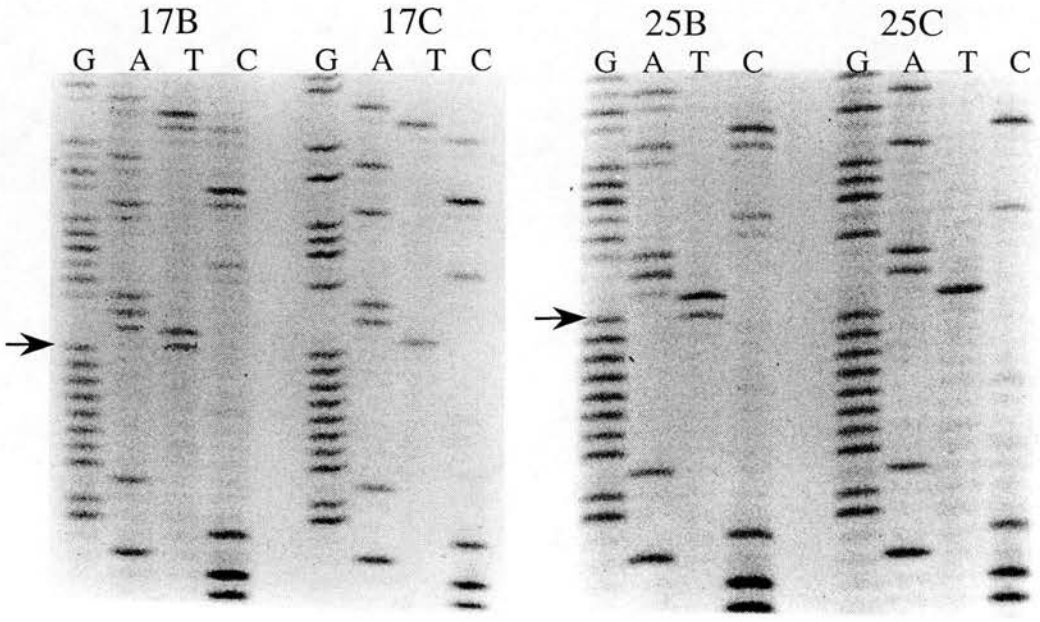


Figure 4.2 **C**



4.4. Discussion

4.4.1. Absence of *Fas* and *Bik* mutation in colorectal cancers

This study identified no mutations in two death pathway genes in 24 colorectal cancers that included 12 RER+ and 12 RER- cancers. The first gene, *Fas* was explored completely for evidence of mutations throughout the coding sequence in all nine exons. The *Fas* gene possesses many mononucleotide repeat sequences within its open reading frame, considered to be potential targets for MMR-deficiency mutations. Other studies have shown that mononucleotide repeat sequences of similar length are susceptible to mutation arising from mismatch repair deficiency (Greenblatt *et al.*, 1996). Longer mononucleotide tandem repeats (e.g. extending to more than 10 base pairs) are rare in coding regions of genes (Parsons *et al.*, 1995; Simms *et al.*, 1997).

The mutation screening method (PCR-SSCP) used is known to detect approximately 80-90% of mutations when applied to single strand DNA between 150-250 nucleotides in length (Humphries *et al.*, 1997). The PCR products generated here ranged in size from 108 to 282 base pairs (bp) (Table 4.1). For 3 of the *Fas* exon PCR assays, the PCR product length was shorter than 150 bp or longer than 250 bp, and in these assays the PCR was designed in this way to avoid false positive results which may arise due to amplifying intronic sequences flanking very short exons such as exon 8 (25 bp) or long exons such as exon 9 (1664 bp in total, 331 bp from the first codon of exon 9 to the stop codon). Hence, it is improbable that mutations in any of the exons in *Fas* or in the tandem repeat region identified in *Bik* are frequent in human colorectal cancers, even when this bears the mutation-susceptible RER+ phenotype. This contrasts with the high incidence of mutation in RER+ colorectal cancers in other genes, notably *TGFβ-RII* (up to 90%) (Parsons *et al.*, 1995), and *Bax* (50%) (Rampino *et al.*, 1997). Recent studies have shown that the low levels or absence of Fas protein expression in colorectal cancer can not be explained on the basis of gene deletion or rearrangement (Butler *et al.*, 1998). The data produced in this thesis demonstrate that *Fas* gene exonic mutations are also not the explanation. The question therefore arises: what is the mechanism of reduction of Fas protein expression following colorectal malignant transformation? Possible speculative answers include transcriptional, translational or post-translational changes affecting functional Fas protein levels.

Fas pathway alterations have been implicated in some forms of human carcinogenesis. Three novel *Fas* mutations were detected in the Canal-Smith Syndrome,

an inherited lymphoproliferative disorder associated with autoimmunity. Two patients followed into adulthood developed neoplasms (e.g. breast adenoma, thyroid adenoma and basal cell carcinoma) and one died of hepatocellular carcinoma (Drappa *et al.*, 1996). These data imply a role for *Fas* mutations in the evolution of these tumours. A recent study has implicated decreased sensitivity to anti-FAS antibody in the evolution of a group of poorly differentiated colorectal carcinoma (Meterissian *et al.*, 1997). In contrast, only 2 poorly differentiated RER+ cancers were studied here. Furthermore, there is evidence that deficiency of the Fas-mediated apoptosis pathway without *Fas* gene mutations can occur in many situations. T-cell lines from patients with autoimmune/lymphoproliferative disease were relatively resistant to apoptosis induced by monoclonal antibody to Fas, while sequencing of the *Fas* gene from the same patients did not identify any causal mutations (Dianzani *et al.*, 1997). Possible explanation for such situation include: expression of apoptosis inhibitors such as the IAP family (Deveraux & Reed, 1999; Suzuki *et al.*, 2000) and FLICE inhibitory protein (FLIP) (Irmler *et al.*, 1997), as well as, expression of decoy receptors, DcR1 and DcR2 (Ashkenazi & Dixit, 1999), or secretion of soluble FasL that binds Fas but does not transmit death signals (Nagao *et al.*, 1999). Recent data support a new role for Fas in driving cell cycle progression (Hiroi *et al.*, 1999; Shinohara *et al.*, 2000). Fas stimulation in HepG2, hepatoma cell line, induces survivin translocation into the nucleus. Survivin then interacts with the cell cycle regulator Cdk4, leading to Cdk2/Cyclin E activation and pRb phosphorylation thus signaling cell cycle progression. As a result of Survivin/Cdk4 complex formation, p21 is released from its complex with Cdk4 and interacts with mitochondrial procaspase 3 to suppress Fas-mediated apoptosis (Suzuki *et al.*, 2000). What triggers this pathway including the negative feed back loop via which Fas suppresses its own apoptotic activity after induction of cell cycle progression remains to be clarified, however.

The role of p53 protein in coupling DNA damage to apoptosis is well established (see Chapter 1 – 1.2.3.2). Fas-mediated apoptosis has been shown to be linked to p53-dependent apoptosis of human carcinoma cells including colon carcinoma (Muller *et al.*, 1997; Mueller *et al.*, 1999). An obvious alternative mechanism for Fas down-regulation would be depressed transcription secondary to reduced availability of wild type p53, since the first intron of *Fas* includes a p53 responsive element (Mueller *et al.*, 1998). Of our 24 cases, 15 showed clear abnormality in p53, exhibited by immunohistochemical stabilisation affecting a high proportion of the nuclei. Although *Fas* down regulation has the capacity to reduce cellular responses to lethal stimuli, it is

significant that mutational inactivation of the gene is not observed in cancer tissues in which mutational events in other genes are common and clonally expanded. We conclude that mutational inactivation of the *Fas* gene is unlikely to be a critical event in colorectal carcinogenesis.

The situation is quite different in the case of the *Bik* gene, as little is known about its tissue expression, biological activity, and its potential role in carcinogenesis. However, compared with the high mutation rate for the *Bax* gene in RER+ colorectal cancers, it can only be concluded that mutations affecting the CTG tandem repeat element of the *Bik* gene are not important in the development of sporadic RER+ colorectal cancers.

4.4.2 Patterns of *Bax* and *TGFβRII* mutations in sporadic colorectal cancers

It is clear that deficiency in mismatch repair permits the generation of large numbers of mutations throughout the genome, mainly in microsatellite loci and repetitive polynucleotide tracts. In sporadic colorectal tumours, the microsatellite instability is generally found in carcinomas but rather little in adenomas, whereas both adenomas and carcinomas from HNPCC patients may show a high proportion of such instability (Thibodeau *et al.*, 1993; Jacoby *et al.*, 1995; Bubb *et al.*, 1996; Samowitz & Slattery, 1997; Grady *et al.*, 1998). These observations strongly suggest that microsatellite instability is acquired at the adenoma-carcinoma interface in the evolution of sporadic tumours, but can appear at an earlier stage in patients who carry germline mutations in MMR genes. Previous studies have shown clonal expansion of shifts of different amplitude at the same microsatellite locus sampled from different sites in the same tumour (Chung *et al.*, 1997), as observed in the present work also. This indicates that a proportion of these microsatellite mutations are acquired as clonal variants throughout the process of tumour formation and expansion and reflect but do not cause the evolution of such tumours. In contrast, mutations at some genetic loci have been found with great consistency in RER+ tumours. An outstanding example is transforming growth factor beta-type 2 receptor (TGFβRII), which is mutated in upwards of 90% of all tested RER+ colorectal cancers (Markowitz *et al.*, 1995; Myeroff *et al.*, 1995; Parsons *et al.*, 1995). In this series, more than 75% of RER+ cancers showed a mutation in the one site within the TGFβRII gene which was tested. Together with independent evidence that TGFβ exerts a suppressive effect on colorectal epithelial growth (Wrana *et al.*, 1994; Polyak, 1996), these

observations have been interpreted as indicative of a causal role for TGFβRII inactivation in colorectal carcinogenesis.

Frame-shift mutations have been detected in the [(G)8] tract of exon 3 of the *Bax* gene in 48% of 63 RER+ sporadic colorectal cancers in a total of 2 separate studies (Rampino *et al.*, 1997; Ouyang *et al.*, 1998), and in a similar proportion of RER+ colorectal cancers from HNPCC patients (Yagi *et al.*, 1998; Yamamoto *et al.*, 1998). The results obtained here are entirely concordant with these data: *Bax* mutations were detected in six out of 12 (50%) RER+ carcinomas. Two patterns for *Bax* mutations were identified in this work. In four of the six tumours bearing *Bax* mutations, identical alterations in the [(G)8] tract were found in all sites sampled within each cancer, supporting the hypothesis that *Bax* mutation was present in the founder malignant clone. This pattern is also consistent with that described for *Bax* mutations in gastric cancers (Chung *et al.*, 1997). However, in two of six cases, a second pattern was demonstrated in which *Bax* mutation is not shared by all the tumour sites of the same cancer. In both these cases, the tumour site in which *Bax* [(G)8] was unchanged showed unequivocal evidence of instability at most of the other tested microsatellite loci. Therefore, sampling errors and inadequate representation of the malignant tissue in the sample are unlikely causes of the observed heterogeneity. There is a possibility that the apparently unaltered [(G)8] tract found in subclones of these tumours represents a reversion, through a second mutation, of the [(G)8] mutation present elsewhere. This possibility, albeit less likely, can in the future be tested in appropriate cell culture models to determine the frequency of occurrence. Another and more likely explanation, however, is that in these tumours the *Bax* [(G)8] mutation was not present in the founder malignant clone but was acquired later in cancer expansion. In these tumours, it is difficult to sustain the view that mutational inactivation of *Bax* could have been a critical event early in carcinogenesis. These data therefore raise some doubt as to the significance of loss of Bax-dependent apoptosis pathways in colorectal carcinogenesis. Rather than indicating that failure of apoptosis exerts a critical role in carcinogenesis, some *Bax* mutations in colorectal tumours may merely aid tumour progression or expansion, or indeed may simply reflect the consequences of mismatch repair deficiency without a functional connotation.

4.5.Conclusions

Bax gene mutations were detected in 50% of RER+ tumours, but were not always present at all tumour sites in 2 out of 6 cases and hence have arisen either during tumour progression rather than in the founder clone or reflect reversion mutation, the former apparently more likely than the latter. In contrast, *TGFβRII* mutations were found in 75% of the RER+ tumours, and were present in all the sampled sites: these mutations must have arisen in the founder clone. No mutations were found in *Fas* or *Bik* genes in RER+ and RER- cancers. Other causes that might account for Fas down regulation or loss of the Fas-mediated apoptosis in colorectal carcinogensis have been discussed. However, it is significant that mutational inactivation of the gene is not observed in cancer tissues in which mutational events in other genes are common and clonally expanded.

CHAPTER 5

EFFECTS OF TUMOUR GENOTYPE ON TREATMENT RESPONSIVENESS IN COLORECTAL CANCER CELLS

5.1. Introduction

Because genetic alterations are in large part responsible for the generation and biological properties of tumours, it is reasonable to hypothesise that the specific alterations in tumours, including changes to the ability to induce apoptosis by certain stimuli, determine or influence the tumour responsiveness to therapeutic agents. Thus, absence of wild type p53, the most frequent founder clone defect in cancers of the colon, rectum and other sites (Hollstein *et al.*, 1991; Purdie *et al.*, 1991; Cripps *et al.*, 1994), was shown to permit propagation of mutant cells following genotoxic damage (Griffiths *et al.*, 1997). Wild type p53 is a critical regulator of cellular responses to genomic damage. Subsequent to DNA damage, p53 is functionally activated and induces G1 cell cycle arrest (Kastan *et al.*, 1992; Lane, 1992), and via this G1 arrest, p53 has been suggested to facilitate DNA repair (Kastan *et al.*, 1992; Hartwell & Kastan, 1994; Linke *et al.*, 1997). p53 is also essential for maintaining the G2 check point in human cells (Bunz *et al.*, 1998). In contrast, restoration of wild-type p53 function in p53 mutant cells can directly induce apoptosis (Yonish-Rouach *et al.*, 1991; Shaw *et al.*, 1992; Polyak *et al.*, 1997). However, the underlying mechanisms of this p53-induced apoptosis are not fully understood (Polyak *et al.*, 1997; Albrechtsen *et al.*, 1999). By enhancing DNA repair, wild-type p53 could potentially enhance the resistance of cells to killing by agents that induce DNA damage. Alternatively, by potentiating apoptosis, wild-type p53 can increase the sensitivity of cells to killing by agents that induce DNA damage. Both of these effects have been supported by findings in different cell line systems both *in vitro* and *in vivo* (Clarke *et al.*, 1993; Lowe *et al.*, 1993; Slichenmyer *et al.*, 1993; Clarke *et al.*, 1994; Lowe *et al.*, 1994; Bracey *et al.*, 1995; Malcomson *et al.*, 1995; Hawkins *et al.*, 1996; Pellegata *et al.*, 1996; Yang *et al.*, 1996; O'Connor *et al.*, 1997; Palazzo *et al.*, 1997; Blandino *et al.*, 1999).

Recent studies have shown that loss of mismatch repair is an important mechanism of resistance to a variety of clinically important cytotoxic drugs including busulphan (Friedman *et al.*, 1997), carboplatin, oxaliplatin (Fink *et al.*, 1996), cisplatin (Aebi *et al.*, 1996; Anthoney *et al.*, 1996; Drummond *et al.*, 1996; Fink *et al.*, 1996; Toft *et al.*, 1999),

Doxorubicin (Drummond *et al.*, 1996), and the alkylating agents: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Kat *et al.*, 1993; Koi *et al.*, 1994; Toft *et al.*, 1999), *N*-methyl-*N*-nitrosourea (MNU) (Friedman *et al.*, 1997), procarbazine (Friedman *et al.*, 1997) and temozolomide (Liu *et al.*, 1996; Friedman *et al.*, 1997; D' Atri *et al.*, 1998; Toft *et al.*, 1999). This wide range of resistance is due in part to the fact that the mismatch repair system can recognize and bind to various types of adducts in DNA as well as to mismatches (Kat *et al.*, 1993; Hawn *et al.*, 1995). Rather than being a major effector in the removal of such adducts, the main role of the mismatch repair system seems to be as a detector of specific types of DNA damage (Fink *et al.*, 1998a). Methylating agents form a variety of adducts in DNA, among which O^6-methylguanine is the most cytotoxic. Although the mismatch repair system does not seem to be able to recognize the O^6-methylguanine directly (Moggs *et al.*, 1997), it does recognize the O^6-methylguanine-thymine mispair that occurs after erroneous incorporation of a thymine rather than a cytosine opposite the O^6-methylguanine during the next cycle of DNA replication (Griffin *et al.*, 1994). One hypothesis is that having recognized the mismatch, the mismatch repair system incises the thymine-containing strand; excises the thymine and surrounding bases creating a gap; and then fills in the gap via repair synthesis. However, because a thymine is again incorporated opposite the persisting O^6-methylguanine, the site is once again recognized and a new round of attempted repair is triggered. This futile cycling is envisioned as increasing the risk of a double-strand break at the time of the next S phase that could then trigger apoptosis (Karran & Bignami, 1994). Recent data support this link between loss of mismatch repair function and the failure to engage apoptosis in response to methylating agents (Toft *et al.*, 1999).

However, many of these data may be specific to the particular cell lineage or system used in the experiment and extrapolation of these results to clinical settings is far from straightforward. This series of experiments, therefore, set out to determine the effects of different combinations of p53 and RER status, as can be found in sporadic colorectal cancers, on the responsiveness to treatment, both *in vitro* and *in vivo*. Three therapeutic agents were used for this study: 5–fluorouracil (5–FU), ionizing radiation (IR) and temozolomide.

5-FU and its derivatives are widely used in the chemotherapy of gastrointestinal cancers, in particular colorectal cancers (Laffer *et al.*, 1995; Erlichman *et al.*, 1999; Schmoll *et al.*, 1999). The molecular mechanisms underlying 5-FU action are still not clear, however, two main pathways for 5-FU anti-tumour effects have been suggested.

First, the drug is metabolised to 5-fluorodeoxyuridine monophosphate (FdUMP), which suppresses thymidylate synthase (TS), an enzyme, which catalyses the methylation of deoxuridylate to thymidylate. 5-FU thus interferes with an essential step in DNA synthesis (Parker & Cheng, 1990). Subsequently, some studies linked the TS levels in tumours to the sensitivity to 5-FU (Leichman *et al.*, 1997; van Triest *et al.*, 1999), however, other studies failed to demonstrate such association (Findlay *et al.*, 1997; Mirjolet *et al.*, 1998). Second, 5-FU is metabolised to fluorouridine triphosphate (FUTP) which can be incorporated into cellular RNA and DNA, resulting in adverse effects on their structures and functions (Parker & Cheng, 1990; Geoffroy *et al.*, 1994). 5-FU was shown to induce apoptosis of FM3A mouse cells *in vitro* (Yoshioka *et al.*, 1987) as well as human tumour cells *in vivo* (Inada *et al.*, 1997). The apoptotic positive fraction in 5-FU treated human cancers correlated with the overall tumour growth sensitivity to 5-FU (Inada *et al.*, 1997). The apoptotic response to 5-FU was suggested to be mediated via activation of the Fas (Houghton *et al.*, 1997) and/or Bax (Koshiji *et al.*, 1997) apoptotic pathways.

IR causes perturbation throughout the cell cycle subsequent to DNA double strand breaks (Maity *et al.*, 1994). Irradiation was shown to induce apoptosis through p53-dependent or p53-independent pathways in many cell systems (Clarke *et al.*, 1993; Lowe *et al.*, 1993; Slichenmyer *et al.*, 1993; Clarke *et al.*, 1994; Bracey *et al.*, 1995; Bristow *et al.*, 1996; Bracey *et al.*, 1997; Held, 1997; Palazzo *et al.*, 1997). The nature of the p53-independent apoptotic pathways in response to IR is still not clear.

Temozolomide is a well-tolerated, orally bio-available, DNA-methylating agent that has significant activity in metastatic malignant melanoma, brain tumours, and, in particular, high grade astrocytomas (Stevens *et al.*, 1987; Newlands *et al.*, 1996; Newlands *et al.*, 1997). Recently, the early apoptotic response to temozolomide was shown to be dependent on p53 in addition to mismatch repair in mouse intestinal epithelium (Toft *et al.*, 1999). This raises the possibility that many tumour genotypes in colorectal cancers might not benefit from this drug.

The following series of experiments was carried out in two model systems. First, two representative cell lines of the commonly seen patterns (p53 wild type/RER+ and p53 mutant/RER-) were tested for their responsiveness to a wide dose range of the three therapeutic agents *in vitro*. Responses were detected by counting apoptotic cells after staining with Acridine Orange and also by the clonogenic survival assay. Further to this, fresh primary tumours representing all possible genetic patterns in colorectal carcinomas in terms of p53 and RER status were selected. They were established as subcutaneous

xenografts in SCID mice and tested for their apoptotic response to therapy *in vivo*. The tumour xenografts were grown through several passages *in vivo* and displayed a remarkable consistency of many genetic, histological, and biological features of the tumours from which they were derived (Shorthouse *et al.*, 1980; Lefrancois *et al.*, 1989; McQueen *et al.*, 1991; Georgiades *et al.*, 1999). This SCID mouse human tumour model reflects the clinical situation in that clinically active therapies are similarly active in the SCID models (PaineMurrieta *et al.*, 1997). The data presented in this chapter thus provides useful information on the effects of combinations of tumour genetic alterations on responsiveness to therapy. These data on the roles of p53 and the mismatch repair pathway in the responses to 5-FU, IR, and temozolomide may be useful in helping to design more effective therapeutic protocols.

5.2. Experimental design

5.2.1. *In vitro* response

The two cell lines, HT29 (p53 mutant/RER-) and LoVo (p53 wild type/RER+) (see Chapter 3 for references), were grown to obtain enough stock. Their responses to each of the therapeutic agents were studied by two methods:

i. The clonogenic survival assay (as described in Chapter 2)

ii. Their apoptotic responses were studied after 1, 2, and 3.5 days of treatment by Acridine Orange staining (see Chapter 2) and counting apoptosis using fluorescent microscopy (Zeiss, Axiovert S100T.V, UK).

5.2.2. *In vivo* response

5.2.2.1 Selection of tumours

For the purposes of this study, four colorectal carcinomas representing all possible combinations of p53 and RER status were selected. Primary tumours and their corresponding xenografts were previously characterized for their p53 status as determined by immunohistochemistry, and exon 5-8 mutation screening by PCR-SSCP analysis. Further data on chromosome arm 17p gain /loss, as determined by CGH, were also available. However, As stated previously, there is a minor possibility that these methods may not detect all p53 abnormalities and, conversely, absence of detectable abnormalities at this level of analysis might not prove that the cells have wild type p53. RER status was determined by testing for microsatellite instability at 5 microsatellite loci as described in Chapter 4 (4.2.2). Ploidy was determined by flow cytometry and analysis of chromosomal gains and losses was done by CGH (Table 5.1). The tumour genotype profiles of the four xenografts are as follow: ANSC (p53 wild type/RER-), CABA (p53 mutant/RER+), JOTH (p53 wild type/RER+), and HEKI (p53 mutant/RER-). It should be noted that mutations were not directly demonstrated in CABA and HEKI and the word 'mutant' is used here to denote the abnormalities observed by immunohistochemistry and CGH (Table 5.1). The word 'mutant' is retained here for consistency in discussing these patterns, which also include the HT29 cell line that shows unequivocal evidence of p53 mutation (Table 3.1). However, because matching normal mucosae were immunohistochemically negative in these cases (CABA and HEKI), it is most unlikely that causes other than p53 mutations could account for the observed protein stabilization. Previous studies have shown that immunocytochemically-detected stabilization of p53 correlates with p53 mutation in colorectal carcinomas (Cripps *et al.*, 1994; Cottu *et al.*, 1996).

Table 5.1

Profiles of the four tumour xenografts

	ANSC	CABA	JOTH	HEKI
Patient age	81	60	70	81
Sex	M	F	F	F
Site of primary	Sig	Asc	Asc	Asc
Histology	Md/ac	Pd/c	Pd/c	Md/ad
Dukes' stage	B	C	C	C
p53 IHC	-	+	-	+
p53 SSCP	NAD	ND	NAD	ND
17p CGH	NAD	loss	NAD	loss
RER	-	+	+	-
Ploidy	aneuploid	diploid	diploid	aneuploid

P53 IHC, detection of stabilized p53 by immunohistochemistry; p53 SSCP, p53 exon 5-8 mutation analysis using PCR-SSCP; 17p CGH, detection of 17p loss by comparative genomic hybridisation; RER, replication error phenotype; Sig, sigmoid colon; Asc, ascending colon; Md/ac, moderately differentiated adenocarcinoma; Pd/c, poorly differentiated carcinoma; NAD, no abnormality detected; ND, not done.

5.2.2.2 Therapeutic doses

SCID mice (Bosma *et al.*, 1983; Custer *et al.*, 1985) are known to bear several defects including DNA double strand break repair deficiency; hence these mice are hypersensitive to the effects of IR (Biedermann *et al.*, 1991; Disney *et al.*, 1992; Chang *et al.*, 1993; Kirchgessner *et al.*, 1995), and probably other drugs. Therefore, an initial experiment was performed to determine the sub–lethal doses of the therapeutic agents (*i.e.* the maximum dose of each agents that SCID mice can tolerate and survive for 10 days). Duplicate SCID mice, not harbouring tumours, were exposed to a range of doses for each therapeutic agent and monitored for their response. Animals were killed if they were suffering or if they survived for 10 days. To minimize unnecessary animal suffering, this experiment was performed at consecutive steps starting by the highest dose in the treatment ranges suggested in the literature (Biedermann *et al.*, 1991; Blumenthal *et al.*, 1994). The highest dose for each therapeutic agent, which was tolerated for 10 days, was chosen.

5.2.2.3 Preparation of mice and treatment application

Tumours were allowed to grow subcutaneously until an externally visible tumour diameter of about 1cm was reached, or when signs of poor health of the mouse were apparent, for typical durations of 28-56 days. Mice were then killed and tumours were cut into small pieces (2 x 2 mm), which were re-grown in a group of 8 female SCID mice all at the same time, as described in Chapter 2. Tumours were allowed to grow until an externally visible tumour diameter of about 0.5 cm was reached. This time varied between tumours from 3 to 8 weeks. The 8 mice were subdivided into two groups of fours. One group of 4 mice were treated with either 5-FU, γ-irradiation, or temozolomide. The other group of 4 SCID mice was used to provide controls by leaving them unexposed to γ-irradiation or injecting them by vehicle not containing the drug in case of 5-FU and temozolomide. A total of 96 female SCID mice were used for this experiment (8 mice per therapeutic agent per tumour xenograft). This figure (96) does not include mice used to establish and maintain these tumours xenografts. All treatments were applied as a single shot of the sub-lethal dose. 5-FU was dissolved in PBS and mice received a single dose of 80 mg kg^{-1} body weight by intra-peritoneal (i.p.) injection. Temozolomide was dissolved in 10% DMSO in PBS and mice received a single i.p. dose of 80 mg kg^{-1}. Mice were exposed to 5 Gy total body IR from a ^{137}Cs source at 0.27 Gy min^{-1}.

5.2.2.4 Detection of treatment responses

At each of four time points (0.75, 3.5, 7 and 10 days), two animals were killed: one from the treated and one from the control groups, tumours were harvested and fixed for haematoxylin and eosin staining. Treatment responses were estimated by counting apoptosis in five high power fields (HPFs) (x400 total magnification). The fields were chosen around the circumference of the tumour where viable tumour cells were abundant. The necrotic tumour centre and the margins of the necrotic areas were avoided. Apoptotic cells inside glandular lumina were not counted. Mitoses were counted in the same 5 HPFs. Semi-quantitative estimates of the overall extent of necrosis were made at low power (x50) using a scale of 10%, 20%, 30%, 40%, 50% and so on. Xenograft tumour diameters were measured before killing the animals. Samples of the middle portion of the small intestine of the mice were harvested with each tumour, and these were fixed, and sections were stained with haematoxylin and eosin. Apoptosis in the murine small intestines were counted in 10 crypts for both the treated and control mice. This small intestinal apoptotic count served as a positive control for the therapeutic agents, especially relevant in the cases of the non-responsive xenografts.

5.3. Results

5.3.1 *In vitro* clonogenic and apoptotic responses of cell lines

The results of the clonogenic survival assays are plotted in graphic forms in Figure 5.1. Treatment with 5-FU induced a significantly greater reduction of clonogenic survival of LoVo compared to HT29 at 150 μM 5-FU ($p = 0.03$, Student's t-test). IR induced significant reductions in the clonogenic survival of LoVo compared to HT29 at 1,3 and 5 Gy IR ($p < 0.03$ for all comparisons, Student's t-test), with the most evident difference at 3 Gy. HT29 formed 2 different colony types, small and large colonies (Figure 5.2), the small colonies were more resistant to therapies, particularly IR at lower doses as shown in Figure 5.1 D. Significant reductions in colony number were seen for large colonies at 3 Gy ($p = 0.04$, Student's t-test) and 5 Gy ($p = 0.02$, Student's t-test), while significant reductions of small colony numbers were seen at 5 Gy only ($p < 0.01$, Student's t-test) compared to the numbers of colonies without IR treatment. Responses of both LoVo and HT29 to temozolomide were comparable in that both lines showed slight reductions in clonogenic survival at 8 μM and 40 μM temozolomide and almost similar decreases of clonogenic survival at a 100 μM temozolomide.

LoVo and HT29 cell lines showed detectable apoptotic responses by Acridine Orange staining and counting of the proportions of apoptotic cells in 100 cells by fluorescence microscopy only after 3.5 days of treatment application (Figure 5.4, examples of apoptotic cells are shown in Figure 5.3). There was hardly any significant apoptotic response detected by this technique 1 or 2 days after treatment. The apoptotic responses of LoVo and HT29 showed a striking inverse relationship with clonogenic survival assay data (Figure 5.4 D; Correlation $R^2 = 0.8688$, $p < 0.001$). Treatment with 5-FU induced significantly more apoptotic death of LoVo compared to HT29 at 150 μM 5-FU ($p = 0.0005$, Student's t-test). IR induced markedly increased apoptosis of LoVo compared to HT29 at 1 ($p = 0.03$, Student's t-test), 3 ($p = 0.04$, Student's t-test) and 5 Gy ($p = 0.03$, Student's t-test), with the most evident difference at 3 Gy. Responses of both LoVo and HT29 to temozolomide were very similar with both lines showing minimal increase in apoptotic death at 8 uM and 40 uM temozolomide and an almost similar slow increase in apoptotic death up to 100 uM temozolomide.

5.3.2 *In vivo* responses of tumour xenografts to therapeutic agents

Apoptotic and mitotic counts and the overall necrosis score for each tumour xenograft are shown in Tables 5.2. Photomicrographs of representative areas of the

haematoxylin and eosin stained sections are presented in Figures 5.5 – 5.8. Small intestinal apoptosis was maximal at 0.75 day then declined rapidly afterwards. Small intestinal apoptotic counts (Figure 5.9 D) at 0.75 day for all the controls were 2-10/ 10 crypts, but 39-63 after 0.75 day of treatment application for any of the 3 therapeutic agents, and these differences were all statistically significant ($p<0.001$ for all comparisons, Student's t-test).

The apoptotic responses of the four tumour xenografts to therapies are presented as graphs (Figure 5.9). p53 wild type tumours, ANSC and JOTH, showed maximal apoptotic responses to 5-FU at 3.5 and 7 days respectively, which was statistically significant ($p = 0.006$ for ANSC and $p = 0.01$ for JOTH compared to baseline apoptotic figures in controls, Student's t-test), whereas p53 mutant tumours—CABA and HEKI—showed no significant responses at days 3.5 or 7 or 10 (Figure 5.9 A). No correlation was observed between RER status and responses to 5-FU. Thus, responses to 5-FU appeared to be dependent on the presence of wild type p53.

Significant apoptotic responses to IR were observed at 3.5 days—compared to base line levels in controls—in tumours which were wild type for p53 (JOTH), mismatch repair (HEKI), or both p53 and mismatch repair (ANSC) ($p = 0.01$, 0.02 and 0.01 respectvely, Student's t-test). The ANSC tumour xenograft showed a significant apoptotic response earlier, at 0.75 days after IR treatment ($p = 0.01$, Student's t-test) than the other two tumours. The fourth tumour (CABA, p53 mutant/RER+) showed no significant apoptotic responses to IR between 0.75 and 10 days. Therefore, apoptotic responses to IR appeared to be dependent on possession of either wild type p53 or alternatively proficient mismatch repair or both.

Apoptotic responses to temozolomide were lower in amplitude than those for 5-FU and IR and were dependent on the presence of both wild type p53 and proficient mismatch repair together, as in the case of ANSC (p53 wild type/RER-) in which the maximum response occurred at the first time point of 0.75 days and declined rapidly thereafter. This apoptotic response of ANSC at 0.75 days was statistically significant compared to baseline apoptotic levels in controls ($p = 0.05$, Student's t-test). The other 3 tumours showed no significant changes in apoptotic responses to temozolomide between 0.75 and 10 days.

The mitotic counts and overall extent of necrosis (Table 5.2) spanned a wide range, which in many cases included or overlapped the control range, or otherwise, was not significantly different from the control values.

5.3.3 Clinical follow up of the patients

Limited clinical follow up data were available on only two of the four tumour cases studied *in vivo*. The patient with the tumour CABA (p53 mutant/RER+) presented with advanced disease, which progressed despite chemotherapy, including 5-FU, and the patient died 9 months after diagnosis. The patient with the tumour JOTH (p53 wild type/RER+), which was a Dukes' C tumour, received adjuvant chemotherapy, including 5-FU, and remained alive and well three years after diagnosis. These clinical outcomes, in terms of response to chemotherapy, appear to be consistent with the results obtained in these experiments.

Table 5.2 Responses of xenografts to therapies

A: ANSC (p53 wild-type/RER-)

Time (days)	0.75	3.5	7	10	S.I.
Apoptosis Count / 5HPFs (X400)					
Control	89	61	63	93	8
5-FU	92	228	170	103	53
Gamma Rays	205	267	195	142	51
Temozolomide	147	110	102	47	56
Necrosis (percent)					
Control	50	50	50	40	
5FU	40	60	50	95	
Gamma Rays	40	90	60	80	
Temozolamide	80	70	80	40	
Mitosis Count/5HPFs (X400)					
Control	12	18	15	15	
5FU	15	22	8	8	
Gamma Rays	25	5	27	35	
Temozolamide	15	18	17	13	

B: CABA (p53 mutant/RER+)

Time (days)	0.75	3.5	7	10	S.I.
Apoptosis Count/5HPFs (X400)					
Control	76	85	89	64	9
5-FU	71	96	88	died	53
Gamma Rays	64	101	85	84	46
Temozolomide	52	73	62	53	52
Necrosis (percent)					
Control	30	30	50	40	
5-FU	30	40	50	died	
Gamma Rays	40	50	D	50	
Temozolamide	D	30	30	50	
Mitosis Count/5HPFs (X400)					
Control	26	22	24	18	
5-FU	5	18	21	died	
Gamma Rays	15	20	18	21	
Temozolomide	28	16	23	25	

Table 5.2 (continued)
C: JOTH (p53 wild-type/RER+)

Time (days)	0.75	3.5	7	10	S.I.
Apoptosis Count/5HPFs (X400)					
Control	60	53	60	99	1
5-FU	87	241	289	138	49
Gamma Rays	61	209	157	98	53
Temozolamide	65	101	93	91	53
Necrosis (percent)					
Control	35	35	45	55	
5-FU	25	30	85	70	
Gamma Rays	30	40	80	50	
Temozolamide	30	75	50	40	
Mitosis Count/5HPFs (X400)					
Control	22	9	15	15	
5-FU	6	10	20	17	
Gamma Rays	16	43	46	22	
Temozolomide	26	21	24	43	

D: HEKI (p53 mutant/RER-)

Time (days)	0.75	3.5	7	10	S.I.
Apoptosis Count/5HPFs (X400)					
Control	38	40	39	46	3
5-FU	69	54	47	59	63
Gamma Rays	42	98	326	died	40
Temozolomide	41	42	48	56	39
Necrosis (percent)					
Control	20	30	15	50	
5-FU	25	30	85	70	
Gamma Rays	10	30	85	died	
Temozolamide	10	15	20	15	
Mitosis Count/5HPFs (X400)					
Control	10	6	10	5	
5-FU	6	10	20	17	
Gamma Rays	6	3	7	died	
Temozolomide	6	3	4	5	

All the control sets from any xenograft showed the same range of values for any of the three parameters: apoptosis, mitosis and necrosis. Therefore, only one control value showing the widest range is presented for each xenograft. S.I., mouse small intestinal apoptotic count/10 crypts at 0.75 day; D, difficult to assess due to biopsy fragmentation and absence of tumour xenograft full face on H&E-stained sections.

Figure 5.1

Clonogenic survival assay

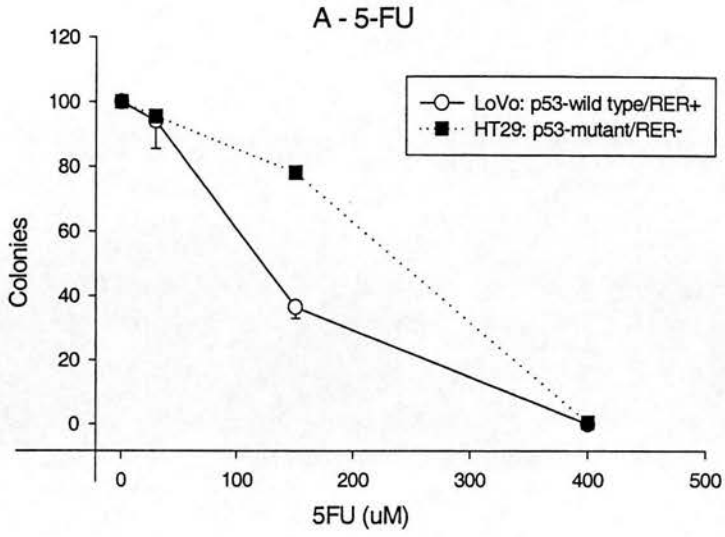
A 5-FU, B ionizing radiation, C temozolomide

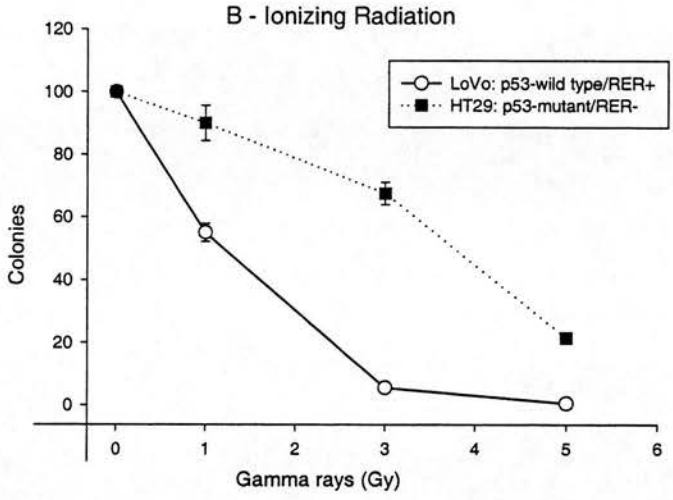
The clonogenic assay was performed in triplicate flasks and treatment responses are shown as mean ± S.E.M of number of surviving colonies relative to 100 colonies in controls. When the control flasks yielded slightly less or more than the standard 100 hundred colonies, the values from treated flask were corrected accordingly.

D Responses of the large and small HT29 colonies to ionizing radiation

Different responses (mean ± SEM)of the large and small HT29 colonies to ionizing radiation are demonstrated. There is progressive reduction in the number of large colnies with increasing doses of ionizing radiation. The small colonies are more resistant to the lower doses of ionizing radiation.

Figure 5.1


A - 5-FU
LoVo: p53-wild type/RER+
HT29: p53-mutant/RER-
Colonies
120
100
80
60
40
20
0
0
100
200
300
400
500
5FU (uM)


B - Ionizing Radiation
LoVo: p53-wild type/RER+
HT29: p53-mutant/RER-
Colonies
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Gamma rays (Gy)

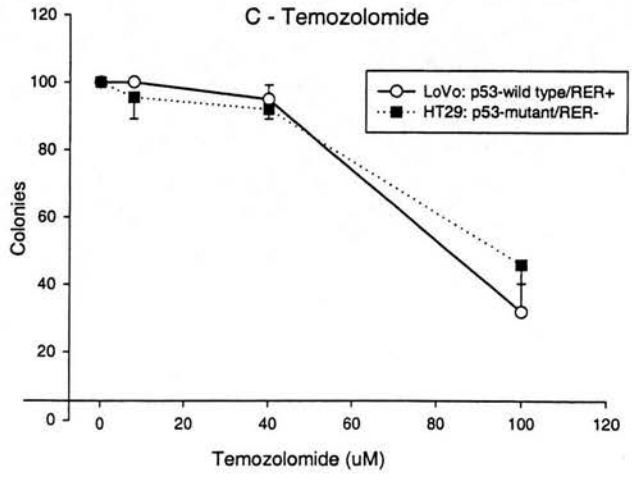

C - Temozolomide
LoVo: p53-wild type/RER+
HT29: p53-mutant/RER-
Colonies
120
100
80
60
40
20
0
0
20
40
60
80
100
120
Temozolomide (uM)

Figure 5.1 D: HT29 response to ionizing radiation according to colony size

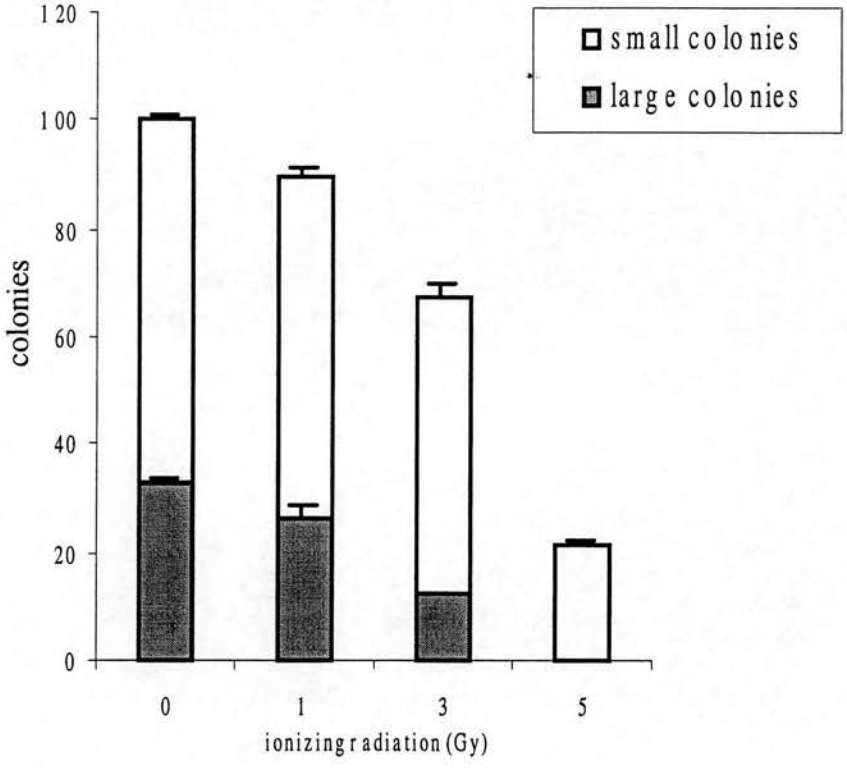


Figure 5.2

Representative flasks showing the clonogenic assay for the HT29 cell line treated with ionizing radiation. Progressive reduction in the number of the large colonies is observed with increasing doses of ionizing radiation, but the small colonies are more resistant to treatment at lower doses.

Figure 5.2

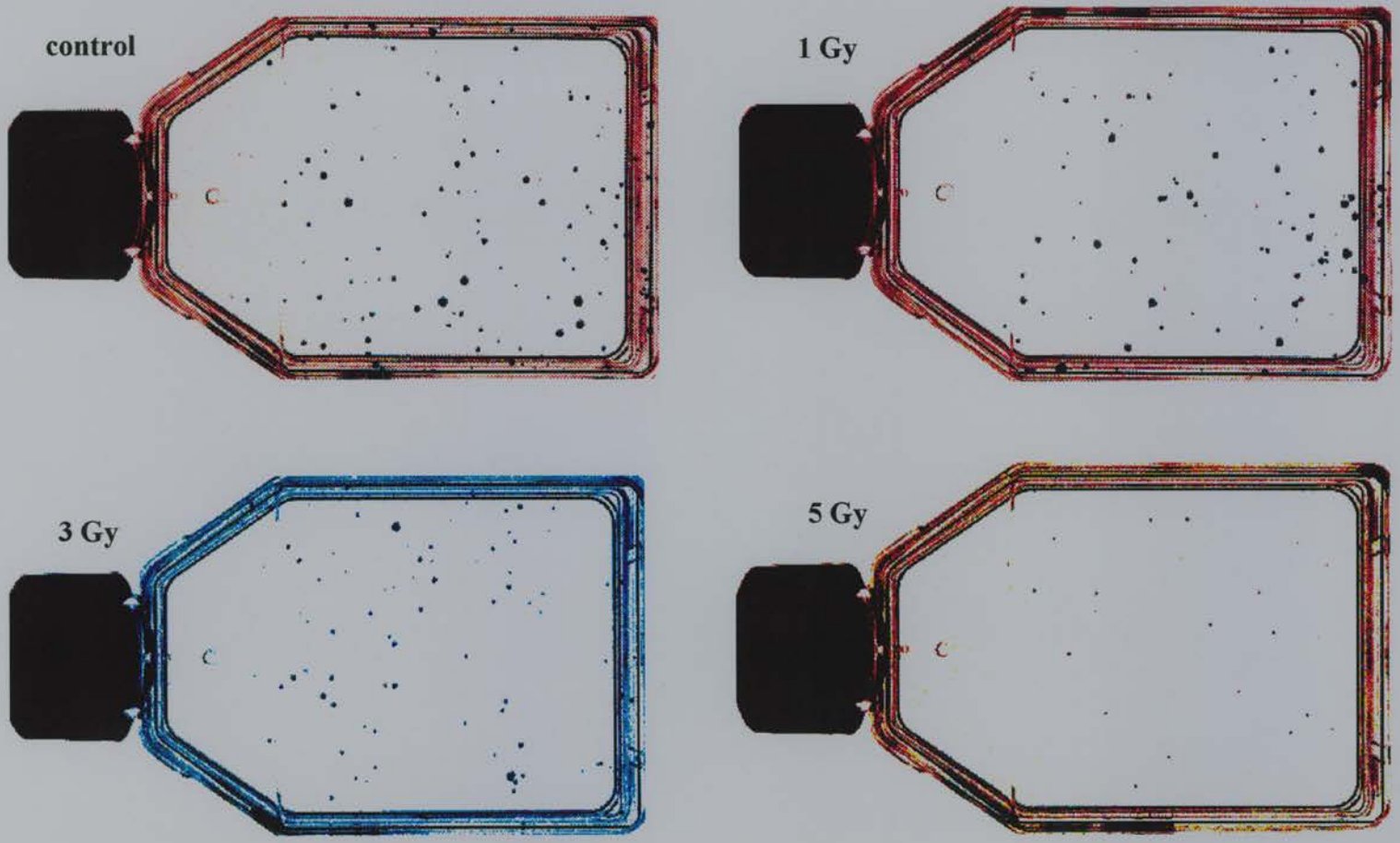


Figure 5.3

Photomicrographs showing different examples of apoptotic cells as detected by Acridine Orange staining and UV fluorescence microscopy of the cell line LoVo after treatment with different therapies (**A – D:** Control, 5-FU, IR and temozolomide respectively). Arrows indicate normal cells and arrow-heads indicate apoptotic cells with condensed nuclear chromatin or nuclear fragmentation (magnification = x400).

Figure 5.3

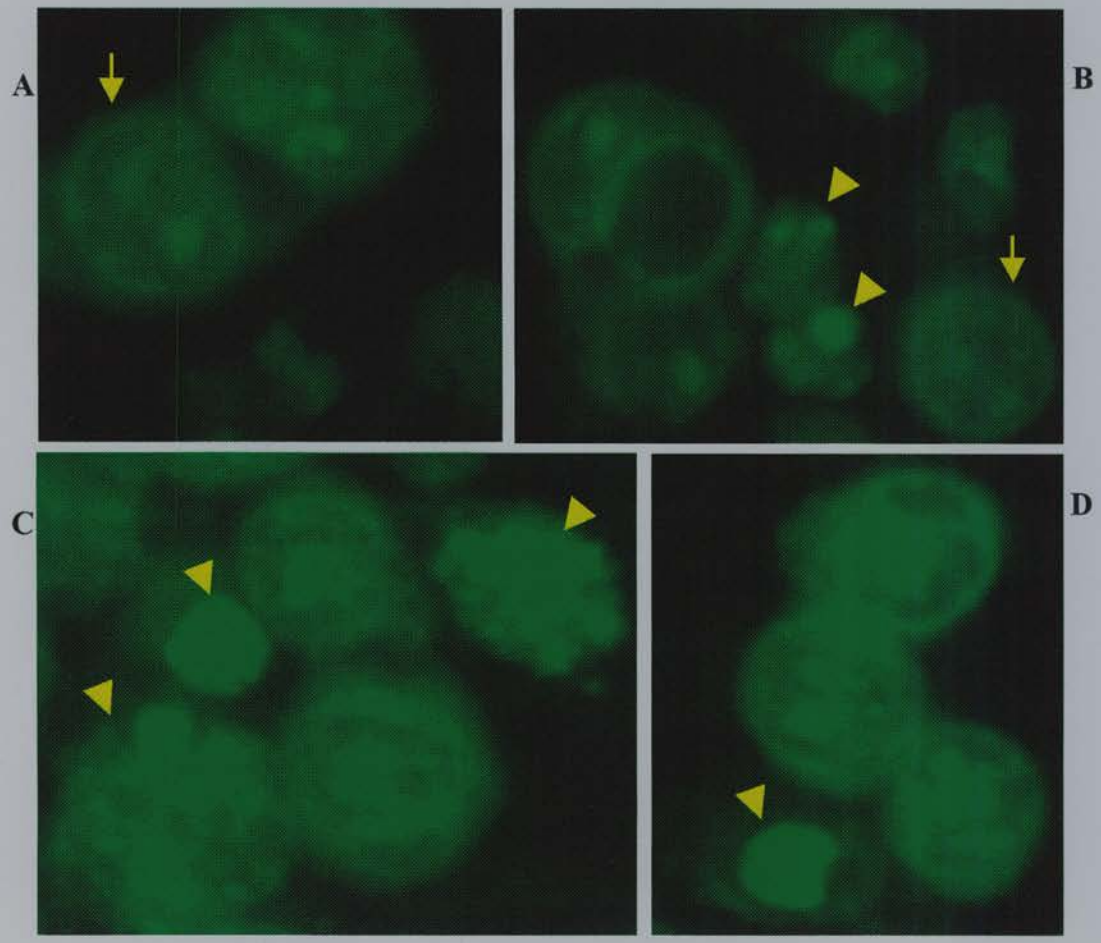


Figure 5.4 A – C

Graphic illustrations of the proportions of apoptotic cells detected by Acridine orange staining of the cell lines LoVo and HT29 3.5 days after treatment with different doses of each therapeutic agent (mean ± SEM of three estimations of apoptotic count).

Figure 5.4 D

Scatter plot comparing apoptotic responses to colony number observed in the clonogenic survival assays of cell lines LoVo and HT29 after treatment with 5-FU, ionizing radiation or temozolomide. The trend-line (regression equation: $y = -2.2565x + 110.19$, $p < 0.001$) indicates a strong inverse relationship between apoptotic counts and clonogenic assay data ($R^2 = 0.8688$).

Figure 5.4

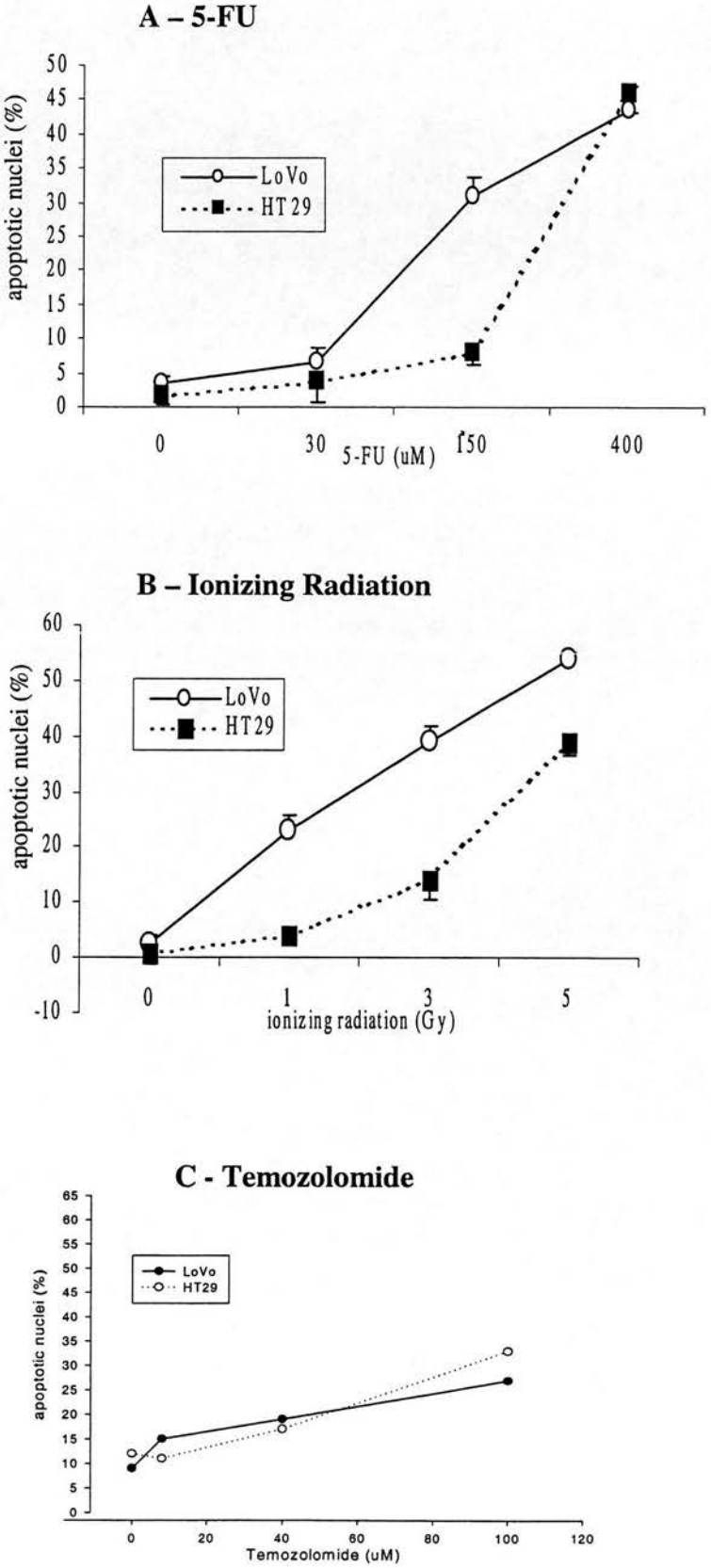

A – 5-FU
apoptotic nuclei (%)
LoVo
HT29
5-FU (uM)
B – Ionizing Radiation
apoptotic nuclei (%)
LoVo
HT29
ionizing radiation (Gy)
C - Temozolomide
apoptotic nuclei (%)
LoVo
HT29
Temozolomide (uM)

Figure 5.4 D: Scatter plot comparing apoptotic responses to clonogenic assay data of LoVo and HT29 after treatment with the three therapeutic agents.

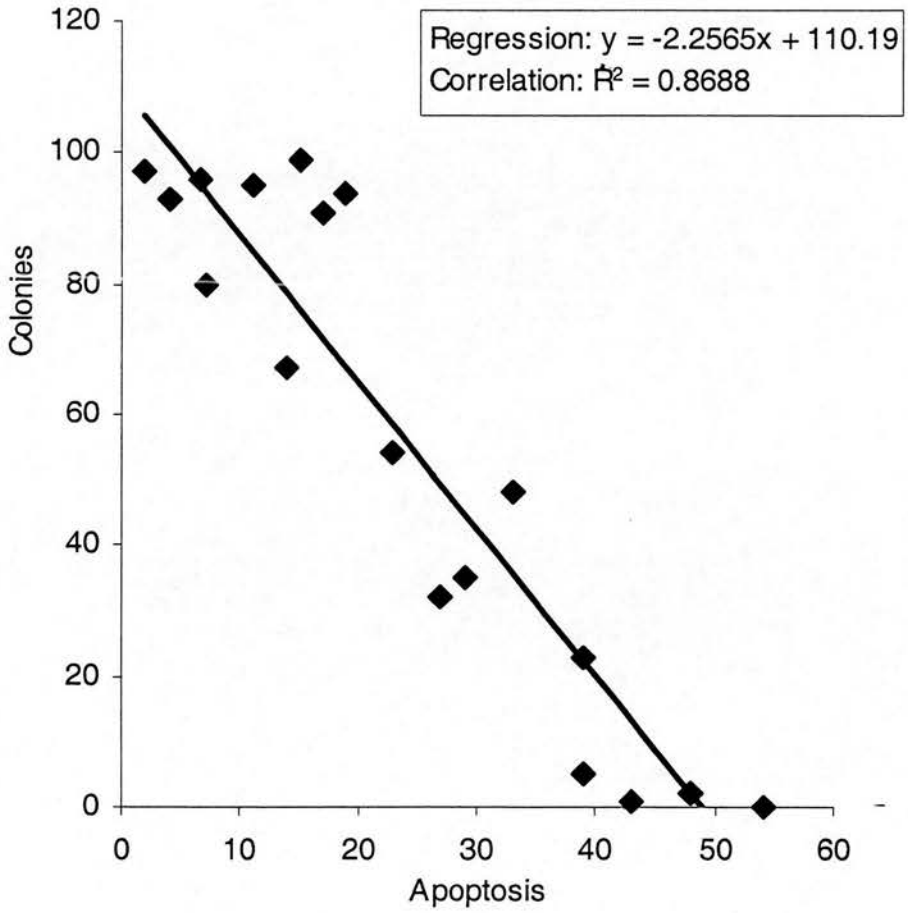


Figure 5.5 ANSC, Figure 5.6 CABA, Figure 5.7 JOTH, Figure 5.8 HEKI

Photomicrographs of representative areas showing the apoptotic responses of the xenografts to therapies *in vivo*. A, control; B, 5-FU; C, γ-irradiation and D, temozolomide (H&E, magnification x400). Arrows indicate apoptotic cells. Double-headed arrows indicate mitoses.

Figure 5.5

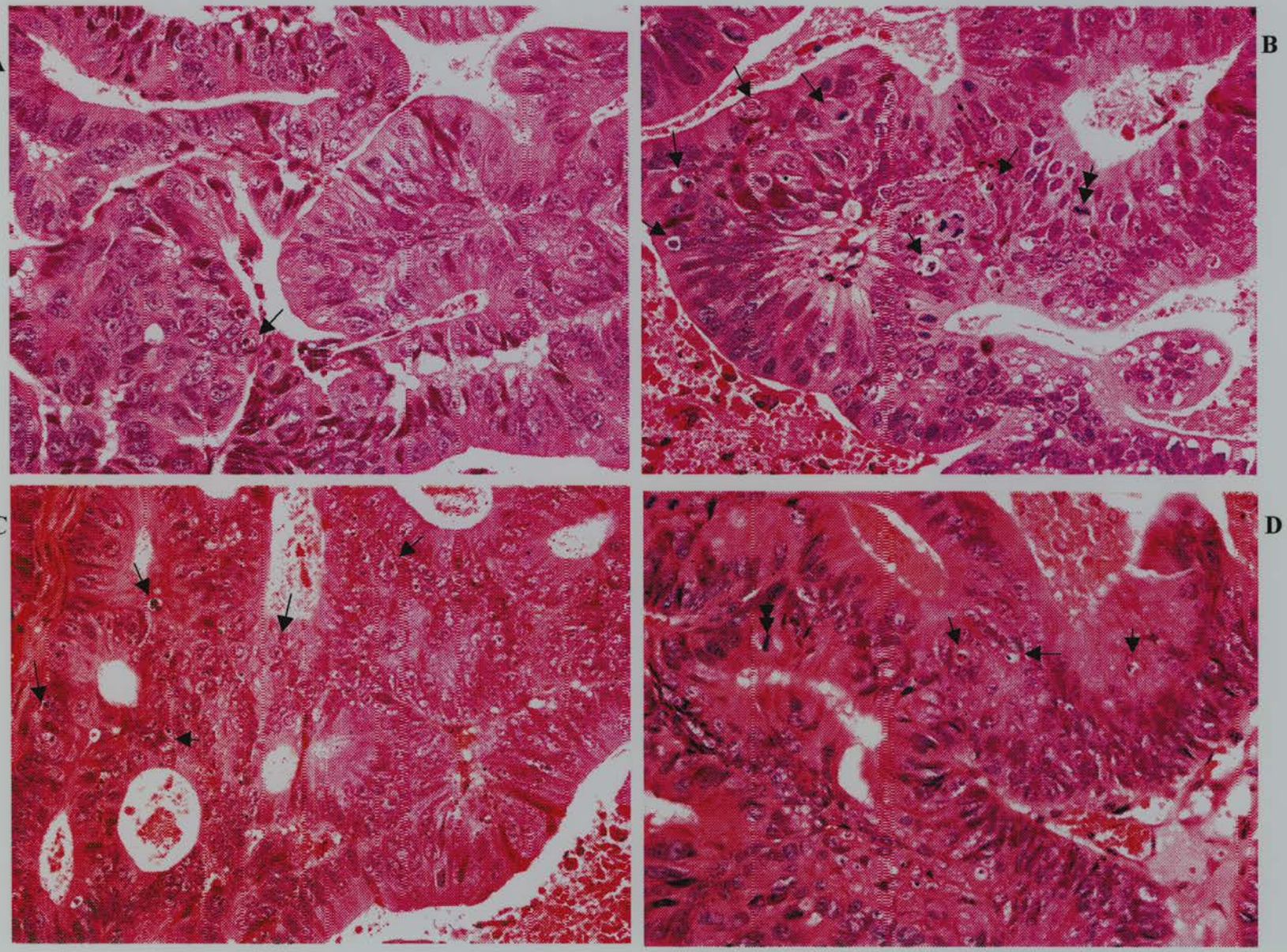


Figure 5.6

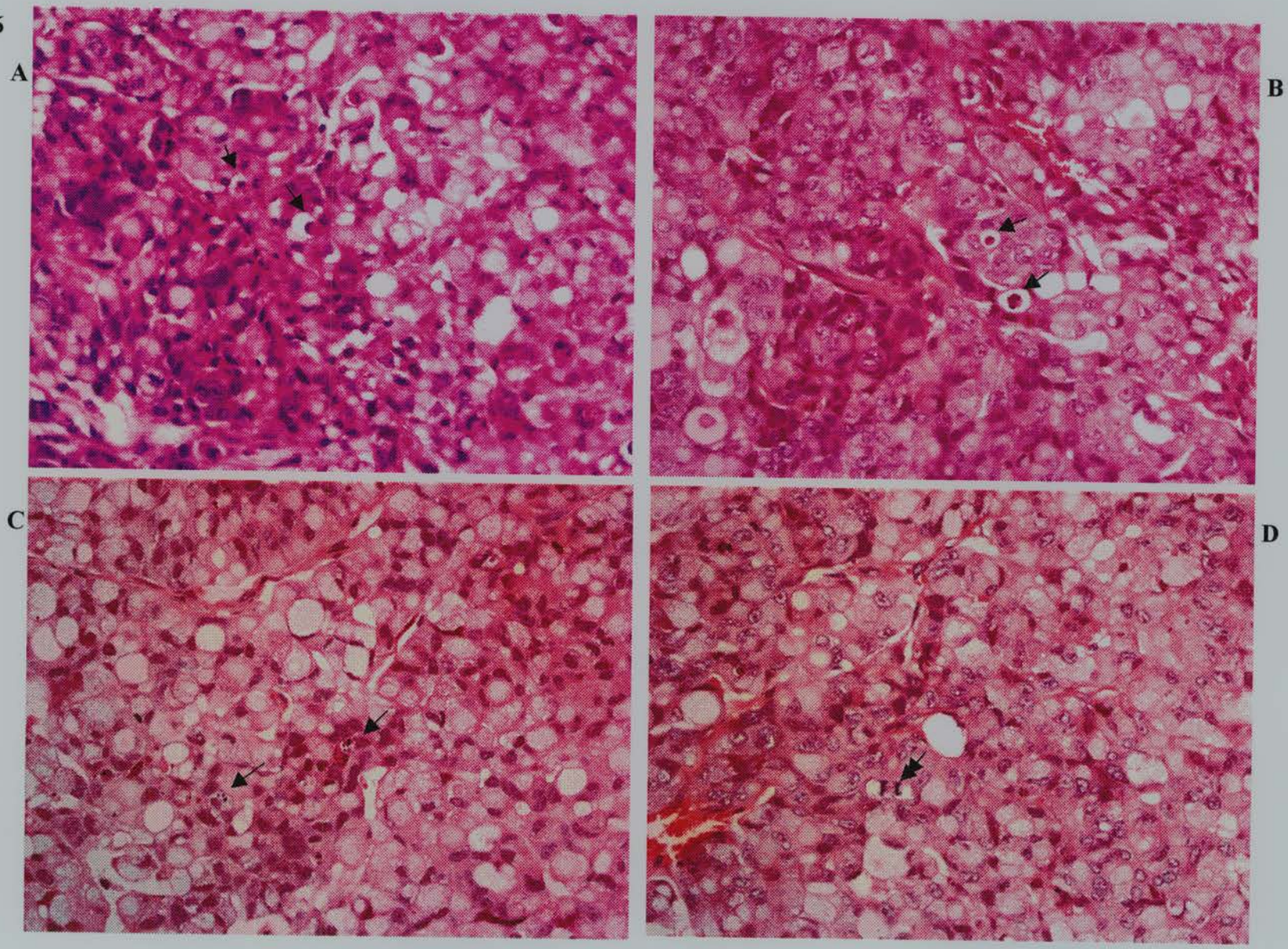


Figure 5.7

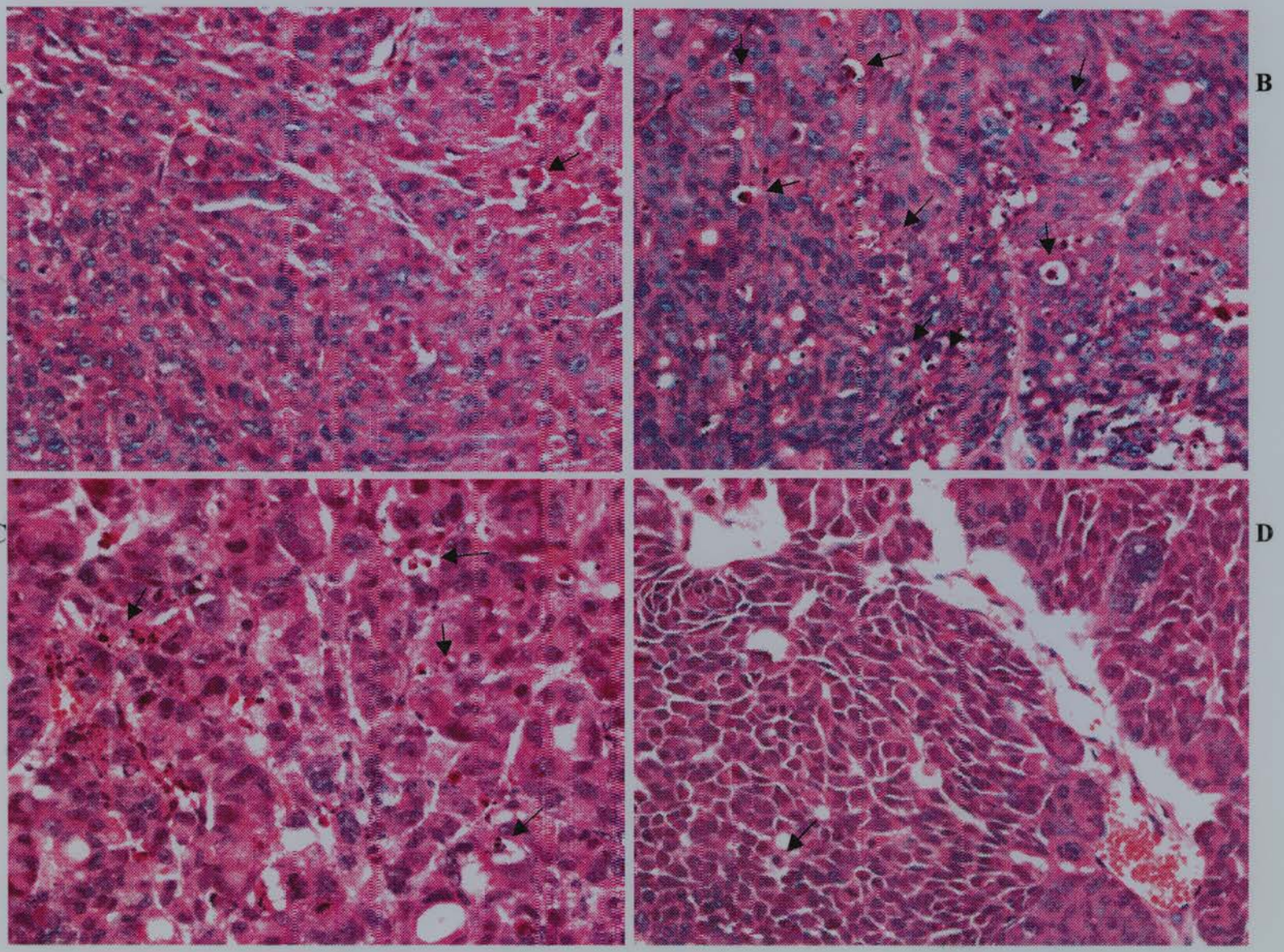


Figure 5.8

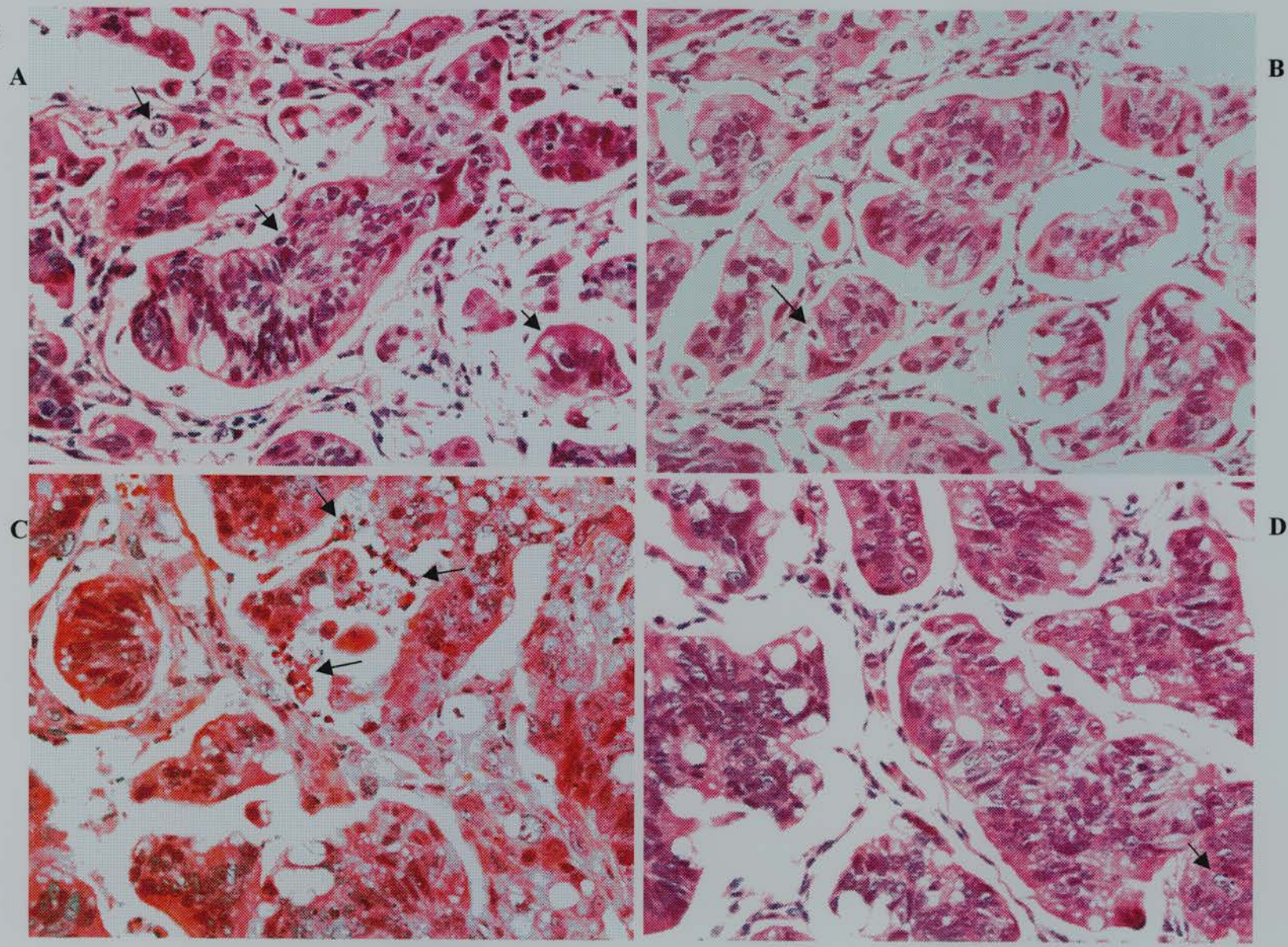


Figure 5.9 A – C

Graphic representation of the apoptotic responses of the tumour xenografts to each therapeutic agent: A 5-FU, B ionizing radiation (IR), C temozolomide

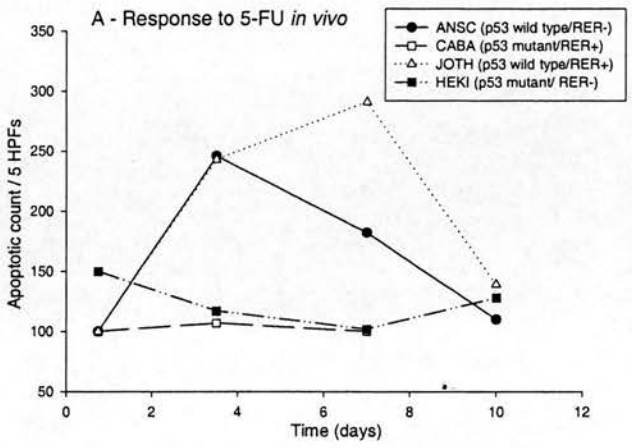
To allow comparison between different tumour xenografts and different therapeutic agents, the test data (number of apoptotic cells/5 high power fields [HPFs] for each tumour xenograft at each time point in response to treatment with 5-FU, IR and temozolomide) were adjusted to take into account the differences between the background apoptotic levels observed in the controls for each tumour xenograft as follows: the highest apoptotic count observed in any one set of controls from a single tumour xenograft treated with one therapeutic agent was converted to 100 and the test apoptotic counts/5 HPFs at each time point from the treated xenografts were converted by the same factor so that they are expressed as a percentage of this control. Two mice died before reaching day 10: the CABA/5-FU-treated mouse and HEKI/IR-treated mouse.

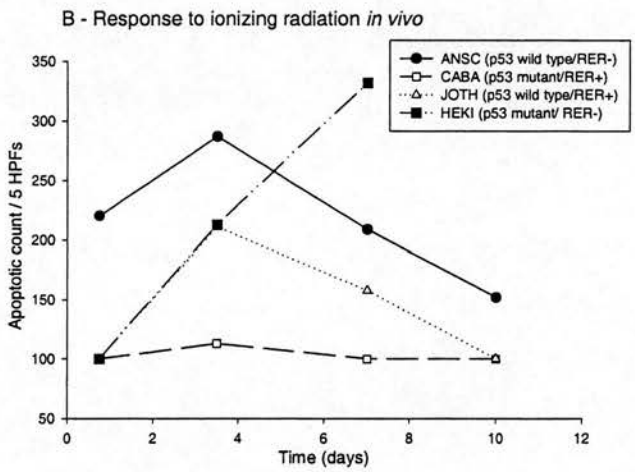
Figure 5.9 D

Apoptotic counts in murine small intestinal epithelium

Murine small intestinal apoptotic count/10 crypts at 0.75 day after application of therapies are shown for mice bearing the 4 tumour xenografts.

Figure 5.9


A - Response to 5-FU *in vivo*
ANSC (p53 wild type/RER-)
CABA (p53 mutant/RER+)
JOTH (p53 wild type/RER+)
HEKI (p53 mutant/ RER-)
Apoptotic count / 5 HPFs
Time (days)


B - Response to ionizing radiation *in vivo*
ANSC (p53 wild type/RER-)
CABA (p53 mutant/RER+)
JOTH (p53 wild type/RER+)
HEKI (p53 mutant/ RER-)
Apoptotic count / 5 HPFs
Time (days)

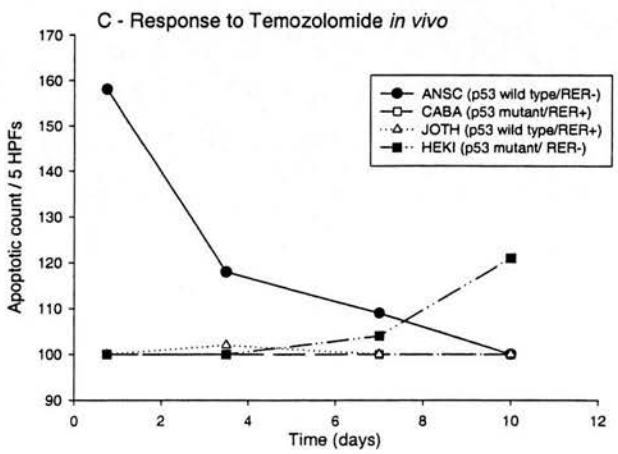
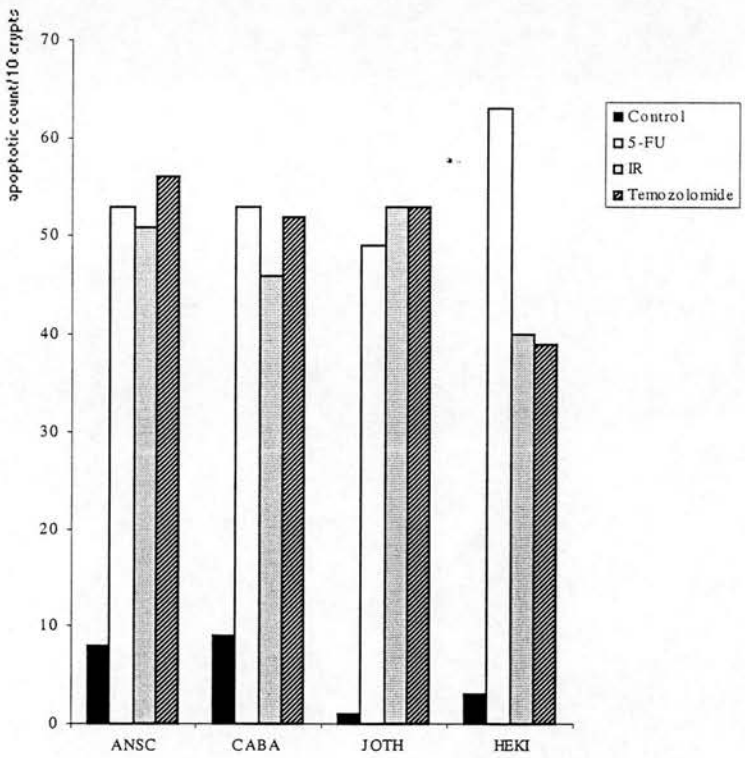

C - Response to Temozolomide *in vivo*
ANSC (p53 wild type/RER-)
CABA (p53 mutant/RER+)
JOTH (p53 wild type/RER+)
HEKI (p53 mutant/ RER-)
Apoptotic count / 5 HPFs
Time (days)

Figure 5.9 D

Apoptotic counts in murine small intestinal epithelium



5.4. Discussion

5.4.1 Comparison of the *in vivo* and *in vitro* responsiveness to cytotoxic treatment

Both of the responses *in vitro* to therapeutic agents determined by counting of apoptosis by fluorescence microscopy with Acridine Orange staining and the reduction of clonogenic survival (Figures 5.1 & 5.4) independently demonstrated that LoVo is more sensitive to both 5-FU and IR and that both cell lines were similarly resistant to temozolomide (Figure 5.4 D). This correlation between apoptotic responses *in vitro* to therapeutic agents and the reduction of clonogenic survival suggest that the apoptotic responses of such tumour cells may be representative of their overall response to therapy. Previous reports have suggested that *in vivo* apoptosis frequency might be, in some cases, a better predictor of drug responsiveness than colony formation (Lamb & Friend, 1997; Waldman *et al.*, 1997; Bunz *et al.*, 1999). In these experiments the apoptotic responses obtained from the two p53/RER combinations studied *in vitro* were broadly similar to the patterns of apoptotic responses obtained *in vivo* from tumours with the equivalent combinations—HT29 (p53 mutant/RER-) compared to HEKI (p53 mutant/RER-) as both showed a low or absent apoptotic responses to 5-FU, and LoVo (p53 wild type/RER+) compared to JOTH (p53 wild type/RER+) as both showed a higher response to 5-FU. This consistency adds weight to the validity of the data obtained in this study and indicates the relevance of combining the results for those p53/RER combinations studied both *in vivo* and *in vitro*.

5.4.2 Apoptotic responses to 5-FU are associated with p53 status

Responses to 5-FU in this study correlated strongly with wild–type p53 status. In the absence of wild type p53 cells were strikingly resistant to the effects of 5-FU both *in vitro* (HT29) and *in vivo* (CABA and HEKI tumour xenografts). The mismatch repair status did not show any significant influence on the responses to 5-FU in the absence of wild type p53. The data obtained here are in accord with those in normal murine intestinal epithelium which showed that p53 deficiency leads to decreased apoptosis after treatment with 5-FU and that the p53-dependent apoptosis after treatment with 5-FU reflected inhibition of RNA metabolism rather than DNA metabolism (Pritchard *et al.*, 1997; Pritchard *et al.*, 1998). These observations in murine intestinal epithelium together with the data presented here in human colorectal cancer cells indicate the fundamental importance of wild type p53 to the apoptotic response to 5-FU. It might be significant that

5-FU effects on RNA metabolism were previously suggested to be independent of TS (Cory *et al.*, 1979; Ghoshal & Jacob, 1997) and clinical studies showed that the effects of 5-FU on RNA metabolism were the most important mode of action for treatment by bolus schedules but not continuous infusion (Harstrick *et al.*, 1998). The data obtained in this experiment are also in strong agreement with a recent report showing that p53 disruption rendered cells strikingly resistant to the cytotoxic effects of 5-FU both *in vitro* and *in vivo* (Bunz *et al.*, 1999). This report also showed that the effects of 5-FU were independent of the cyclin dependent kinase inhibitor p21, and appeared to be the result of perturbation of RNA metabolism rather than inhibition of TS and, subsequently, DNA metabolism. Furthermore, the report demonstrated also that p53–dependent cell death following 5-FU treatment was apoptotic in requiring active biosynthesis of cellular suicide proteins (Bunz *et al.*, 1999). Overall, these data justify further work to elucidate the molecular mechanisms underlying the 5-FU mediated induction of p53-dependent apoptosis.

5.4.3 IR induces apoptosis via alternative pathways

Apoptotic responses and reduction of clonogenicity after IR in these experiments were associated with presence of either wild type p53 or proficient mismatch repair or both together, in that only the CABA xenograft tumour (p53 mutant/RER+) showed no increase in apoptosis above base line levels. Although, it is difficult to draw conclusions regarding the link between the amplitude of the apoptotic response and the underlying p53/RER combination (Figure 5.9), some comments are appropriate. The *in vivo* experiments demonstrated that the combined presence of wild type p53 and proficient mismatch repair (ANSC) gave the earliest response at 0.75 day as well as the strongest response at 3.5 days which subsequently declined; while the p53 mutant–mismatch repair proficient tumour (HEKI) showed a progressive increase in apoptotic response which continued up to day seven (the mouse harbouring the tumour to be harvested at day 10 died before completion of this experiment). This pattern suggested that absence of wild type p53 may sensitise tumours that possess alternative pathways for responses to IR such as the mismatch repair system. This is in accord with, and may further explain, previously published work which has highlighted the conflicting results on the relationship between p53 status and response to IR (Slichenmyer *et al.*, 1993; Lowe *et al.*, 1994; Bracey *et al.*, 1995; Bristow *et al.*, 1996; Yang *et al.*, 1996; Bunz *et al.*, 1999). However, the lower but still increasing *in vitro* apoptotic responses to IR of the HT29 cell line (p53 mutant/RER- tumour) indicate a poorer correlation between *in vitro* and *in vivo* behaviour of p53 mutant tumour cells. The

discrepancy between the *in vivo* and *in vitro* responses even for the same tumour has been documented in the literature and the *in vivo* responses are considered to be more informative (Lamb & Friend, 1997; Waldman *et al.*, 1997; Bunz *et al.*, 1999). Furthermore, it was observed in this work that the HT29 cell line gave rise to different-sized colonies one small and the other large. The large colonies showed progressive reduction in frequency with increasing doses of irradiation, and at 5 Gy they disappeared completely. The small-sized HT29 colonies were more resistant and formed all the surviving colonies at 5 Gy. This observation raises interesting issues regarding the effects of tumour heterogeneity on the responsiveness to treatment and further study of this particular line may reveal the basis for such clonal heterogeneity and its influence on the responses to treatment.

Absence of both wild type p53 and proficient mismatch repair in the same xenograft tumour (CABA) was associated with a very poor apoptotic response to IR. This finding suggests a critical role for the mismatch repair system in the apoptotic response to IR in the absence of wild type p53.

5.4.4 Colorectal cancers appear to be resistant to temozolomide

Apoptotic responses and reduction of clonogenicity after temozolomide treatment were insignificant, apart from the response of the p53 wild type/RER- tumour (ANSC). However, this apoptotic response of ANSC was weaker, and declined faster than those responses observed after the other two therapeutic agents. This may represent the expected response from a single bolus dose considering the very short half-life of temozolomide (Stevens *et al.*, 1987; Newlands *et al.*, 1996). The lack of other tumours of this particular phenotype in this study, and generally in colorectal cancers, makes it difficult to judge the significance of this early apoptotic response. The data obtained here suggest that colorectal cancer responses to temozolomide may be dependent on the presence of wild type p53, together with proficient mismatch repair. Previous studies have shown that DNA methylator damage, as seen with temozolomide treatment, induces hMutSα and hMutLα-dependent phosphorylation of p53 (Duckett *et al.*, 1999). Recent reports in the literature also suggest that wild type p53 potentiates the killing effects of the methylating agents in mouse small intestine (Toft *et al.*, 1999), and in human tumour cells (Tentori *et al.*, 1998; Duckett *et al.*, 1999).

5.5 Conclusions

It is important to bear in mind that responses to therapeutic agents are complex and unlikely to be completely explained by a single molecular alteration in a tumour. However, the data obtained here, combined with the available follow up information on two of these tumours, revealed significant effects of the p53 and RER status on responsiveness to treatments. These effects varied dramatically depending on the therapeutic agent. The data may explain previous reports about the effects of p53 status on the response to IR as it links it to the mismatch repair status: p53 wild type tumours are generally responsive to IR. However, the combined loss of p53 and mismatch repair rendered the tumours studied resistant to IR. The data also suggested a critical role for wild type p53 in the response to 5-FU. A reasonable overall interpretation is that tumours with mutant p53 appear to be less likely to be responsive to 5-FU and temozolomide than tumours with wild type p53. The mismatch repair status, combined with p53, can predict the response to temozolomide. Further experiments based on these data in appropriate cell line systems would help to dissect the complex relationships between the p53 status and the mismatch repair status in treatment responsiveness.

CHAPTER 6

SUMMARY, FINAL DISCUSSION AND FUTURE PROSPECTS

6.1 Summary and final discussion

This thesis has characterized patterns of genomic instability associated with sporadic colorectal cancers. The thesis has also examined mechanisms underlying deregulation of key apoptotic pathways and the impact of some aspects of tumour genotype on treatment responsiveness in sporadic colorectal cancers.

6.1.1 genomic instability in colorectal cancers

Application of FISH-based techniques in this study identified subsets of colorectal cancers characterized by patterns of chromosomal changes. RER- colorectal cancer cell lines usually displayed extensive numerical and structural chromosomal changes with marked intermetaphase heterogeneity. While most of the RER- lines showed a tendency to approach a near-triploid karyotype with multiple trisomies, a minor subgroup retained a near-diploid karyotype. Only one cell line was near-pentaploid and showed clear evidence of endoreduplication in the form of duplication of almost all abnormal chromosomes. The common feature of all of these subgroups of RER- cell lines was the presence of multiple forms of chromosomal instability including numerical chromosomal instability, structural chromosomal instability, numerical inter-metaphase heterogeneity and structural inter-metaphase heterogeneity. These different forms of instabilities were measured by a set of indices: numerical (aneuploidy) index, break index, numerical heterogeneity index and structural heterogeneity index (see Chapter 3, Table 3.4). These indices showed that some lines had a pronounced tendency to acquire particular forms of chromosomal instability but were relatively immune to other forms. This suggests that different forms of chromosomal instability arise through independent mechanisms and possible mechanisms for some of these have been discussed before: abnormalities of the mitotic machinery as a source of numerical chromosomal instability (see Chapter 1: 1.4.7.1), DNA double strand breaks, and changes to telomeric sequences and telomerase expression as a source of structural chromosomal instability (see Chapter 1: 1.4.7.2). However, the situation is much less clear regarding the mechanisms underlying the heterogeneity forms of instability. One possible explanation could be that the mechanisms responsible for numerical and structural chromosomal instabilities are the same ones that produce ongoing intermetaphase

heterogeneity. In that case, the heterogeneity reflects a survival advantage of the new clone or an equal survival capability of the new clone to that of the existing clones. More plausible explanations exist for both forms of heterogeneity, however. The marked numerical heterogeneity is more likely to be associated with particular defects in the mitotic check point proteins including Chfr, the BUB family and EB1 as discussed previously (see 1.4.7.1.4). Marked structural heterogeneity may be due to an increased rate of DNA double-strand break events, which could be due to multiple factors including endogenous or exogenous toxins, drugs and ionizing radiations as well as genetic alterations such as telomerase deficiency. Recent data have shown that telomere attrition in aging telomerase-deficient, *p53* mutant mice promoted the development of epithelial cancers via the formation of complex non-reciprocal translocations (Artandi *et al.*, 2000). This led to the speculation that telomerase dysfunction early in the process of carcinogenesis may lead to tumour development via the generation of multiple structural chromosomal changes if permitted by *p53* mutations (Hanahan, 2000).

The RER+ lines examined here usually showed stability of both chromosome number and structure. However, this turned out to be just an association, as extensive chromosomal instability was identified in 3 out of 8 RER+ lines, one of which showed a near-triploid pattern as commonly seen in RER- tumours. Thus, microsatellite instability did not preclude chromosomal instability, although it may possible that in most instances the combined effect of both instabilities may generate too much instability beyond that which is consistent with tumour cell survival and growth.

Almost half of the cell lines studied here were chosen to have no evidence of *p53* mutations. This group of cell lines demonstrated chromosomal instability suggesting that *p53* mutation by itself is not necessary for the production of chromosomal instability. Furthermore, *p53* mutations were present in some of the RER+ lines, but were not associated with any chromosomal instability, suggesting that *p53* mutations are also not sufficient for the production of chromosomal instability. However, the evidence that has been accumulated in the literature associating p53 abnormalities with aneuploidy could be compatible with p53 abnormalities being permissive for some forms of chromosomal instability, such as those chromosomally unstable tumours that were produced in telomerase-deficient, *p53* mutant mice (Artandi *et al.*, 2000).

SKY has also revealed the existence of a new type of instability in colorectal cancers characterised by multiple reciprocal translocation events. Two RER+ lines were near-diploid but showed a striking and previously unreported tendency to acquire balanced translocations. Previous work has suggested a role for the mismatch repair system in maintaining the fidelity of the homologous recombination repair of DNA double-strand breaks (de Wind *et al.*, 1995; Schimenti, 1999). Therefore, it was possible to speculate that this predisposition to balanced translocations is a consequence of the mismatch repair deficiency leading to subsequent failure to recognize a *homeologous* (in contrast to homologous) recombination repair of DNA breaks joining imperfectly matching DNA sequences (Jasin, 2000). Combined to this, it is also possible that this balanced translocation phenotype may be related to particular types of DNA insults. It has been suggested that the balanced and unbalanced translocations in therapy-related acute myeloid leukaemia develop in different ways depending on the therapeutic agent used. Balanced chromosomal aberrations were the result of illegitimate DNA recombinations related to the activity of DNA-topoisomerase II (Pedersen-Bjergaard & Rowley, 1994). This may be applicable as well to the balanced translocations observed here in the colorectal cancer cells, as the original neoplasms were continuously exposed to various intestinal toxins which might have played a role in their initial development (as explained in 1.1.4). Furthermore, some toxic agents might have been used for the treatment of the two tumours from which these lines were established. Although such data are not available, it could be quite possible in case of the LoVo cell line as it was established from a lymph node metastasis (Drewinko *et al.*, 1976).

Some patterns of chromosomal instability identified here were not possible without events that are likely to cause apoptosis, such as DNA double-strand breaks, mismatches produced through illegitimate DNA recombinations and probably non-disjunction and other mitotic defects responsible for numerical chromosomal alterations. These patterns of chromosomal instabiliy also appear to give rise to repeated genomic alterations, albeit constrained in type, presumably reflecting deficient pathways for recognition and removal of the altered cells by apoptosis. As it has been discussed previously, several molecules appear to link apoptotic pathways to pathways implicated in the production of some forms of chromosomal instability. Thus, survivin, BUB1, Bcl-2 and Fas all feature in pathways linking apoptosis to the mitotic machinery (see Introduction: 1.4.7.1.5). p53 and other downstream apoptotic molecules such as Bax are clearly involved in pathways responsible for removal of cells with damaged DNA, which otherwise are likely to produce different

sorts of structural chromosomal rearrangements (see Introduction: 1.2.3). Hence, tumours arising in this way could be more resistant to cytotoxic therapeutic agents due to loss of key apoptotic pathways—or such therapies may even trigger genetic alteration contributing to further neoplastic progression. No clear relationship was detected here between *p53* mutations and chromosomal instability. *Bax* gene mutations were recently identified in 3 of the RER+ lines used here (LoVo, LS174T, HCT116) (Carethers & Pham, 2000), but 2 of these lines displayed the marked karyotypic stability typical of an RER+ karyotype. However, the status of other candidate pro- or anti-apoptotic molecules that may play a role in chromosomal instability, such as survivin, remains to be explored in common cancers.

6.1.2 Apoptosis deregulation in colorectal cancers

Deregulation of key apoptotic pathways was studied by analysis of three apoptosis genes (*Fas, Bik* and *Bax*) and one growth control gene (*TGFβRII*) for mutations. Mutational inactivation of the *Fas* gene or mutations affecting the CTG tandem repeat element of the *Bik* gene were not found and thus are unlikely to be critical events in colorectal carcinogenesis. Thus, the basis of Fas under-expression in colorectal cancers remains elusive. Recent work showed that p53 controls the levels of Fas expression (Maecker *et al.*, 2000), and this may be an explanation of why Fas receptor is under-expressed in a majority of colorectal carcinomas. However, pre- and post-transcriptional causes may also be responsible for Fas under-expression in colorectal carcinomas.

Although *Bax* gene mutations were found in 50% of the RER+ tumours studied here, they were not always homogeneously present at all sites of the same tumour. This could represent reversion of the original mutation through a second mutational event at the same site, as these tumours already have an elevated rate of mutation (100-300 fold compared to RER- tumours), especially at these sites of repetitive nucleotide tracts. Compared with the results from the *TGFβRII* gene, *Bax* mutations may be interpreted as simply a consequence of mismatch repair deficiency without a functional connotation. It is also possible that these late occurring *Bax* gene mutations help tumour progression by offering better survival advantage in terms of loss of the susceptibility to apoptosis. This could be particularly useful for tumour metastasis or tumours exposed to therapeutic agents. The higher incidence (75% of RER+ cancers) and the ubiquitous intra-tumoral presence of *TGFβRII* mutations found in this work indicate a significant role for such

mutations in RER+ colorectal carcinogenesis. Furthermore, inactivating mutations were found in *TGFβRII* in 15% of RER- tumours and a further 55% of RER- cancers demonstrated a TGFβ signalling blockade distal to TGFβRII, including inactivation of SMAD4 and SMAD2 (Grady *et al.*, 1999). These findings can be interpreted as indicative of a significant causal role for TGFβ pathway inactivation in colorectal carcinogenesis.

6.1.3 Effects of tumour genotype on treatment responsiveness

The combined data obtained from the *in vivo* and *in vitro* experiments in this thesis revealed significant effects of the p53 and RER status on tumour cell responsiveness to treatments. The data showed that wild type p53 is critical for tumour cell killing by the three therapeutic agents used here—5-FU, ionizing radiation (IR) and temozolomide. Wild type p53 was absolutely necessary for the 5-FU anti-tumour effects. Tumours lacking wild type p53 used alternative independent pathways including mismatch repair in response to IR-induced damage, and the combined loss of p53 and mismatch repair rendered the tumours studied here resistant to IR. Responses to temozolomide were dependent on the presence of both wild type p53 and functional mismatch repair. Accordingly, temozolomide or similar agents are less likely to be effective in the treatment of colorectal cancers as most of these tumours are deficient in p53 or mismatch repair or both.

6.2 Future Prospects

The colorectal cancer cell lines studied here were carefully selected to represent different combinations of p53 and RER status. The comprehensive karyotypic information provided here on these colorectal cancer cell lines together with other information assembled in Table 3.1 should serve further research in the field. One immediate aim would be to test if any of these patterns are more or less sensitive to therapy. Equally important would be to test if such treatments stimulate further chromosomal changes in these lines. SKY could be a useful tool to characterise the chromosomal changes after treatment with different therapies and compare it to the original SKY karyotypes. As it has been discussed before, there is evidence that balanced translocations appear to arise following treatment with particular therapeutic agents in haematological malignancies and possibly in one of the colorectal cancer cell lines studied here (see 6.1.1). This possibility raises the serious implication that cytotoxic treatments of such tumours may provoke accumulation of more genetic abnormalities resulting in progression to a more aggressive cancer. This possibility needs to be fully explored by testing the patterns of chromosomal changes in the surviving cells after application of a range of therapies to cells representative of the different patterns described here, particularly the RER+, chromosomally stable group. It may be necessary to test the invasive and metastatic behaviour of such tumours, which may develop via this pathway *in vivo* as well as *in vitro*.

Molecules implicated in the generation of chromosomal instability such as Chfr, the BUB family EB1 and telomerase should be candidates for future studies to understand the mechanisms of chromosomal instability. An attractive study would be analysis of the role of the mismatch repair system in homologous/homeologous recombination events in the generation of balanced translocations. Cloning of the break points involved in the consistently present balanced translocations may reveal sequences that are, for some reason, prone to balanced translocation events or it may reveal hybrid genes generated by these translocations with oncogenic potential similar to those observed in haemato-lymphoid malignancies (Rabbitts, 1994).

There are recognition sequences in the *Fas* promotor for a number of transcriptional proteins including p53, c-myb and NF-KB (Behrmann *et al.*, 1994)—the loss of any of which may reduce or prevent Fas expression. The *Fas* promotor also contains a number of CpG dinucleotides, which are targets for hypermethylation and subsequent reduction or loss of *Fas* expression. Therefore, future work could include

study of the status of the recognition sequences and of CpG hypermethylation in the *Fas* promotor region in colorectal cancers.

Future work could be done to evaluate the significance of *Bax* gene mutations in colorectal carcinogenesis by reintroducing wild type *Bax* into *Bax*-deficient tumour cells. Restoring wild type *TGFβRII* to cell lines lacking functional TGFβRII was found to be potentially growth suppressive (Grady *et al.*, 1999), and *TGFβRII* gene therapy may thus have a future role in the treatment of colorectal carcinoma tumours.

The data obtained in the thesis linking responses to 5-FU to p53 status deserve further work to clarify the basis of such a relationship and how widespread it is in primary or xenografted colorectal cancers. Study of larger number of tumours would be useful to establish the correlation between 5-FU responses and wild type p53. Other factors, which may also affect the response to 5-FU such as thymidylate synthase, could be studied in relation to p53 status in the process of cellular responsiveness to 5-FU in a suitable cell line system.

The accumulated evidence indicates an association between lack of functional p53 and inability or reduced capability of tumour cells to undergo apoptosis in response to therapies. Therefore, restoration of normal p53 functions in tumours might restore the apoptotic pathways and lead to increased responsiveness to conventional therapeutics. In the past few years, the development of methods for the introduction of genes such as *p53* into tumour cells *in vivo* has made gene therapy intervention a possibility for the future (Chang *et al.*, 2000).

The information obtained here from the series of treatment responsiveness experiments suggest that it may be possible to subclassify colorectal tumours into groups regarding the combined status of both p53 and mismatch repair in order to predict which tumours are most likely to benefit from a particular therapeutic agent.

BIBLIOGRAPHY

Aaltonen, L.A., Peltomaki, P., Leach, F.S., Sistonen, P., Pylkkanen, L., Mecklin, J.P., Jarvinen, H., Powell, S.M., Jen, J., Hamilton, S.R., Petersen, G.M., Kinzler, K.W., Vogelstein, B. & Delachapelle, A. (1993). Clues to the pathogenesis of familial colorectal cancer. *Science*, **260,** 812-816.

Adeyinka, A., Kytola, S., Mertens, F., Pandis, N. & Larsson, C. (2000). Spectral karyotyping and chromosome banding studies of primary breast carcinomas and their lymph node metastases. *International Journal of Molecular Medicine*, **5,** 235-240.

Aebi, S., KurdiHaidar, B., Gordon, R., Cenni, B., Zheng, H., Fink, D., Christen, R.D., Boland, C.R., Koi, M., Fishel, R. & Howell, S.B. (1996). Loss of DNA mismatch repair in acquired resistance to cisplatin. *Cancer Research*, **56,** 3087-3090.

Albrechtsen, N., Dornreiter, I., Grosse, F., Kim, E., Wiesmuller, L. & Deppert, W. (1999). Maintenance of genomic integrity by p53: complementary roles for activated and non-activated p53. *Oncogene*, **18,** 7706-7717.

Alderson, M.R., Tough, T.W., Davissmith, T., Braddy, S., Falk, B., Schooley, K.A., Goodwin, R.G., Smith, C.A., Ramsdell, F. & Lynch, D.H. (1995). Fas ligand mediates activation-induced cell death in human T- lymphocytes. *Journal of Experimental Medicine*, **181,** 71-77.

AlMulla, F., Keith, W.N., Pickford, I.R., Going, J.J. & Birnie, G.D. (1999). Comparative genomic hybridization analysis of primary colorectal carcinomas and their synchronous metastases. *Genes Chromosomes and Cancer*, **24,** 306-314.

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. & Yuan, J. (1996). Human ICE/CED-3 protease nomenclature. *Cell*, **87,** 171.

Altieri, D.C. & Marchisio, P.C. (1999). Survivin apoptosis: An interloper between cell death and cell proliferation in cancer. *Laboratory Investigation*, **79,** 1543.

Anthoney, D.A., McIlwrath, A.J., Gallagher, W.M., Edlin, A.R.M. & Brown, R. (1996). Microsatellite instability, apoptosis, and loss of p53 function in drug-resistant tumor cells. *Cancer Research*, **56,** 1374-1381.

Arends, J.W. (2000). Molecular interactions in the Vogelstein model of colorectal carcinoma. *Journal of Pathology*, **190,** 412-416.

Arends, M.J., McGregor, A.H., Toft, N.J., Brown, E.J.H. & Wyllie, A.H. (1993). Susceptibility to apoptosis is differentially regulated by *C-Myc* and mutated *ha-Ras* oncogenes and is associated with endonuclease availability. *British Journal of Cancer*, **68,** 1127-1133.

Arends, M.J. & Wyllie, A.H. (1991). Apoptosis: mechanisms and roles in pathology. *International Review of Experimental Pathology*, **32,** 223-254.

Artandi, S.E., Chang, S., S-L, L., Alson, S., Gottlieb, G.J., Chin, L. & DePinho, R.A. (2000). Teleomere Dysfunction Promotes non-reciprocal translocations and epithelial cancers in mice. *Nature*, **446,** 641-645.

Arvand, A., Bastians, H., Welford, S.M., Thompson, A.D., Ruderman, J.V. & Denny, C.T. (1998). EWS/FLI1 up regulates mE2-C, a cyclin-selective ubiquitin conjugating enzyme involved in cyclin B destruction. *Oncogene*, **17,** 2039-2045.

Asada, M., Yamada, T., Ichijo, H., Delia, D., Miyazono, K., Fukumuro, K. & Mizutani, S. (1999). Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. *EMBO Journal*, **18,** 1223-1234.

Ashkenazi, A. & Dixit, V.M. (1999). Apoptosis control by death and decoy receptors. *Current Opinion in Cell Biology*, **11,** 255-260.

Bakalkin, G., Selivanova, G., Yakovleva, T., Kiseleva, E., Kashuba, E., Magnusson, K.P., Szekely, L., Klein, G., Terenius, L. & Wiman, K.G. (1995). p53 binds single-stranded DNA ends through the C-terminal domain and internal DNA segments via the middle domain. *Nucleic Acids Research*, **23,** 362-369.

Baker, S.J., Preisinger, A.C., Jessup, J.M., Paraskeva, C., Markowitz, S., Willson, J.K., Hamilton, S. & Vogelstein, B. (1990). p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Research*, **50,** 7717-7722.

Bardi, G., Johansson, B., Pandis, N., Mandahl, N., Bak Jensen, E., Lindstrom, C., Tornqvist, A., Frederiksen, H., Andren Sandberg, A., Mitelman, F. & et al. (1993a). Cytogenetic analysis of 52 colorectal carcinomas--non-random aberration pattern and correlation with pathologic parameters. *International Journal of Cancer*, **55,** 422-428.

Bardi, G., Pandis, N., Fenger, C., Kronborg, O., Bomme, L. & Heim, S. (1993b). Deletion of 1p36 as a primary chromosomal aberration in intestinal tumorigenesis. *Cancer Research*, **53,** 1895-1898.

Bardi, G., Parada, L.A., Bomme, L., Pandis, N., Johansson, B., Willen, R., Fenger, C., Kronborg, O., Mitelman, F. & Heim, S. (1997). Cytogenetic findings in metastases from colorectal cancer. *International Journal of Cancer*, **72,** 604-607.

Bardi, G., Sukhikh, T., Pandis, N., Fenger, C., Kronborg, O. & Heim, S. (1995). Karyotypic characterization of colorectal adenocarcinomas. *Genes Chromosomes and Cancer*, **12,** 97-109.

Barlow, C., Brown, K.D., Deng, C.X., Tagle, D.A. & Wynshaw Boris, A. (1997). ATMselectively regulates distinct p53-dependent cell-cycle checkpoint and apoptotic pathways [published erratum appears in Nat Genet 1998 Mar;18(3):298]. *Nature Genetics*, **17,** 453-456.

Barnes, C.J., Cameron, I.L., Hardman, W.E. & Lee, M. (1998). Non-steroidol anti-inflammatory drug effect on crypt cell proliferation and apoptosis during initiation of rat colon carcinogenesis. *British Journal of Cancer*, **77,** 573-580.

Bassam, B.J., Caetanoanolles, G. & Gresshoff, P.M. (1991). Fast and sensitive silver staining of DNA in polyacrylamide gels. *Analytical Biochemistry*, **196,** 80-83.

Bates, S. & Vousden, K.H. (1999). Mechanisms of p53-mediated apoptosis. *Cellular and Molecular Life Sciences*, **55,** 28-37.

Beck, N.E., Tomlinson, I.P.M., Homfray, T., Frayling, I., Hodgson, S.V., Harocopos, C. & Bodmer, W.F. (1997). Use of SSCP analysis to identify germline mutations in HNPCC families fulfilling the Amsterdam criteria. *Human Genetics*, **99,** 219-224.

Beham, A., Marin, M.C., Fernandez, A., Herrmann, J., Brisbay, S., Tari, A.M., Lopez Berestein, G., Lozano, G., Sarkiss, M. & McDonnell, T.J. (1997). Bcl-2 inhibits p53 nuclear import following DNA damage. *Oncogene*, **15,** 2767-2772.

Behrmann, I., Walczak, H. & Krammer, P.H. (1994). Structure of the human APO-1 gene. *European Journal of Immunology*, **24,** 3057-3062.

Benchimol, S., Lamb, P., Crawford, L.V., Sheer, D., Shows, T.B., Bruns, G.A. & Peacock, J. (1985). Transformation associated p53 protein is encoded by a gene on human chromosome 17. *Somatic Cell Molecular Genetics*, **11,** 505-510.

Bennett, M., Macdonald, K., Chan, S.W., Luzio, J.P., Simari, R. & Weissberg, P. (1998). Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science*, **282,** 290-293.

Biedermann, K.A., Sun, J.R., Giaccia, A.J., Tosto, L.M. & Brown, J.M. (1991). SCID mutation in mice confers hypersensitivity to ionizing radiation and a deficiency in DNA double strand break repair. *Proceedings of the National Academy of Sciences of the United States of America*, **88,** 1394-1397.

Bingham, S.A., Pignatelli, B., Pollock, J.R., Ellul, A., Malaveille, C., Gross, G., Runswick, S., Cummings, J.H. & O'Neill, I.K. (1996). Does increased endogenous formation of N-nitroso compounds in the human colon explain the association between red meat and colon cancer? *Carcinogenesis*, **17,** 515-523.

Bissonnette, N., Wasylyk, B. & Hunting, D.J. (1997). The apoptotic and transcriptional transactivation activities of p53 can be dissociated. *Biochemistry and Cell Biology*, **75,** 351-358.

Blandino, G., Levine, A.J. & Oren, M. (1999). Mutant p53 gain of function: differential effects of different p53 mutants on resistance of cultured cells to chemotherapy. *Oncogene*, **18,** 477-485.

Blumenthal, R.D., Sharkey, R.M., Natale, A.M., Kashi, R., Wong, G. & Goldenberg, D.M. (1994). Comparison of equitoxic radioimmunotherapy and chemotherapy in the treatment of human colonic cancer xenografts. *Cancer Research*, **54,** 142-151.

Bodmer, W. (1997). The somatic evolution of cancer - The Harveian Oration of 1996. *Journal of the Royal College of Physicians of London*, **31,** 82-89.

Bodmer, W.F., Bailey, C.J., Bodmer, J., Bussey, H.J., Ellis, A., Gorman, P., Lucibello, F.C., Murday, V.A., Rider, S.H., Scambler, P. & et al. (1987). Localization of the gene for familial adenomatous polyposis on chromosome 5. *Nature*, **328,** 614-616.

Boland, C.R. (1996). Roles of the DNA mismatch repair genes in colorectal tumorigenesis. *International Journal of Cancer*, **69,** 47-49.

Bomme, L., Bardi, G., Pandis, N., Fenger, C., Kronborg, O. & Heim, S. (1994). Clonal karyotypic abnormalities in colorectal adenomas: clues to the early genetic events in the adenoma-carcinoma sequence. *Genes Chromosomes and Cancer*, **10,** 190-196.

Bomme, L., Bardi, G., Pandis, N., Fenger, C., Kronborg, O. & Heim, S. (1996). Chromosome abnormalities in colorectal adenomas: two cytogenetic subgroups characterized by deletion of 1p and numerical aberrations. *Human Pathology*, **27,** 1192-1197.

Bomme, L., Heim, S., Bardi, G., Fenger, C., Kronborg, O., Brogger, A. & Lothe, R.A. (1998). Allelic imbalance and cytogenetic deletion of 1p in colorectal adenomas: a target region identified between DIS199 and DIS234. *Genes Chromosomes and Cancer*, **21,** 185-194.

Bos, J.L. (1988). The ras gene family and human carcinogenesis. *Mutation Research*, **195,** 255-271.

Bos, J.L., Fearon, E.R., Hamilton, S.R., Verlaan de Vries, M., van Boom, J.H., van der Eb, A.J. & Vogelstein, B. (1987). Prevalence of ras gene mutations in human colorectal cancers. *Nature*, **327,** 293-297.

Bosma, G.C., Custer, R.P. & Bosma, M.J. (1983). A severe combined immunodeficiency mutation in the mouse. *Nature*, **301,** 527-230.

Bouffler, S.D., Morgan, W.F., Pandita, T.K. & Slijepcevic, P. (1996). The involvement of telomeric sequences in chromosomal aberrations. *Mutation Research*, **366,** 129-135.

Boyd, J.M., Gallo, G.J., Elangovan, B., Houghton, A.B., Malstrom, S., Avery, B.J., Ebb, R.G., Subramanian, T., Chittenden, T., Lutz, R.J. & Chinnadurai, G. (1995). Bik, a novel death-inducing protein shares a distinct sequence motif with Bcl-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene*, **11,** 1921-1928.

Bracey, T.S., Miller, J.C., Preece, A. & Paraskeva, C. (1995). Gamma radiation-induced apoptosis in human colorectal adenoma and carcinoma cell lines can occur in the absence of wild type p53. *Oncogene*, **10,** 2391-2396.

Bracey, T.S., Williams, A.C. & Paraskeva, C. (1997). Inhibition of radiation-induced G(2) delay potentiates cell death by apoptosis and/or the induction of giant cells in colorectal tumor cells with disrupted p53 function. *Clinical Cancer Research*, **3,** 1371-1381.

Brady, H.J.M., Salomons, G.S., Bobeldijk, R.C. & Berns, A.J.M. (1996). T cells from bax alpha transgenic mice show accelerated apoptosis in response to stimuli but do not show restored DNA damage-induced cell death in the absence of p53. *Embo Journal*, **15,** 1221-1230.

Brattain, M.G., Fine, W.D., Khaled, F.M., Thompson, J. & Brattain, D.E. (1981). Heterogeneity of malignant cells from a human colonic carcinoma. *Cancer Research*, **41,** 1751-1756.

Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J. & Kouzarides, T. (1998). Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*, **391,** 597-601.

Bristow, R.G., Benchimol, S. & Hill, R.P. (1996). The p53 gene as a modifier of intrinsic radiosensitivity: Implications for radiotherapy. *Radiotherapy and Oncology*, **40,** 197-223.

Brown, R. (1997). The Bcl-2 family of proteins. *British Medical Bulletin*, **53,** 466-477.

Bruderlein, S., van der Bosch, K., Schlag, P. & Schwab, M. (1990). Cytogenetics and DNA amplification in colorectal cancers. *Genes Chromosomes and Cancer*, **2,** 63-70.

Bubb, V.J., Curtis, L.J., Cunningham, C., Dunlop, M.G., Carothers, A.D., Morris, R.G., White, S., Bird, C.C. & Wyllie, A.H. (1996). Microsatellite instability and the role of hMSH2 in sporadic colorectal cancer. *Oncogene*, **12,** 2641-2649.

Buckbinder, L., Talbott, R., Velasco Miguel, S., Takenaka, I., Faha, B., Seizinger, B.R. & Kley, N. (1995). Induction of the growth inhibitor IGF-binding protein 3 by p53. *Nature*, **377,** 646-649.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J.P., Sedivy, J.M., Kinzler, K.W. & Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science*, **282,** 1497-1501.

Bunz, F., Hwang, P.M., Torrance, C., Waldman, T., Zhang, Y.G., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K.W. & Vogelstein, B. (1999). Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *Journal of Clinical Investigation*, **104,** 263-269.

Buroker, T.R., O'Connell, M.J., Wieand, H.S., Krook, J.E., Gerstner, J.B., Mailliard, J.A., Schaefer, P.L., Levitt, R., Kardinal, C.G. & Gesme, D.H., Jr. (1994). Randomized comparison of two schedules of fluorouracil and leucovorin in the treatment of advanced colorectal cancer [see comments]. *Journal of ClinicalOncology*, **12,** 14-20.

Butler, L.M., Hewett, P.J., Butler, W.J. & Cowled, P.A. (1998). Down regulation of *Fas* gene expression in colon cancer is not a result of allelic loss or gene rearrangement. *British Journal of Cancer*, **77,** 1454-1459.

Cahill, D.P., Kinzler, K.W., Vogelstein, B. & Lengauer, C. (1999a). Genetic instability and darwinian selection in tumours. *Trends in Cell Biology*, **9,** M57-M60.

Cahill, D.P., Kinzler, K.W., Vogelstein, B. & Lengauer, C. (1999b). Genetic instability and Darwinian selection in tumours. *Trends in Biochemical·Sciences*, **24,** M57-M60.

Cahill, D.P., Lengauer, C., Yu, J., Riggins, G.J., Willson, J.K.V., Markowitz, S.D., Kinzler, K.W. & Vogelstein, B. (1998). Mutations of mitotic checkpoint genes in human cancers. *Nature*, **392,** 300-303.

Cajot, J.F., Sordat, I., Silvestre, T. & Sordat, B. (1997). Differential display cloning identifies motility-related protein (MRP1/CD9) as highly expressed in primary compared to metastatic human colon carcinoma cells. *Cancer Research*, **57,** 2593-2597.

Cancer Research Campaign. (1993). Cancer Research Campaign Factsheet 18.1: Cambridge.

Cancer Research Campaign. (1999a). Cancer Research Campaign Statistics - Sources: 1 - Office for National Statistics, Mortality Statistics: Cause. England and Wales 1996 (1997) - 2 - General Register Office for Scotland, Annual Report for Scotland 1996 - 3 - General Register Office for Northern Ireland, Annual Report 1996.

Cancer Research Campaign. (1999b). CRC CancerStats: Survival England and Wales 1971-1995.

Carder, P., Wyllie, A.H., Purdie, C.A., Morris, R.G., White, S., Piris, J. & Bird, C.C. (1993). Stabilized p53 facilitates aneuploid clonal divergence in colorectal cancer. *Oncogene*, **8,** 1397-1401.

Carder, P.J., Cripps, K.J., Morris, R., Collins, S., White, S., Bird, C.C. & Wyllie, A.H. (1995). Mutation of the *P53* gene precedes aneuploid clonal divergence in colorectal carcinoma. *British Journal of Cancer*, **71,** 215-218.

Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S. & Reed, J.C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. *Science*, **282,** 1318-1321.

Carethers, J.M. & Pham, T.T. (2000). Mutations of transforming growth factor beta 1 type II receptor, BAX, and insulin-like growth factor II receptor genes in microsatellite_unstable cell lines. *In Vivo*, **14,** 13-20.

Carney, J.P. (1999). Chromosomal breakage syndromes. *Current Opinion in Immunology*, **11,** 443-447.

Carney, J.P., Maser, R.S., Olivares, H., Davis, E.M., Le Beau, M., Yates, J.R., 3rd, Hays, L., Morgan, W.F. & Petrini, J.H. (1998). The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell*, **93,** 477-486.

Chadeneau, C., Hay, K., Hirte, H.W., Gallinger, S. & Bacchetti, S. (1995). Telomerase activity associated with acquisition of malignancy in human colorectal cancer. *Cancer Research*, **55,** 2533-2536.

Chan, T.A., Hermeking, H., Lengauer, C., Kinzler, K.W. & Vogelstein, B. (1999). 14-3-3 sigma is required to prevent mitotic catastrophe after DNA damage. *Nature*, **401,** 616-620.

Chang, C., Biedermann, K.A., Mezzina, M. & Brown, J.M. (1993). Characterization of the DNA double strand break repair defect in SCID mice. *Cancer Research*, **53,** 1244-1248.

Chang, E.H., Pirollo, K.F. & Bouker, K.B. (2000). *Tp53* gene therapy: a key to modulating resistance to anticancer therapies? *MOLECULAR MEDICINE TODAY*, **6,** 358-364.

Chen, J., Giovannucci, E., Kelsey, K., Rimm, E.B., Stampfer, M.J., Colditz, G.A., Spiegelman, D., Willett, W.C. & Hunter, D.J. (1996). A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. *Cancer Research*, **56,** 4862-4864.

Chen, J., Stampfer, M.J., Hough, H.L., Garcia Closas, M., Willett, W.C., Hennekens, C.H., Kelsey, K.T. & Hunter, D.J. (1998). A prospective study of N-acetyltransferase genotype, red meat intake, and risk of colorectal cancer. *Cancer Research*, **58,** 3307-3311.

Chen, J., Wu, W., Tahir, S.K., Kroeger, P.E., Rosenberg, S.H., Cowsert, L.M., Bennett, F., Krajewski, S., Krajewska, M., Welsh, K., Reed, J.C. & Ng, S.C. (2000). Down-regulation of survivin by antisense oligonucleotides increases apoptosis, inhibits cytokinesis and anchorage-independent growth. *Neoplasia*, **2,** 235-241.

Chen, T.R., Dorotinsky, C.S., McGuire, L.J., Macy, M.L. & Hay, R.J. (1995). DLD-1 and HCT-15 cell lines derived separately from colorectal carcinomas have totally different chromosome changes but the same genetic origin. *Cancer Genetics and Cytogenetics*, **81,** 103-108.

Chen, T.R., Hay, R.J. & Macy, M.L. (1982). Karyotype consistency in human colorectal carcinoma cell lines established *in vitro*. *Cancer Genetics and Cytogenetics*, **6,** 93-117.

Chung, Y.J., Park, S.W., Song, J.M., Lee, K.Y., Seo, E.J., Choi, S.W. & Rhyu, M.G. (1997). Evidence of genetic progression in human gastric carcinomas with microsatellite instability. *Oncogene*, **15,** 1719-1726.

Cimini, D., Antoccia, A., Tanzarella, C. & Degrassi, F. (1997). Topoisomerase II inhibition in mitosis produces numerical and structural chromosomal aberrations in human fibroblasts. *Cytogenetics and Cell Genetics*, **76,** 61-67.

Clarke, A.R., Gledhill, S., Hooper, M.L., Bird, C.C. & Wyllie, A.H. (1994). p53 dependence of early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma irradiation. *Oncogene*, **9,** 1767-1773.

Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L. & Wyllie, A.H. (1993). Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, **362,** 849-852.

Cohen, A.M., Minsky, B.D. & Schilsky, R.L. (1997). Cancer of the colon and rectum. In *Cancer prinbciples and practice of oncology*, DeVita, V.T., Jr, Hellman, S. & Rosenberg, S.A. (eds), Vol. 1. pp. 1144-1234. Lippincott-Raven: Philadelphia, New York.

Coquelle, A., Pipiras, E., Toledo, F., Buttin, G. & Debatisse, M. (1997). Expression of fragile sites triggers intrachromosomal mammalian gene amplification and sets boundaries to early amplicons. *Cell*, **89,** 215-225.

Coquelle, A., Toledo, F., Stern, S., Bieth, A. & Debatisse, M. (1998). A new role for hypoxia in tumor progression: induction of fragile site triggering genomic rearrangements and formation of complex DMs and HSRs. *Molecular cell*, **2,** 259-265.

Cory, J.G., Breland, J.C. & Carter, G.L. (1979). Effect of 5-fluorouracil on RNA metabolism in Novikoff hepatoma cells. *Cancer Research*, **39,** 4905-4913.

Cottu, P.H., Muzeau, F., Estreicher, A., Flejou, J.F., Iggo, R., Thomas, G. & Hamelin, R. (1996). Inverse correlation between RER(+) status and p53 mutation in colorectal cancer cell lines. *Oncogene*, **13,** 2727-2730.

Cripps, K.J., Curtis, L.J. & Wyllie, A.H. (1995). Mutational analysis of the MCC gene by single-strand conformational polymorphism analysis. *European Journal of Cancer*, **31a,** 853-855.

Cripps, K.J., Purdie, C.A., Carder, P.J., White, S., Komine, K., Bird, C.C. & Wyllie, A.H. (1994). A study of stabilization of p53 protein versus point mutation in colorectal-carcinoma. *Oncogene*, **9,** 2739-2743.

Cunningham, C. & Dunlop, M.G. (1994). Genetics of colorectal cancer. *British Medical Bullten*, **50,** 640-655.

Cunningham, C., Dunlop, M.G., Bird, C.C. & Wyllie, A.H. (1994). Deletion analysis of chromosome 8p in sporadic colorectal adenomas. *British Journal of Cancer*, **70,** 18-20.

Cunningham, C., Dunlop, M.G., Wyllie, A.H. & Bird, C.C. (1993). Deletion mapping in colorectal cancer of a putative tumour suppressor gene in 8p22-p21.3. *Oncogene*, **8,** 1391-1396.

Cunningham, J., Lust, J.A., Schaid, D.J., Bren, G.D., Carpenter, H.A., Rizza, E., Kovach, J.S. & Thibodeau, S.N. (1992). Expression of p53 and 17p allelic loss in colorectal carcinoma. *Cancer Research*, **52,** 1974-1980.

Curtis, L.J., Bubb, V.J., Gledhill, S., Morris, R.G., Bird, C.C. & Wyllie, A.H. (1994). Loss of heterozygosity of MCC is not associated with mutation of the retained allele in sporadic colorectal cancer. *Human Molecular Genetics*, **3,** 443-446.

Curtis, L.J., Georgiades, I.B., White, S., Bird, C.C., J., H.D. & Wyllie, A.H. (2000). Specific patterns of abnormalities are associated with RE status in sporadic colorectal cancer. *Submitted.*

Custer, R.P., Bosma, G.C. & Bosma, M.J. (1985). Severe combined immunodeficiency (SCID) in the mouse. Pathology, reconstitution, neoplasms. *American Journal of Pathology*, **120,** 464-477.

D' Atri, S., Tentori, L., Lacal, P.M., Graziani, G., Pagani, E., Benincasa, E., Zambruno, G., Bonmassar, E. & Jiricny, J. (1998). Involvement of the mismatch repair system in temozolomide-induced apoptosis. *Molecular Pharmacology*, **54,** 334-341.

Dasika, G.K., Lin, S.C., Zhao, S., Sung, P., Tomkinson, A. & Lee, E.Y. (1999). DNA damage-induced cell cycle checkpoints and DNA strand break repair in development and tumorigenesis. *Oncogene*, **18,** 7883-7899.

Davidson, J.M., Gorringe, K.L., Chin, S.-F., Orsetti, B., Besret, C., Courtay-Cahen, C., Roberts, I., Theillet, C., Caldas, C. & Edwards, P.A.W. (2000). Molecular cytogetic analysis of breast cancer cell lines. *British Journal of Cancer*, **in press.**

Day, J.P., Limoli, C.L. & Morgan, W.F. (1998). Recombination involving interstitial telomere repeat-like sequences promotes chromosomal instability in Chinese hamster cells. *Carcinogenesis*, **19,** 259-265.

de Caestecker, M.P., Parks, W.T., Frank, C.J., Castagnino, P., Bottaro, D.P., Roberts, A.B. & Lechleider, R.J. (1998). Smad2 transduces common signals from receptor serine-threonine and tyrosine kinases. *Genes and Development*, **12,** 1587-1592.

de Wind, N., Dekker, M., Berns, A., Radman, M. & Riele, H.T. (1995). Inactivation of the mouse *Msh2* gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell*, **82,** 321-330.

DeAngelis, P.M., Clausen, O.P.F., Schjolberg, A. & Stokke, T. (1999). Chromosomal gains and losses in primary colorectal carcinomas detected by CGH and their associations with tumour DNA ploidy, genotypes and phenotypes. *British Journal of Cancer*, **80,** 526-535.

DeAngelis, P.M., Stokke, T., Thorstensen, L., Lothe, R.A. & Clausen, O.P.F. (1998). Apoptosis and expression of Bax, Bcl-x, and Bcl-2 apoptotic regulatory proteins in colorectal carcinomas, and associations with p53 genotype/phenotype. *Journal of Clinical Pathology-Molecular Pathology*, **51,** 254-261.

Deveraux, Q.L. & Reed, J.C. (1999). IAP family proteins--suppressors of apoptosis. *Genes and Development*, **13,** 239-252.

Dianzani, U., Bragardo, M., DiFranco, D., Alliaudi, C., Scagni, P., Buonfiglio, D., Redoglia, V., Bonissoni, S., Correra, A., Dianzani, I. & Ramenghi, U. (1997). Deficiency of the Fas apoptosis pathway without *Fas* gene mutations in pediatric patients with autoimmunity/lymphoproliferation. *Blood*, **89,** 2871-2879.

Dietmaier, W., Wallinger, S., Bocker, T., Kullmann, F., Fishel, R. & Ruschoff, J. (1997). Diagnostic microsatellite instability: definition and correlation with mismatch repair protein expression. *Cancer Research*, **57,** 4749-4756.

Ding, H.F., McGill, G., Rowan, S., Schmaltz, C., Shimamura, A. & Fisher, D.E. (1998). Oncogene-dependent regulation of caspase activation by p53 protein in a cell-free system. *Journal of Biological Chemistry*, **273,** 28378-28383.

Disney, J.E., Barth, A.L. & Shultz, L.D. (1992). Defective repair of radiation-induced chromosomal damage in SCID/SCID mice. *Cytogenetics and Cell Genetics*, **59,** 39-44.

Doxsey, S. (1998). The centrosome--a tiny organelle with big potential. *Nature Genetics*, **20,** 104-106.

Dragovich, T., Rudin, C.M. & Thompson, C.B. (1998). Signal transduction pathways that regulate cell survival and cell death. *Oncogene*, **17,** 3207-3213.

Drappa, J., Vaishnaw, A.K., Sullivan, K.E., Chu, J.L. & Elkon, K.B. (1996). *Fas* gene mutations in the Canale-Smith Syndrome, an Inherited lymphoproliferative disorder associated with autoimmunity. *New England Journal of Medicine*, **335,** 1643-1649.

Drewinko, B., Romsdahl, M.M., Yang, L.Y., Ahearn, M.J. & Trujillo, J.M. (1976). Establishment of a human carcinoembryonic antigen-producing colon adenocarcinoma cell line. *Cancer Research*, **36,** 467-475.

Drummond, J.T., Anthoney, A., Brown, R. & Modrich, P. (1996). Cisplatin and adriamycin resistance are associated with MutL alpha and mismatch repair deficiency in an ovarian tumor cell line. *Journal of Biological Chemistry*, **271,** 19645-19648.

Duckett, D.R., Bronstein, S.M., Taya, Y. & Modrich, P. (1999). hMutS alpha- and hMutL alpha-dependent phosphorylation of p53 in response to DNA methylator damage. *Proceedings of the National Academy of Sciences of the United States of America*, **96,** 12384-12388.

Dumaz, N., Duthu, A., Ehrhart, J.C., Drougard, C., Appella, E., Anderson, C.W., May, P., Sarasin, A. & Daya Grosjean, L. (1997). Prolonged p53 protein accumulation in trichothiodystrophy fibroblasts dependent on unrepaired pyrimidine dimers on the transcribed strands of cellular genes. *Molecular Carcinogenesis*, **20,** 340-347.

Dutrillaux, B. (1995). Pathways of chromosome alteration in human epithelial cancers. *Advances in Cancer Research*, **67,** 59-82.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. *Genes and Development*, **12,** 2245-2262.

Elghezal, H., RadfordWeiss, I., Perot, C., VanderAkker, J., Macintyre, E.A., Eydoux, P., Vekemans, M. & Romana, S.P. (1999). Specificity and sensitivity of spectral karyotyping (SKY) for the detection of chromosomal rearrangements in acute lymphoblastic leukemia (ALL). *Blood*, **94,** 2226.

Eppert, K., Scherer, S.W., Ozcelik, H., Pirone, R., Hoodless, P., Kim, H., Tsui, L.C., Bapat, B., Gallinger, S., Andrulis, I.L., Thomsen, G.H., Wrana, J.L. & Attisano, L. (1996). MADR2 maps to 18q21 and encodes a TGFbeta-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell*, **86,** 543-552.

Erisman, M.D., Litwin, S., Keidan, R.D., Comis, R.L. & Astrin, S.M. (1988a). Noncorrelation of the expression of the c-myc oncogene in colorectal carcinoma with recurrence of disease or patient survival. *Cancer Research*, **48,** 1350-1355.

Erisman, M.D., Scott, J.K., Watt, R.A. & Astrin, S.M. (1988b). The c-myc protein is constitutively expressed at elevated levels in colorectal carcinoma cell lines. *Oncogene*, **2,** 367-378.

Erlichman, C., Oconnell, M., Kahn, M., Marsoni, S., Torri, V., Tardio, B., Zaniboni, A., Pancera, G., Martignoni, G., Labianca, R., Barni, A., Seitz, J.F., Milan, C., Bedenne, L., Giovannini, M., Letreut, Y.P., Skillings, J., Shepard, L., Zee, B., Petrioli, R. & Francini, G. (1999). Efficacy of adjuvant fluorouracil and folinic acid in B2 colon cancer. *Journal of Clinical Oncology*, **17,** 1356-1363.

Eshleman, J.R., Casey, G., Kochera, M.E., Sedwick, W.D., Swinler, S.E., Veigl, M.L., Willson, J.K.V., Schwartz, S. & Markowitz, S.D. (1998). Chromosome number and structure both are markedly stable in RER colorectal cancers and are not destabilized by mutation of p53. *Oncogene*, **17,** 719-725.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z. & Hancock, D.C. (1992). Induction of apoptosis in fibroblasts by C-Myc Protein. *Cell*, **69,** 119-128.

Facchini, L.M. & Penn, L.Z. (1998). The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. *FASEB Journal*, **12,** 633-651.

Fan, Y.S., Siu, V.M., Jung, J.H. & Xu, J. (2000). Sensitivity of multiple color spectral karyotyping in detecting small interchromosomal rearrangements. *Genetic Testing*, **4,** 9-14.

Fearon, E.R. & Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell*, **61,** 759-767.

Feinberg, A.P. & Vogelstein, B. (1983). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*, **301,** 89-92.

Feinberg, A.P. & Vogelstein, B. (1987). Alterations in DNA methylation in human colon neoplasia. *Seminars in Surgical Oncology*, **3,** 149-151.

Field, C., Li, R. & Oegema, K. (1999). Cytokinesis in eukaryotes: a mechanistic comparison. *Current Opinion in Cell Biology*, **11,** 68-80.

Findlay, M.P., Cunningham, D., Morgan, G., Clinton, S., Hardcastle, A. & Aherne, G.W. (1997). Lack of correlation between thymidylate synthase levels in primary colorectal tumours and subsequent response to chemotherapy. *British Journal of Cancer*, **75,** 903-909.

Fink, D., Aebi, S. & Howell, S.B. (1998a). The role of DNA mismatch repair in drug resistance. *Clinical Cancer Research*, **4,** 1-6.

Fink, D., Nebel, S., Aebi, S., Zheng, H., Cenni, B., Nehme, A., Christen, R.D. & Howell, S.B. (1996). The role of DNA mismatch repair in platinum drug resistance. *Cancer Research*, **56,** 4881-4886.

Fink, D., Nebel, S., Norris, P.S., Aebi, S., Kim, H.K., Haas, M. & Howell, S.B. (1998b). The effect of different chemotherapeutic agents on the enrichment of DNA mismatch repair-deficient tumour cells. *British Journal of Cancer*, **77,** 703-708.

Fink, D., Zheng, H., Nebel, S., Norris, P.S., Aebi, S., Lin, T.P., Nehme, A., Christen, R.D., Haas, M., MacLeod, C.L. & Howell, S.B. (1997). In vitro and in vivo resistance to cisplatin in cells that have lost DNA mismatch repair. *Cancer Research*, **57,** 1841-1845.

Flaman, J.M., Robert, V., Lenglet, S., Moreau, V., Iggo, R. & Frebourg, T. (1998). Identification of human p53 mutations with differential effects on the bax and p21 promoters using functional assays in yeast. *Oncogene*, **16,** 1369-1372.

Fogh, J. & Trempe, G. (1975). New human tumour cell lines. In *Human Tumour Cells in vitro*, Fogh, J. (ed) pp. 115-159. Plenum Press: NY.

Ford, J.M. & Hanawalt, P.C. (1995). Li-Fraumeni syndrome fibroblasts homozygous for p53 mutations are deficient in global DNA-repair but exhibit normal transcription-coupled repair and enhanced UV resistance. *Proceedings of the National Academy of Sciences of the United States of America*, **92,** 8876-8880.

Ford, J.M. & Hanawalt, P.C. (1997). Expression of wild-type p53 is required for efficient global genomic nucleotide excision repair in UV-irradiated human fibroblasts. *Journal of Biological Chemistry*, **272,** 28073-28080.

Foulds, L. (1958). The natural history of cancer. *Journal of Chronic Diseases*, **8,** 2-37.

Friedman, H.S., Johnson, S.P., Dong, Q., Schold, S.C., Rasheed, B.K.A., Bigner, S.H., AliOsman, F., Dolan, E., Colvin, O.M., Houghton, P., Germain, G., Drummond, J.T., Keir, S., Marcelli, S., Bigner, D.D. & Modrich, P. (1997). Methylator resistance mediated by mismatch repair deficiency in a glioblastoma multiforme xenograft. *Cancer Research*, **57,** 2933-2936.

Friesen, C., Herr, I., Krammer, P.H. & Debatin, K.M. (1996). Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. *Nature Medicine*, **2,** 574-577.

Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S. & Vande Woude, G.F. (1996). Abnormal centrosome amplification in the absence of p53. *Science*, **271,** 1744-1747.

Gagos, S., Hopwood, V.L., Iliopoulos, D., Kostakis, A., Karayannakos, P., Yatzides, H., Skalkeas, G.D. & Pathak, S. (1995). Chromosomal markers associated with metastasis in two colon cancer cell lines established from the same patient. *Anticancer Research*, **15,** 369-378.

Gagos, S., Iliopoulos, D., TseleniBalafouta, S., Agapitos, M., Antachopoulos, C., Kostakis, A., Karayannakos, P. & Skalkeas, G. (1996). Cell senescence and a mechanism of clonal evolution leading to continuous cell proliferation, loss of heterozygosity, and tumor heterogeneity: Studies on two immortal colon cancer cell lines. *Cancer Genetics and Cytogenetics*, **90,** 157-165.

Garro, A.J. & Lieber, C.S. (1990). Alcohol and cancer. *Annual Review of Pharmacologic Toxicology*, **30,** 219-249.

Geoffroy, F.J., Allegra, C.J., Sinha, B. & Grem, J.L. (1994). Enhanced cytotoxicity with nterleukin-1-alpha and 5-fluorouracil in HCT116 colon cancer. *Oncology Research*, **6,** 581-591.

Georgiades, I.B., Curtis, L.J., Morris, R.M., Bird, C.C. & Wyllie, A.H. (1999). Heterogeneity studies identify a subset of sporadic colorectal cancers without evidence for chromosomal or microsatellite instability. *Oncogene*, **18,** 7933-7940.

Ghadimi, B.M., Sackett, D.L., Difilippantonio, M.J., Schrock, E., Neumann, T., Jauho, A., Auer, G. & Ried, T. (2000). Centrosome amplification and instability occurs exclusively in aneuploid, but not in diploid colorectal cancer cell lines, and correlates with numerical chromosomal aberrations. *Genes Chromosomes and Cancer*, **27,** 183-190.

Ghadimi, B.M., Schrock, E., Walker, R.L., Wangsa, D., Jauho, A., Meltzer, P.S. & Ried, T. (1999). Specific chromosomal aberrations and amplification of the *AIB1* nuclear receptor coactivator gene in pancreatic carcinomas. *American Journal of Pathology*, **154,** 525-536.

Ghoshal, K. & Jacob, S.T. (1997). An alternative molecular mechanism of action of 5-fluorouracil, a potent anticancer drug. *Biochemical Pharmacology*, **53,** 1569-1575.

Glover, T.W. (1998). Instability at chromosomal fragile sites. *Recent Results in Cancer Research*, **154,** 185-199.

Goelz, S.E., Hamilton, S.R. & Vogelstein, B. (1985). Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. *Biochemical and Biophysical Research Communications*, **130,** 118-126.

Gottlieb, T.M. & Oren, M. (1998). p53 and apoptosis. *Seminars in Cancer Biology*, **8,** 359-368.

Grady, W.M., Myeroff, L.L., Swinler, S.E., Rajput, A., Thiagalingam, S., Lutterbaugh, J.D., Neumann, A., Brattain, M.G., Chang, J., Kim, S.J., Kinzler, K.W., Vogelstein, B., Willson, J.K.V. & Markowitz, S. (1999). Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. *Cancer Research*, **59,** 320-324.

Grady, W.M., Rajput, A., Myeroff, L., Liu, D.F., Kwon, K.H., Willis, J. & Markowitz, S. (1998). Mutation of the type II transforming growth factor-beta receptor is coincident with the transformation of human colon adenomas to malignant carcinomas. *Cancer Research*, **58,** 3101-3104.

Greenblatt, M.S., Grollman, A.P. & Harris, C.C. (1996). Deletions and insertions in the *P53* tumor-suppressor gene in human cancers: confirmation of the DNA-polymerase slippage misalignment model. *Cancer Research*, **56,** 2130-2136.

Griffin, S., Branch, P., Xu, Y.Z. & Karran, P. (1994). DNA mismatch binding and incision at modified guanine bases by extracts of mammalian cells implications for tolerance to DNA methylation damage. *Biochemistry*, **33,** 4787-4793.

Griffiths, S.D., Clarke, A.R., Healy, L.E., Ross, G., Ford, A.M., Hooper, M.L., Wyllie, A.H. & Greaves, M. (1997). Absence of p53 permits propagation of mutant cells following genotoxic damage. *Oncogene*, **14,** 523-531.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M. & et al. (1991). Identification and characterization of the familial adenomatous polyposis coli gene. *Cell*, **66,** 589-600.

Hague, A. & Paraskeva, C. (1995). The short-chain fatty acid butyrate induces apoptosis in colorectal tumour cell lines. *European Journal of Cancer Previews*, **4,** 359-364.

Hahn, S.A., Schutte, M., Hoque, A.T., Moskaluk, C.A., da Costa, L.T., Rozenblum, E., Weinstein, C.L., Fischer, A., Yeo, C.J., Hruban, R.H. & Kern, S.E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science*, **271,** 350-353.

Haldar, S., Basu, A. & Croce, C.M. (1997). Bcl2 is the guardian of microtubule integrity. *Cancer Research*, **57,** 229-233.

Hale, A.J., Smith, C.A., Satherland, L.C., Stoneman, V.E.A., Longthorne, V., Culhane, A.C. & Williams, G.T. (1996). Apoptosis - molecular regulation of cell death. *European Journal of Biochemistry*, **237,** 884-884.

Han, J., Sabbatini, P. & White, E. (1996). Induction of apoptosis by human Nbk/Bik, a BH3-containing protein that interacts with E1B 19K. *Molecular and Cellular Biology*, **16,** 5857-5864.

Hanahan, D. (2000). Benifits of bad telomers. *Nature*, **406,** 573-574.

Hansen, R. & Oren, M. (1997). p53; From inductive signal to cellular effect. *Current Opinion in Genetics & Development*, **7,** 46-51.

Harstrick, A., Gonzales, A., Schleucher, N., Vanhoefer, U., Lu, K., Formento, J.L., Milano, G., Wilke, H., Seeber, S. & Rustum, Y. (1998). Comparison between short or long exposure to 5-fluorouracil in human gastric and colon cancer cell lines: biochemical mechanism of resistance. *Anticancer Drugs*, **9,** 625-634.

Hartwell, L.H. & Kastan, M.B. (1994). Cell cycle control and cancer. *Science*, **266,** 1821-1828.
Hastie, N.D., Dempster, M., Dunlop, M.G., Thompson, A.M., Green, D.K. & Allshire, R.C. (1990). Telomere reduction in human colorectal carcinoma and with ageing. *Nature*, **346,** 866-868.
Haupt, Y., Rowan, S., Shaulian, E., Vousden, K.H. & Oren, M. (1995). Induction of apoptosis in HeLa cells by trans-activation-deficient p53. *Genes and Development*, **9,** 2170-2183.
Hawkins, D.S., Demers, G.W. & Galloway, D.A. (1996). Inactivation of p53 enhances sensitivity to multiple chemotherapeutic agents. *Cancer Research*, **56,** 892-898.
Hawn, M.T., Umar, A., Carethers, J.M., Marra, G., Kunkel, T.A., Boland, C.R. & Koi, M. (1995). Evidence for a connection between the mismatch repair system and the G(2) cell cycle checkpoint. *Cancer Research*, **55,** 3721-3725.
He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B. & Kinzler, K.W. (1998). Identification of c-MYC as a target of the APC pathway [see comments]. *Science*, **281,** 1509-1512.
Hecht, F., Tajara, E.H., Lockwood, D., Sandberg, A.A. & Hecht, B.K. (1988). New common fragile sites. *Cancer Genetics and Cytogenetics*, **33,** 1-9.
Held, K.D. (1997). Radiation-induced apoptosis and its relationship to loss of clonogenic survival. *Apoptosis*, **2,** 265-282.
Herman, J.G., Umar, A., Polyak, K., Graff, J.R., Ahuja, N., Issa, J.P.J., Markowitz, S., Willson, J.K.V., Hamilton, S.R., Kinzler, K.W., Kane, M.F., Kolodner, R.D., Vogelstein, B., Kunkel, T.A. & Baylin, S.B. (1998). Incidence and functional consequences of *hMLH1* promoter hypermethylation in colorectal carcinoma. *Proceedings of the National Academy of Sciences of the United States of America*, **95,** 6870-6875.
Hermeking, H., Lengauer, C., Polyak, K., He, T.C., Zhang, L., Thiagalingam, S., Kinzler, K.W. & Vogelstein, B. (1997). 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Molecular Cell*, **1,** 3-11.
Hinchcliffe, E.H., Li, C., Thompson, E.A., Maller, J.L. & Sluder, G. (1999). Requirement of Cdk2-cyclin E activity for repeated centrosome reproduction in Xenopus egg extracts. *Science*, **283,** 851-854.
Hiroi, N., Maruta, H. & Tanuma, S. (1999). Fas-mediated apoptosis in Jurkat cells is suppressed in the pre-G2/M phase. *Apoptosis*, **4,** 255-261.
Hoang, J.M., Cottu, P.H., Thuille, B., Salmon, R.J., Thomas, G. & Hamelin, R. (1997). BAT-26, an indicator of the replication error phenotype in colorectal cancers and cell lines. *Cancer Research*, **57,** 300-303.
Hockenbery, D.M., Zutter, M., Hickey, W., Nahm, M. & Korsmeyer, S.J. (1991). BCL2 protein is topographically restricted in tissues characterized by apoptotic cell death. *Proceedings of the National Academy of Sciences of the United States of America*, **88,** 6961-6965.
Hollstein, M., Sidransky, D., Vogelstein, B. & Harris, C.C. (1991). *P53* mutations in human cancers. *Science*, **253,** 49-53.
Homfray, T.F.R., Cottrell, S.E., Ilyas, M., Rowan, A., Talbot, I.C., Bodmer, W.F. & Tomlinson, I.P.M. (1998). Defects in mismatch repair occur after *APC* mutations in the pathogenesis of sporadic colorectal tumours. *Human Mutation*, **11,** 114-120.
Houghton, J.A., Harwood, F.G. & Tillman, D.M. (1997). Thymineless death in colon carcinoma cells is mediated via fas signaling. *Proceedings of the National Academy of Science of the United States of America*, **94,** 8144-8149.
Howe, J.R. & Guillem, J.G. (1997). The genetics of colorectal cancer. *Surgical Clinics of North America*, **77,** 175-195.
Howe, J.R., Mitros, F.A. & Summers, R.W. (1998). The risk of gastrointestinal carcinoma in familial juvenile polyposis. *Annals of Surgical Oncology*, **5,** 751-756.
Huang, J., Papadopoulos, N., McKinley, A.J., Farrington, S.M., Curtis, L.J., Wyllie, A.H., Zheng, S., Willson, J.K.V., Markowitz, S.D., Morin, P., Kinzler, K.W., Vogelstein, B. & Dunlop, M.G. (1996). *APC* mutations in colorectal tumors with mismatch repair deficiency. *Proceedings of the National Academy of Sciences of the United States of America*, **93,** 9049-9054.

Hubbard, A.L., Harrison, D.J., Moyes, C., Wyllie, A.H., Cunningham, C., Mannion, E. & Smith, C.A. (1997). N-acetyltransferase 2 genotype in colorectal cancer and selective gene retention in cancers with chromosome 8p deletions. *Gut*, **41,** 229-34.

Hubbard, A.L., Moyes, C., Wyllie, A.H., Smith, C.A. & Harrison, D.J. (1998). N-acetyl transferase 1: two polymorphisms in coding sequence identified in colorectal cancer patients. *Br J Cancer*, **77,** 913-6.

Hueber, A.O., Zornig, M., Lyon, D., Suda, T., Nagata, S. & Evan, G.I. (1997). Requirement for the CD95 receptor-ligand pathway in C-MYC-induced apoptosis. *Science*, **278,** 1305-1309.

Humphries, S.E., Gudnason, V., Whittall, R. & Day, I.N.M. (1997). Single-strand conformation polymorphism analysis with high throughput modifications, and its use in mutation detection in familial hypercholesterolemia. *Clinical Chemistry*, **43,** 427-435.

Hwang, B.J., Ford, J.M., Hanawalt, P.C. & Chu, G. (1999). Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. *Proceedings of the National Academy of Sciences of the United States of America*, **96,** 424-428.

Ilyas, M., Straub, J., Tomlinson, I.P.M. & Bodmer, W.F. (1999). Genetic pathways in colorectal and other cancers. *European Journal of Cancer*, **35,** 335-351.

Inada, T., Ichikawa, A., Kubota, T., Ogata, Y., Moossa, A. & Hoffman, R.M. (1997). 5-FU-induced apoptosis correlates with efficacy against human gastric and colon cancer xenografts in nude mice. *Anticancer Research*, **17,** 1965-1971.

Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D. & Perucho, M. (1993). Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature*, **363,** 558-561.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J.L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L.E. & Tschopp, J. (1997). Inhibition of death receptor signals by cellular FLIP. *Nature*, **388,** 190-5.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y. & Nagata, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*, **66,** 233-243.

Jacoby, R.F., Marshall, D.J., Kailas, S., Schlack, S., Harms, B. & Love, R. (1995). Genetic instability associated with adenoma to carcinoma progression in Hereditary Nonpolyposis Colon Cancer. *Gastroenterology*, **109,** 73-82.

Janus, F., Albrechtsen, N., Knippschild, U., Wiesmuller, L., Grosse, F. & Deppert, W. (1999). Different regulation of the p53 core domain activities 3'-to-5' exonuclease and sequence-specific DNA binding. *Molecular and Cellular Biology*, **19,** 2155-2168.

Jasin, M. (2000). Chromosome breaks and genomic instability. *Cancer Investigation*, **18,** 78-86.

Jass, J.R., Do, K.A., Simms, L.A., Iino, H., Wynter, C., Pillay, S.P., Searle, J., RadfordSmith, G., Young, J. & Leggett, B. (1998). Morphology of sporadic colorectal cancer with DNA replication errors. *Gut*, **42,** 673-679.

Jass, J.R., Smyrk, T.C., Stewart, S.M., Lane, M.R., Lanspa, S.J. & Lynch, H.T. (1994). Pathology of hereditary non-polyposis colorectal cancer. *Anticancer Research*, **14,** 1631-1634.

Jen, J., Kim, H., Piantadosi, S., Liu, Z.F., Levitt, R.C., Sistonen, P., Kinzler, K.W., Vogelstein, B. & Hamilton, S.R. (1994a). Allelic loss of chromosome 18q and prognosis in colorectal cancer. *New England Journal of Medicine*, **331,** 213-221.

Jen, J., Powell, S.M., Papadopoulos, N., Smith, K.J., Hamilton, S.R., Vogelstein, B. & Kinzler, K.W. (1994b). Molecular determinants of dysplasia in colorectal lesions. *Cancer Research*, **54,** 5523-5526.

Ju, S.T., Panka, D.J., Cui, H.L., Ettinger, R., Elkhatib, M., Sherr, D.H., Stanger, B.Z. & Marshakrothstein, A. (1995). Fas(Cd95)-FasL interactions required for programmed cell death after T-Cell activation. *Nature*, **373,** 444-448.

Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J.C., Valent, A., Minty, A., Chalon, P., Lelias, J.M., Dumont, X., Ferrara, P., McKeon, F. & Caput, D. (1997). Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell*, **90,** 809-819.

Kakiuchi, H., Watanabe, M., Ushijima, T., Toyota, M., Imai, K., Weisburger, J.H., Sugimura, T. & Nagao, M. (1995). Specific 5'-GGGA-3'-->5'-GGA-3' mutation of the Apc gene in rat

colon tumors induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Proceedings of the National Academy of Sciences of the United States of America*, **92,** 910-914.

Kalgutkar, A.S., Crews, B.C., Rowlinson, S.W., Garner, C., Seibert, K. & Marnett, L.J. (1998). Aspirin-like molecules that covalently inactivate cyclooxygenase-2. *Science*, **280,** 1268-1270.

Kallioniemi, A., Kallioniemi, O.P., Piper, J., Tanner, M., Stokke, T., Chen, L., Smith, H.S., Pinkel, D., Gray, J.W. & Waldman, F.M. (1994). Detection and mapping of amplified DNA-sequences in breast cancer by Comparative Genomic Hybridization. *Proceedings of the National Academy of Sciences of the United States of America*, **91,** 2156-2160.

Kanaar, R. & Hoeijmakers, J.H. (1997). Recombination and joining: different means to the same ends. *Genes Functions*, **1,** 165-174.

Kanaar, R., Hoeijmakers, J.H. & van Gent, D.C. (1998). Molecular mechanisms of DNA double strand break repair. *Trends in Cell Biology*, **8,** 483-489.

Karran, P. & Bignami, M. (1994). DNA damage tolerance, mismatch repair and genome instability. *Bioessays*, **16,** 833-839.

Kastan, M.B., Zhan, Q.M., Eldeiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B. & Fornace, A.J. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia telangiectasia. *Cell*, **71,** 587-597.

Kat, A., Thilly, W.G., Fang, W.H., Longley, M.J., Li, G.M. & Modrich, P. (1993). An alkylation-tolerant, mutator human cell line is deficient in strand specific mismatch repair. *Proceedings of the National Academy of Sciences of the United States of America*, **90,** 6424-6428.

Kawada, M., Yamagoe, S., Murakami, Y., Suzuki, K., Mizuno, S. & Uehara, Y. (1997). Induction of p27Kip1 degradation and anchorage independence by Ras through the MAP kinase signaling pathway. *Oncogene*, **15,** 629-637.

Keino Masu, K., Masu, M., Hinck, L., Leonardo, E.D., Chan, S.S., Culotti, J.G. & Tessier Lavigne, M. (1996). Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell*, **87,** 175-85.

Kerr, J.F., Wyllie, A.H. & Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, **26,** 239-257.

Kinzler, K.W., Nilbert, M.C., Su, L.K., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hedge, P., McKechnie, D. & et al. (1991a). Identification of FAP locus genes from chromosome 5q21. *Science*, **253,** 661-665.

Kinzler, K.W., Nilbert, M.C., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hamilton, S.R., Hedge, P., Markham, A. & et al. (1991b). Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. *Science*, **251,** 1366-1370.

Kinzler, K.W. & Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. *Cell*, **87,** 159-170.

Kirchgessner, C.U., Patil, C.K., Evans, J.W., Cuomo, C.A., Fried, L.M., Carter, T., Oettinger, M.A. & Brown, J.M. (1995). DNA-dependent kinase (p350) as a candidate gene for the murine SCID defect. *Science*, **267,** 1178-1183.

Kirkland, S.C. (1985). Dome formation by a human colonic adenocarcinoma cell line (HCA-7). *Cancer Research*, **45,** 3790-3795.

Kirkland, S.C. & Bailey, I.G. (1986). Establishment and characterization of 6 human colorectal adenocarcinoma cell lines. *British Journal of Cancer*, **53,** 779-785.

Kluck, R.M., Bossy Wetzel, E., Green, D.R. & Newmeyer, D.D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science*, **275,** 1132-1136.

Knudson, A.G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proceedings of the National Academy of Sciences of the United States of America*, **68,** 820-823.

Koi, M., Umar, A., Chauhan, D.P., Cherian, S.P., Carethers, J.M., Kunkel, T.A. & Boland, C.R. (1994). Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N'-nitro-N- nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. *Cancer Research*, **54,** 4308-4312.

Konishi, M., Kikuchiyanoshita, R., Tanaka, K., Muraoka, M., Onda, A., Okumura, Y., Kishi, N., Iwama, T., Mori, T., Koike, M., Ushio, K., Chiba, M., Nomizu, S., Konishi, F., Utsunomiya, J. & Miyaki, M. (1996). Molecular nature of colon tumors in hereditary nonpolyposis colon cancer, familial polyposis, and sporadic colon cancer. *Gastroenterology*, **111,** 307-317 30 Jul 98 18:47:05 +0000.

Konstantinova, L.N., Fleischman, E.W., Knisch, V.I., Perevozchikov, A.G. & Kopnin, B.P. (1991). Karyotype pecularities of human colorectal adenocarcinomas. *Human Genetics*, **86,** 491-496.

Korn, W.M., Yasutake, T., Kuo, W.L., Warren, R.S., Collins, C., Tomita, M., Gray, J. & Waldman, F.M. (1999). Chromosome arm 20q gains and other genomic alterations in colorectal cancer metastatic to liver, as analyzed by comparative genomic hybridization and fluorescence in situ hybridization. *Genes Chromosomes and Cancer*, **25,** 82-90.

Koshiji, M., Adachi, Y., Taketani, S., Takeuchi, K., Hioki, K. & Ikehara, S. (1997). Mechanisms underlying apoptosis induced by combination of 5-fluorouracil and interferon-gamma. *Biochemical and Biophysical Research Communications*, **240,** 376-81.

Kroemer, G. (1997). The proto-oncogene Bcl-2 and its role in regulating apoptosis. *Nature Medicine*, **3,** 614-620.

Laffer, U.T., Metzger, U., Aeberhard, P., Mailbach, R., Castiglione, M., Goldhirsch, A., Weber, W., Gloor, F., Stamm, B., Waltzer, U., Egeli, R., Arma, S., Barras, J.P., Lampert, V.D., Harder, F., Martinoli, S., Muller, W., Schroder, R. & Vonhuben, R. (1995). Long-term results of single course of adjuvant intraportal chemotherapy for colorectal cancer. *Lancet*, **345,** 349-353.

Lamb, J.R. & Friend, S.H. (1997). Which guesstimate is the best guesstimate? Predicting chemotherapeutic outcomes [news; comment] [see comments]. *Nature Medicine*, **3,** 962-963.

Lane, D.P. (1992). Cancer - P53, guardian of the genome. *Nature*, **358,** 15-16.

Lane, N. (1976). The precursor tissue of ordinary large bowel cancer. *Cancer Research*, **36,** 2669-2672.

Lasserre, C., Sabatier, L., Beaumatin, J., Luccioni, C., Lefrancois, D., Muleris, M. & Dutrillaux, B. (1994). Gene dosage and expression, and enzyme activity of thymidine kinase and thymidylate synthase in xenografted colorectal adenocarcinomas. *International Journal of Cancer*, **56,** 506-511.

Laurent Puig, P., Olschwang, S., Delattre, O., Remvikos, Y., Asselain, B., Melot, T., Validire, P., Muleris, M., Girodet, J., Salmon, R.J. & et al. (1992). Survival and acquired genetic alterations in colorectal cancer. *Gastroenterology*, **102,** 1136-1141.

Leach, F.S., Nicolaides, N.C., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomaki, P., Sistonen, P., Aaltonen, L.A., Nystrom Lahti, M. & et al. (1993). Mutations of a mutS homolog in hereditary nonpolyposis colorectal cancer. *Cell*, **75,** 1215-1225.

Lee, C.W., Sorensen, T.S., Shikama, N. & La Thangue, N.B. (1998). Functional interplay between p53 and E2F through co-activator p300. *Oncogene*, **16,** 2695-2710.

Lee, S., Cavallo, L. & Griffith, J. (1997). Human p53 binds Holliday junctions strongly and facilitates their cleavage. *Journal of Biological Chemistry*, **272,** 7532-7539.

Lee, S., Elenbaas, B., Levine, A. & Griffith, J. (1995). p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. *Cell*, **81,** 1013-1020.

Lefrancois, D., Olschwang, S., Delattre, O., Muleris, M., Dutrillaux, A.M., Thomas, G. & Dutrillaux, B. (1989). Preservation of chromosome and DNA characteristics of human colorectal adenocarcinomas after passage in nude mice. *International Journal of Cancer*, **44,** 871-878.

Leibovitz, A., Stinson, J.C., McCombs, W.B., 3rd, McCoy, C.E., Mazur, K.C. & Mabry, N.D. (1976). Classification of human colorectal adenocarcinoma cell lines. *Cancer Research*, **36,** 4562-4569.

Leichman, C.G., Lenz, H.J., Leichman, L., Danenberg, K., Baranda, J., Groshen, S., Boswell, W., Metzger, R., Tan, M. & Danenberg, P.V. (1997). Quantitation of intratumoral thymidylate synthase expression predicts for disseminated colorectal cancer response and resistance to

protracted-infusion fluorouracil and weekly leucovorin. *Journal of Clinical Oncology*, **15,** 3223-3229.

Leister, I., Weith, A., Bruderlein, S., Cziepluch, C., Kangwanpong, D., Schlag, P. & Schwab, M. (1990). Human colorectal cancer: high frequency of deletions at chromosome 1p35. *Cancer Research*, **50,** 7232-7235.

Lengauer, C., Kinzler, K.W. & Vogelstein, B. (1997). Genetic instability in colorectal cancers. *Nature*, **386,** 623-627.

Lengauer, C., Kinzler, K.W. & Vogelstein, B. (1998). Genetic instabilities in human cancers. *Nature*, **396,** 643-649.

Leppert, M., Dobbs, M., Scambler, P., O'Connell, P., Nakamura, Y., Stauffer, D., Woodward, S., Burt, R., Hughes, J., Gardner, E. & et al. (1987). The gene for familial polyposis coli maps to the long arm of chromosome 5. *Science*, **238,** 1411-1413.

Leveillard, T., Andera, L., Bissonnette, N., Schaeffer, L., Bracco, L., Egly, J.M. & Wasylyk, B. (1996). Functional interactions between p53 and the TFIIH complex are affected by tumour-associated mutations. *Embo Journal*, **15,** 1615-1624.

Levin, K.E. & Dozois, R.R. (1991). Epidemiology of large bowel cancer. *World Journal of Surgery*, **15,** 562-567.

Levine, A.J. (1997). p53, the cellular gatekeeper for growth and division. *Cell*, **88,** 323-3231.

Lewis, J. & Bird, A. (1991). DNA methylation and chromatin structure. *FEBS Letters*, **285,** 155-159.

Li, F., Ambrosini, G., Chu, E.Y., Plescia, J., Tognin, S., Marchisio, P.C. & Altieri, D.C. (1998). Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature*, **396,** 580-584.

Li, F.Z., Ackermann, E.J., Bennett, C.F., Rothermel, A.L., Plescia, J., Tognin, S., Villa, A., Marchisio, P.C. & Altieri, D.C. (1999). Pleiotropic cell-division defects and apoptosis induced by interference with survivin function. *Nature Cell Biology*, **1,** 461-466.

Li, G. & Ho, V.C. (1998). p53-dependent DNA repair and apoptosis respond differently to high- and low-dose ultraviolet radiation. *BritishJournal of Dermatology*, **139,** 3-10.

Lin, Y. & Benchimol, S. (1995). Cytokines inhibit p53-mediated apoptosis but not p53-mediated G1 arrest. *Molecular Cell Biology*, **15,** 6045-6054.

Linke, S.P., Clarkin, K.C. & Wahl, G.M. (1997). p53 mediates permanent arrest over multiple cell cycles in response to gamma-irradiation. *Cancer Research*, **57,** 1171-1179.

Liu, B., Nicolaides, N.C., Markowitz, S., Willson, J.K.V., Parsons, R.E., Jen, J., Papadopolous, N., Peltomaki, P., Delachapelle, A., Hamilton, S.R., Kinzler, K.W. & Vogelstein, B. (1995). Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nature Genetics*, **9,** 48-55.

Liu, L.L., Markowitz, S. & Gerson, S.L. (1996). Mismatch repair mutations override alkyltransferase in conferring resistance to temozolomide but not to 1,3-bis(2-chloroethyl)nitrosourea. *Cancer Research*, **56,** 5375-5379.

Liu, Y.A., Bohn, S.A. & Sherley, J.L. (1998). Inosine-5'-monophosphate dehydrogenase is a rate-determining factor for p53-dependent growth regulation. *Molecular Biology of the Cell*, **9,** 15-28.

Loda, M., Cukor, B., Tam, S.W., Lavin, P., Fiorentino, M., Draetta, G.F., Jessup, J.M. & Pagano, M. (1997). Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nature Medicine*, **3,** 231-234.

Loeb, L.A. (1994). Microsatellite instability - marker of a mutator phenotype in cancer. *Cancer Research*, **54,** 5059-5063.

Loffler, M. (1987). Restimulation of cell cycle progression by hypoxic tumour cells with deoxynucleosides requires ppm oxygen tension. *Experimental Cell Research*, **169,** 255-261.

Lowe, S.W., Bodis, S., McClatchey, A., Remington, L., Ruley, H.E., Fisher, D.E., Housman, D.E. & Jacks, T. (1994). P53 status and the efficacy of cancer therapy *in vivo*. *Science*, **266,** 807-810.

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A. & Jacks, T. (1993). P53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, **362,** 847-849.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C. & Wang, X. (1998). Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell*, **94,** 481-490.

Lynch, A.M., Gooderham, N.J., Davies, D.S. & Boobis, A.R. (1998). Genetic analysis of PHIP intestinal mutations in MutaMouse. *Mutagenesis*, **13,** 601-605.

Ma, J., Stampfer, M.J., Giovannucci, E., Artigas, C., Hunter, D.J., Fuchs, C., Willett, W.C., Selhub, J., Hennekens, C.H. & Rozen, R. (1997). Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. *Cancer Research*, **57,** 1098-1102.

Macville, M., Schrock, E., PadillaNash, H., Keck, C., Ghadimi, B.M., Zimonjic, D., Popescu, N. & Ried, T. (1999). Comprehensive and definitive molecular cytogenetic characterization of HELA cells by spectral karyotyping. *Cancer Research*, **59,** 141-150.

Macville, M., Veldman, T., PadillaNash, H., Wangsa, D., Obrien, P., Schrock, E. & Ried, T. (1997). Spectral karyotyping: a 24-colour FISH technique for the identification of chromosomal rearrangements. *Histochemistry and Cell Biology*, **108,** 299-305.

Maecker, H.L., Koumenis, C. & Giaccia, A.J. (2000). p53 promotes selection for Fas-mediated apoptotic resistance. *Cancer Research*, **60,** 4638-4644.

Maity, A., McKenna, W.G. & Muschel, R.J. (1994). The molecular basis for cell cycle delays following ionizing radiation - a review. *Radiotherapy and Oncology*, **31,** 1-13.

Malcomson, R.D.G., Oren, M., Wyllie, A.H. & Harrison, D.J. (1995). p53-independent death and p53-induced protection against apoptosis in fibroblasts treated with chemotherapeutic drugs. *British Journal of Cancer*, **72,** 952-957.

Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L.Z., Lutterbaugh, J., Fan, R.S., Zborowska, E., Kinzler, K.W., Vogelstein, B., Brattain, M. & Willson, J.K.V. (1995). Inactivation of the type-II TGF-Beta receptor in colon cancer cells with microsatellite instability. *Science*, **268,** 1336-1338.

Marsh, K.A., Stamp, G.W.H. & Kirkland, S.C. (1993). Isolation and characterization of multiple cell types from a single human colonic carcinoma - Tumorigenicity of these cell types in a xenograft system. *Journal of Pathology*, **170,** 441-450.

Maze, R., Carney, J.P., Kelley, M.R., Glassner, B.J., Williams, D.A. & Samson, L. (1996). Increasing DNA repair methyltransferase levels via bone marrow stem cell transduction rescues mice from the toxic effects of 1,3-bis(2-chloroethyl)-1-nitrosourea, a chemotherapeutic alkylating agent. *Proceedings of the National Academy of Sciences of the United States of America*, **93,** 206-210.

McBain, J.A., Weese, J.L., Meisner, L.F., Wolberg, W.H. & Willson, J.K.V. (1984). Establishment and characterization of human colorectal cancer cell lines. *Cancer Research*, **44,** 5813-5821.

McCormick, F. (1999). Signalling networks that cause cancer. *Trends Cell Biology*, **9,** M53-56.

McCurrach, M.E., Connor, T.M., Knudson, C.M., Korsmeyer, S.J. & Lowe, S.W. (1997). bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. *Proceedings of the National Academy of Sciences of the United States of America*, **94,** 2345-2349.

McKay, B.C., Ljungman, M. & Rainbow, A.J. (1998). Persistent DNA damage induced by ultraviolet light inhibits p21(waf1) and bax expression: implications for DNA repair, UV sensitivity and the induction of apoptosis. *Oncogene*, **17,** 545-555.

McKeown-Eyssen, G. (1994). Epidemiology of colorectal cancer revisited: are serum triglycerides and/or plasma glucose associated with risk? *Cancer Epidemiology Biomarkers Preview*, **3,** 687-695.

McQueen, H.A., Wyllie, A.H., Piris, J., Foster, E. & Bird, C.C. (1991). Stability of critical genetic lesions in human colorectal carcinoma xenografts. *Britsh Journal of Cancer*, **63,** 94-96.

Meijer, G.A., Hermsen, M., Baak, J.P.A., vanDiest, P.J., Meuwissen, S.G.M., Belien, J.A.M., Hoovers, J.M.N., Joenje, H., Snijders, P.J.F. & Walboomers, J.M.M. (1998). Progression from colorectal adenoma to carcinoma is associated with non-random chromosomal gains as detected by comparative genomic hybridisation. *Journal of Clinical Pathology*, **51,** 901-909.

Melcher, R., Steinlein, C., Feichtinger, W., Muller, C.R., Menzel, T., Luhrs, H., Scheppach, W. & Schmid, M. (2000). Spectral karyotyping of the human colon cancer cell lines SW480 and SW620. *Cytogenetics and Cell Genetics*, **88,** 145-152.

Mertens, F., Jin, Y., Heim, S., Mandahl, N., Jonsson, N., Mertens, O., Persson, B., Salemark, L., Wennerberg, J. & Mitelman, F. (1992). Clonal structural chromosome aberrations in nonneoplastic cells of the skin and upper aerodigestive tract. *Genes Chromosomes and Cancer*, **4,** 235-240.

Mertens, F., Johansson, B., Hoglund, M. & Mitelman, F. (1997). Chromosomal imbalance maps of malignant solid tumors: A cytogenetic survey of 3185 neoplasms. *Cancer Research*, **57,** 2765-2780.

Meterissian, S.H., Kontogiannea, M., Po, J., Jensen, G. & Ferdinand, B. (1997). Apoptosis induced in human colorectal carcinoma by anti-Fas antibody. *Annals of Surgical Oncology*, **4,** 169-175.

Mirjolet, J.F., Barberi Heyob, M., Merlin, J.L., Marchal, S., Etienne, M.C., Milano, G. & Bey, P. (1998). Thymidylate synthase expression and activity: relation to S-phase parameters and 5-fluorouracil sensitivity. *British Journal of Cancer*, **78,** 62-68.

Mitelman, F. (1995). ISCN 1995: An internatioinal system for human cytogenetic nomenclature. S. Karger: Basel.

Mitelman, F., Mertens, F. & Johansson, B. (1997). A breakpoint map of recurrent chromosomal rearrangements in human neoplasia. *Nature Genetics*, **15,** 417-474.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B. & Reed, J.C. (1994). Tumor-suppressor p53 is a regulator of *BCL-2* and *Bax* gene expression *in vitro* and *in vivo*. *Oncogene*, **9,** 1799-1805.

Miyashita, T. & Reed, J.C. (1995). Tumor-suppressor p53 is a direct transcriptional activator of the human *Bax* gene. *Cell*, **80,** 293-299.

Moertel, C.G. (1994). Chemotherapy for colorectal cancer. *New England Journal of Medicine*, **330,** 1136-1142.

Moertel, C.G., Fleming, T.R., Macdonald, J.S., Haller, D.G., Laurie, J.A., Goodman, P.J., Ungerleider, J.S., Emerson, W.A., Tormey, D.C., Glick, J.H. & et al. (1990). Levamisole and fluorouracil for adjuvant therapy of resected colon carcinoma. *New England Journal of Medicine*, **322,** 352-358.

Moertel, C.G., Fleming, T.R., Macdonald, J.S., Haller, D.G., Laurie, J.A., Tangen, C.M., Ungerleider, J.S., Emerson, W.A., Tormey, D.C., Glick, J.H. & et al. (1995). Fluorouracil plus levamisole as effective adjuvant therapy after resection of stage III colon carcinoma: a final report. *Annals of Internationl Medicine*, **122,** 321-326.

Moggs, J.G., Szymkowski, D.E., Yamada, M., Karran, P. & Wood, R.D. (1997). Differential human nucleotide excision repair of paired and mispaired cisplatin-DNA adducts. *Nucleic Acids Research*, **25,** 480-490.

Moller, P., Koretz, K., Leithauser, F., Bruderlein, S., Henne, C., Quentmeier, A. & Krammer, P.H. (1994). Expression of Apo-1 (Cd95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. *International Journal of Cancer*, **57,** 371-377.

Morgan, W.F., Corcoran, J., Hartmann, A., Kaplan, M.I., Limoli, C.L. & Ponnaiya, B. (1998). DNA double-strand breaks, chromosomal rearrangements, and genomic instability. *Mutation Research*, **404,** 125-128.

Morin, P.J., Vogelstein, B. & Kinzler, K.W. (1996). Apoptosis and APC in colorectal tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America*, **93,** 7950-7794.

Moskaluk, C.A. & Kern, S.E. (1996). Cancer gets Mad: DPC4 and other TGFbeta pathway genes in human cancer. *Biochemica et Biophysica ACTA*, **1288,** M31-33.

Mueller, M., Kairat, A., SchulzeBergkamen, H., Friedman, S.L., Oren, M., Krammer, P.H. & Stremmel, W. (1999). An intact intronic p53-binding site is a precondition for p53-dependent CD95 gene expression in hepatoma cells. *Hepatology*, **30,** 1021.

Mueller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., LiWeber, M., Friedman, S.L., Galle, P.R., Stremmel, W., Oren, M. & Krammer, P.H. (1998). p53 activates the CD95

(APO-1/Fas) gene in response to DNA damage by anticancer drugs. *Journal of Experimental Medicine*, **188,** 2033-2045.

Muhua, L., Adames, N.R., Murphy, M.D., Shields, C.R. & Cooper, J.A. (1998). A cytokinesis checkpoint requiring the yeast homologue of an APC-binding protein. *Nature*, **393,** 487-491.

Muleris, M., Dutrillaux, A.M., Olschwang, S., Salmon, R.J. & Dutrillaux, B. (1995). Predominance of normal karyotype in colorectal tumors from hereditary nonpolyposis colorectal cancer patients. *Genes Chromosomes and Cancer*, **14,** 223-226.

Muleris, M., Salmon, R.J. & Dutrillaux, B. (1988). Existence of two distinct processes of chromosomal evolution in near diploid colorectal tumors. *Cancer Genetics and Cytogenetics*, **32,** 43-50.

Muleris, M., Salmon, R.J. & Dutrillaux, B. (1990). Cytogenetics of colorectal adenocarcinomas. *Cancer Genetics and Cytogenetics*, **46,** 143-156.

Muller, M., Strand, S., Hug, H., Heinemann, E.M., Walczak, H., Hofmann, W.J., Stremmel, W., Krammer, P.H. & Galle, P.R. (1997). Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild type p53. *Journal of Clinical Investigation*, **99,** 403-413.

Muto, T., Bussey, H.J. & Morson, B.C. (1975). The evolution of cancer of the colon and rectum. *Cancer*, **36,** 2251-2270.

Myeroff, L.L., Parsons, R., Kim, S.J., Hedrick, L., Cho, K.R., Orth, K., Mathis, M., Kinzler, K.W., Lutterbaugh, J., Park, K., Bang, Y.J., Lee, H.Y., Park, J.G., Lynch, H.T., Roberts, A.B., Vogelstein, B. & Markowitz, S.D. (1995). A transforming growth factor beta receptor type II gene mutation common in colon and gastric but rare in endometrial cancers with microsatellite instability. *Cancer Research*, **55,** 5545-5547.

Nagao, M., Nakajima, Y., Hisanaga, M., Kayagaki, N., Kanehiro, H., Aomatsu, Y., Ko, S., Yagita, H., Yamada, T., Okumura, K. & Nakano, H. (1999). The alteration of Fas receptor and ligand system in hepatocellular carcinomas: how do hepatoma cells escape from the host immune surveillance in vivo? *Hepatology*, **30,** 413-421.

Nagengast, F.M., Grubben, M.J. & van Munster, I.P. (1995). Role of bile acids in colorectal carcinogenesis. *European Journal of Cancer*, **31a,** 1067-1070.

Nakamura, Y., Nishisho, I., Kinzler, K.W., Vogelstein, B., Miyoshi, Y., Miki, Y., Ando, H. & Horii, A. (1992). Mutations of the *APC* (Adenomatous Polyposis Coli) gene in FAP (Familial Polyposis Coli) patients and in sporadic colorectal tumors. *Tohoku Journal of Experimental Medicine*, **168,** 141-147.

Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R.J., Matsuda, H. & Tsujimoto, Y. (1998). Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. *Proceedings of the National Academy of Sciences of the United States of America*, **95,** 14681-14686.

Newlands, E.S., Oreilly, S.M., Glaser, M.G., Bower, M., Evans, H., Brock, C., Brampton, M.H., Colquhoun, I., Lewis, P., RiceEdwards, J.M., Illingworth, R.D. & Richards, P.G. (1996). The Charing Cross Hospital experience with temozolomide in patients with gliomas. *European Journal of Cancer*, **32A,** 2236-2241.

Newlands, E.S., Stevens, M.F.G., Wedge, S.R., Wheelhouse, R.T. & Brock, C. (1997). Temozolomide: A review of its discovery, chemical properties, pre- clinical development and clinical trials. *Cancer Treatment Reviews*, **23,** 35-61.

Nucci, M.R., Robinson, C.R., Longo, P., Campbell, P. & Hamilton, S.R. (1997). Phenotypic and genotypic characteristics of aberrant crypt foci in human colorectal mucosa. *Human Pathology*, **28,** 1396-407.

Nystromlahti, M., Parsons, R., Sistonen, P., Pylkkanen, L., Aaltonen, L.A., Leach, F.S., Hamilton, S.R., Watson, P., Bronson, E., Cavalieri, J., Lynch, J., Lanspa, S., Smyrk, T., Lynch, P., Drouhard, T., Kinzler, K.W., Vogelstein, B., Lynch, H.T., Delachapelle, A. & Peltomaki, P. (1994). Mismatch repair genes on chromosomes 2p and 3p account for a major share of hereditary nonpolyposis colorectal cancer families evaluable by linkage. *American Journal of Human Genetics*, **55,** 659-665.

O'Connell, M.J., Laurie, J.A., Kahn, M., Fitzgibbons, R.J., Jr., Erlichman, C., Shepherd, L., Moertel, C.G., Kocha, W.I., Pazdur, R., Wieand, H.S., Rubin, J., Vukov, A.M., Donohue, J.H., Krook, J.E. & Figueredo, A. (1998). Prospectively randomized trial of postoperative adjuvant chemotherapy in patients with high-risk colon cancer. *Journal of Clinical Oncology*, **16,** 295-300.

O'Connell, M.J., Martenson, J.A., Wieand, H.S., Krook, J.E., Macdonald, J.S., Haller, D.G., Mayer, R.J., Gunderson, L.L. & Rich, T.A. (1994). Improving adjuvant therapy for rectal cancer by combining protracted-infusion fluorouracil with radiation therapy after curative surgery. *New England Journalof Medicine*, **331,** 502-507.

O'Connor, L., Harris, A.W. & Strasser, A. (2000). CD95 (Fas/APO-1) and p53 signal apoptosis independently in diverse cell types. *Cancer Research*, **60,** 1217-1220.

O'Connor, P.M., Jackman, J., Bae, I., Myers, T.G., Fan, S.J., Mutoh, M., Scudiero, D.A., Monks, A., Sausville, E.A., Weinstein, J.N., Friend, S., Fornace, A.J. & Kohn, K.W. (1997). Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. *Cancer Research*, **57,** 4285-4300.

Oehm, A., Behrmann, I., Falk, W., Pawlita, M., Maier, G., Klas, C., Liweber, M., Richards, S., Dhein, J., Trauth, B.C., Ponstingl, H. & Krammer, P.H. (1992). Purification and molecular cloning of the Apo-1 cell surface antigen, a member of the tumor necrosis factor nerve growth factor receptor superfamily sequence identity with the Fas antigen. *Journal of Biological Chemistry*, **267,** 10709-10715.

Ofner, D., Riehemann, K., Maier, H., Riedmann, B., Nehoda, H., Totsch, M., Bocker, W., Jasani, B. & Schmid, K.W. (1995). Immunohistochemically detectable bcl-2 expression in colorectal carcinoma: correlation with tumour stage and patient survival. *British Journal of Cancer*, **72,** 981-985.

Olsen, J., Kronborg, O., Lynggaard, J. & Ewertz, M. (1994). Dietary risk factors for cancer and adenomas of the large intestine. A case-control study within a screening trial in Denmark. *European Journal of Cancer*, **30a,** 53-60.

Oltvai, Z.N., Milliman, C.L. & Korsmeyer, S.J. (1993). Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74,** 609-619.

Ookawa, K., Sakamoto, M., Hirohashi, S., Yoshida, Y., Sugimura, T., Terada, M. & Yokota, J. (1993). Concordant p53 and DCC alterations and allelic losses on chromosomes 13q and 14q associated with liver metastases of colorectal carcinoma. *International Journal of Cancer*, **53,** 382-387.

Otto, E., McCord, S. & Tlsty, T.D. (1989). Increased incidence of CAD gene amplification in tumorigenic rat lines as an indicator of genomic instability of neoplastic cells. *Journal of Biological Chemistry*, **264,** 3390-339.

Ouyang, H., Furukawa, T., Abe, T., Kato, Y. & Horii, A. (1998). The*BAX* gene, the promoter of apoptosis, is mutated in genetically unstable cancers of the colorectum, stomach, and endometrium. *Clinical Cancer Research*, **4,** 1071-1074.

Owen, D.A. (1996). Flat adenoma, flat carcinoma, and de novo carcinoma of the colon. *Cancer*, **77,** 3-6.

Owen-schaub, L.B., Zhang, W., Cusack, J.C., Angelo, L.S., Santee, S.M., Fujiwara, T., Roth, J.A., Deisseroth, A.B., Zhang, W.W., Kruzel, E. & Radinsky, R. (1995). Wild-type human P53 and a temperature-sensitive mutant induce Fas/Apo-1 expression. *Molecular and Cellular Biology*, **15,** 3032-3040.

PaineMurrieta, G.D., Taylor, C.W., Curtis, R.A., Lopez, M.H.A., Dorr, R.T., Johnson, C.S., Funk, C.Y., Thompson, F. & Hersh, E.M. (1997). Human tumor models in the severe combined immune deficient (scid) mouse. *Cancer Chemotherapy and Pharmacology*, **40,** 209-214.

Palazzo, J.P., Kafka, N.J., Grasso, L., Chakrani, F., Hanau, C., Cuesta, K.H. & Mercer, W.E. (1997). The role of p53, p21(WAF1)/(C1P1), and bcl-2 in radioresistant colorectal carcinoma. *Human Pathology*, **28,** 1189-1195.

Palmer, S. & Bakshi, K. (1983). Diet, nutrition, and cancer: interim dietary guidelines. *Jopurnal of the National Cancer Institute*, **70,** 1151-70.

Parker, W.B. & Cheng, Y.C. (1990). Metabolism and mechanism of action of 5-fluorouracil. *Pharmacology and Therapeutics*, **48,** 381-395.

Parsons, R., Li, G.M., Longley, M.J., Fang, W.H., Papadopoulos, N., Jen, J., Delachapelle, A., Kinzler, K.W., Vogelstein, B. & Modrich, P. (1993). Hypermutability and mismatch repair deficiency in RER+ tumor cells. *Cell*, **75,** 1227-1236.

Parsons, R., Myeroff, L.L., Liu, B., Willson, J.K.V., Markowitz, S.D., Kinzler, K.W. & Vogelstein, B. (1995). Microsatellite instability and mutations of the transforming growth factor beta type II receptor gene in colorectal cancer. *Cancer Research*, **55,** 5548-5550.

Pedersen-Bjergaard, J. & Rowley, J.D. (1994). The balanced and the unbalanced chromosome aberrations of acute myeloid leukemia may develop in different ways and may contribute differently to malignant transformation. *Blood*, **83,** 2780-2986.

Pellegata, N.S., Antoniono, R.J., Redpath, J.L. & Stanbridge, E.J. (1996). DNA damage and p53-mediated cell cycle arrest: A reevaluation. *Proceedings of the National Academy of Sciences of the United States of America*, **93,** 15209-15214.

Peltomaki, P. (1995). Microsatellite instability and hereditary non-polyposis colon cancer. *Journal of Pathology*, **176,** 329-30.

Peng, C.Y., Graves, P.R., Thoma, R.S., Wu, Z., Shaw, A.S. & Piwnica Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science*, **277,** 1501-1505.

Perry, I., Hardy, R., Jones, T. & Jankowski, J. (1999). A colorectal cell line with alterations in E-cadherin and epithelial biology may be an in vitro model of colitis. *Journal of Clinical Pathology-Molecular Pathology*, **52,** 231-242.

Phillips, A.C., Ernst, M.K., Bates, S., Rice, N.R. & Vousden, K.H. (1999). E2F-1 potentiates cell death by blocking antiapoptotic signaling pathways. *Molecular Cell*, **4,** 771-781.

Pihan, G.A. & Doxsey, S.J. (1999). The mitotic machinery as a source of genetic instability in cancer. *Seminars in Cancer Biology*, **9,** 289-302.

Pihan, G.A., Purohit, A., Wallace, J., Knecht, H., Woda, B., Quesenberry, P. & Doxsey, S.J. (1998). Centrosome defects and genetic instability in malignant tumors. *Cancer Research*, **58,** 3974-85.

Pinkoski, M.J., Brunner, T., Green, D.R. & Lin, T. (2000). Fas and Fas ligand in gut and liver. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, **278,** G354-366.

Polyak, K. (1996). Negative regulation of cell growth by TGF beta. *Biochimica Et Biophysica Acta-Reviews On Cancer*, **1242,** 185-199.

Polyak, K., Xia, Y., Zweier, J.L., Kinzler, K.W. & Vogelstein, B. (1997). A model for p53-induced apoptosis. *Nature*, **389,** 300-305.

Potter, J.D. (1999). Colorectal cancer: molecules and populations. *Journal of the National Cancer Institute*, **91,** 916-32.

Potter, J.D., Slattery, M.L., Bostick, R.M. & Gapstur, S.M. (1993). Colon cancer: a review of the epidemiology. *Epidemiology Review*, **15,** 499-545.

Powell, S.M., Zilz, N., Beazerbarclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B. & Kinzler, K.W. (1992). *APC* mutations occur early during colorectal tumorigenesis. *Nature*, **359,** 235-237.

Praml, C., Finke, L.H., Herfarth, C., Schlag, P., Schwab, M. & Amler, L. (1995a). Deletion mapping defines different regions in 1p34.2-pter that may harbor genetic information related to human colorectal cancer. *Oncogene*, **11,** 1357-1362.

Praml, C., Savelyeva, L., Le Paslier, D., Siracusa, L.D., Buchberg, A.M., Schwab, M. & Amler, L.C. (1995b). Human homologue of a candidate for the Mom1 locus, the secretory type II phospholipase A2 (PLA2S-II), maps to 1p35-36.1/D1S199. *Cancer Research*, **55,** 5504-5506.

Prisco, M., Hongo, A., Rizzo, M.G., Sacchi, A. & Baserga, R. (1997). The insulin-like growth factor I receptor as a physiologically relevant target of p53 in apoptosis caused by interleukin-3 withdrawal. *Molecular Cell Biology*, **17,** 1084-1092.

Pritchard, D.M., Potten, C.S. & Hickman, J.A. (1998). The relationships between p53-dependent apoptosis, inhibition of proliferation, and 5-fluorouracil-induced histopathology in murine intestinal epithelia. *Cancer Research*, **58,** 5453-5465.

Pritchard, D.M., Watson, A.J.M., Potten, C.S., Jackman, A.L. & Hickman, J.A. (1997). Inhibition by uridine but not thymidine of p53-dependent intestinal apoptosis initiated by 5-fluorouracil: Evidence for the involvement of RNA perturbation. *Proceedings of the National Academy of Sciences of the United States of America*, **94,** 1795-1799.

Purdie, C.A., Ogrady, J., Piris, J., Wyllie, A.H. & Bird, C.C. (1991). P53 expression in colorectal tumors. *American Journal of Pathology*, **138,** 807-813.

Rabbitts, T.H. (1994). Chromosomal translocations in human cancer. *Nature*, **372,** 143-149.

Rampino, N., Yamamoto, H., Ionov, Y., Li, Y., Sawai, H., Reed, J.C. & Perucho, M. (1997). Somatic frameshift mutations in the *BAX* gene in colon cancers of the microsatellite mutator phenotype. *Science*, **275,** 967-969.

Rao, P.H., Cigudosa, J., Ning, Y., Calasanz, M.J., Tagawa, S., Michaele, S., Klein, B., DallaFavera, R., Ried, T. & Chaganti, R.S.K. (1997). Karyotypic complexity of multiple myeloma defined by multicolor spectral karyotyping (SKY). *Blood*, **90,** 381.

Reddy, B., Engle, A., Katsifis, S., Simi, B., Bartram, H.P., Perrino, P. & Mahan, C. (1989). Biochemical epidemiology of colon cancer: effect of types of dietary fiber on fecal mutagens, acid, and neutral sterols in healthy subjects. *Cancer Research*, **49,** 4629-4635.

Reichmann, A., Levin, B. & Martin, P. (1982). Human large bowel cancer - Correlation of clinical and histopathological features with banded chromosomes. *International Journal of Cancer*, **29,** 625-629.

Reichmann, A., Martin, P. & Levin, B. (1981). Chromosomal banding patterns in human large bowel cancer. *International Journal of Cancer*, **28,** 431-440.

Reichmann, A., Martin, P. & Levin, B. (1984). Chromosomes in human large bowel tumors - a study of chromosome one. *Cancer Genetics and Cytogenetics*, **12,** 295-301.

Reinke, V. & Lozano, G. (1997). Differential activation of p53 targets in cells treated with ultraviolet radiation that undergo both apoptosis and growth arrest. *Radiation Research*, **148,** 115-122.

Remvikos, Y., Muleris, M., Salmon, R.J. & Dutrillaux, B. (1997). Colorectal carcinogenesis: from chromosomal evolution pathways to molecular pathogenesis. *Cancer Genetics and Cytogenetics*, **93,** 63-73.

Remvikos, Y., Vogt, N., Muleris, M., Salmon, R.J., Malfoy, B. & Dutrillaux, B. (1995). DNA-repeat instability is associated with colorectal cancers presenting minimal chromosome rearrangements. *Genes Chromosomes and Cancer*, **12,** 272-276.

Ricardson, C. & Jasin, M. (2000). Frequent chromosomal translocations induced by DNA double-strand breaks. *Nature*, **405,** 697-700.

Rice, G.C., Hoy, C. & Schimke, R.T. (1986). Transient hypoxia enhances the frequency of dihydrofolate reductase gene amplification in Chinese hamster ovary cells. *Proceedings of the National Academy of Sciences of the United States of America*, **83,** 5978-5982.

Ried, T. (1998). Analysis of chromosomal aberrations by spectral karyotyping. *European Journal of Human Genetics*, **6,** L26.

Ried, T., Knutzen, R., Steinbeck, R., Blegen, H., Schrock, E., Heselmeyer, K., duManoir, S. & Auer, G. (1996). Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. *Genes Chromosomes and Cancer*, **15,** 234-245.

Ried, T., Macville, M., Veldman, T., PadillaNash, H., Roschke, A., Ning, Y., duManoir, S., BarAm, I., Garini, Y., Liyanage, M. & Schrock, E. (1997). Spectral karyotyping. *Cytogenetics and Cell Genetics*, **77,** L4.

Robertson, K.D. & Jones, P.A. (2000). DNA methylation: past, present and future directions. *Carcinogenesis*, **21,** 461-467.

Roperch, J.P., Alvaro, V., Prieur, S., Tuynder, M., Nemani, M., Lethrosne, F., Piouffre, L., Gendron, M.C., Israeli, D., Dausset, J., Oren, M., Amson, R. & Telerman, A. (1998). Inhibition of presenilin 1 expression is promoted by p53 and p21(WAF- 1) and results in apoptosis and tumor suppression. *Nature Medicine*, **4,** 835-838.

Rowan, A.J., Lamlum, H., Ilyas, M., Wheeler, J., Straub, J., Papadopoulou, A., Bicknell, D., Bodmer, W.F. & Tomlinson, I.P.M. (2000). APC mutations in sporadic colorectal tumors:

A mutational "hotspot" and interdependence of the "two hits". *Proceedings of the National Academy of Sciences of the United States of America*, **97,** 3352-3357.

Ruley, H.E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature*, **304,** 602-606.

Ruschoff, J., Wallinger, S., Dietmaier, W., Bocker, T., Brockhoff, G., Hofstadter, F. & Fishel, R. (1998). Aspirin suppresses the mutator phenotype associated with hereditary nonpolyposis colorectal cancer by genetic selection. *Proceedings of the National Academy of Sciences of the United States of America*, **95,** 11301-11306.

Rutzky, L.P., Kaye, C.I., Siciliano, M.J., Chao, M. & Kahan, B.D. (1980). Longitudinal karyotype and genetic signature analysis of cultured human colon adenocarcinoma cell lines LS180 and LS174T. *Cancer Research*, **40,** 1443-1448.

Samowitz, W.S. & Slattery, M.L. (1997). Transforming growth factor beta receptor type 2 mutations and microsatellite instability in sporadic colorectal adenomas and carcinomas. *American Journal of Pathology*, **151,** 33-35.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H. & Peter, M.E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *EMBO Journal*, **17,** 1675-1687.

Scherer, S.J., Welter, C., Zang, K.D. & Dooley, S. (1996). Specific in vitro binding of p53 to the promoter region of the human mismatch repair gene hMSH2. *Biochemical and Biophysical Research Communications*, **221,** 722-728.

Schimenti, J.C. (1999). Mice and the role of unequal recombination in gene-family evolution. *American Journal of Human Genetics*, **64,** 40-45.

Schmid, M., Haaf, T. & Grunert, D. (1984). 5-Azacytidine-induced undercondensations in human chromosomes. *Human Genetics*, **67,** 257-163.

Schmoll, H.J., Buchele, T., Grothey, A. & Dempke, W. (1999). Where do we stand with 5-fluorouracil? *Seminars in Oncology*, **26,** 589-605.

Schneider, E., Montenarh, M. & Wagner, P. (1998). Regulation of CAK kinase activity by p53. *Oncogene*, **17,** 2733-41.

Schrock, E., duManoir, S., Veldman, T., Schoell, B., Wienberg, J., FergusonSmith, M.A., Ning, Y., Ledbetter, D.H., BarAm, I., Soenksen, D., Garini, Y. & Ried, T. (1996). Multicolor spectral karyotyping of human chromosomes. *Science*, **273,** 494-497.

Scolnick, D.M. & Halazonetis, T.D. (2000). Chfr defines a mitotic stress checkpoint that delays entry into metaphase. *Nature*, **406,** 430-435.

Shackney, S.E., Smith, C.A., Miller, B.W., Burholt, D.R., Murtha, K., Giles, H.R., Ketterer, D.M. & Pollice, A.A. (1989). Model for the genetic evolution of human solid tumors. *Cancer Research*, **49,** 3344-3354.

Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B. & Costa, J. (1992). Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proceedings of the National Academy of Sciences of the United States of America*, **89,** 4495-4499.

Shinohara, H., Yagita, H., Ikawa, Y. & Oyaizu, N. (2000). Fas drives cell cycle progression in glioma cells via extracellular signal-regulated kinase activation. *Cancer Research*, **60,** 1766-1772.

Shirasawa, S., Furuse, M., Yokoyama, N. & Sasazuki, T. (1993). Altered growth of human colon cancer cell lines disrupted at activated *Ki-ras*. *Science*, **260,** 85-88.

Shorthouse, A.J., Smyth, J.F., Steel, G.G., Ellison, M., Mills, J. & Peckham, M.J. (1980). The human tumour xenograft---a valid model in experimental chemotherapy? *Britsh Journal of Surgery*, **67,** 715-722.

Sikora, K., Chan, S., Evan, G., Gabra, H., Markham, N., Stewart, J. & Watson, J. (1987). c-myc oncogene expression in colorectal cancer. *Cancer*, **59,** 1289-1295.

Simms, L.A., Zou, T.T., Young, J., Shi, Y.Q., Lei, J.Y., Appel, R., Rhyu, M.G., Sugimura, H., ChenevixTrench, G., Souza, R.F., Meltzer, S.J. & Leggett, B.A. (1997). Apparent protection from instability of repeat sequences in cancer- related genes in replication error positive gastrointestinal cancers. *Oncogene*, **14,** 2613-2618.

Sionov, R.V. & Haupt, Y. (1998). Apoptosis by p53: mechanisms, regulation, and clinical implications. *Springer Seminars in Immunopathology*, **19,** 345-362.

Sionov, R.V. & Haupt, Y. (1999). The cellular response to p53: the decision between life and death. *Oncogene*, **18,** 6145-6157.

Slattery, M.L., Schaffer, D., Edwards, S.L., Ma, K.N. & Potter, J.D. (1997). Are dietary factors involved in DNA methylation associated with colon cancer? *Nutrition and Cancer*, **28,** 52-62.

Slichenmyer, W.J., Nelson, W.G., Slebos, R.J. & Kastan, M.B. (1993). Loss of a p53-associated G1 checkpoint does not decrease cell survival following DNA damage. *Cancer Research*, **53,** 4164-4168.

Slijepcevic, P. & Bryant, P.E. (1998). Chromosome healing, telomere capture and mechanisms of radiation-induced chromosome breakage. *International Journal of Radiation Biology*, **73,** 1-13.

Smith, A.J., Stern, H.S., Penner, M., Hay, K., Mitri, A., Bapat, B.V. & Gallinger, S. (1994a). Somatic *APC* and *K-ras* codon 12 mutations in aberrant crypt foci from human colons. *Cancer Research*, **54,** 5527-5530.

Smith, C.A., Farrah, T. & Goodwin, R.G. (1994b). The TRF receptor superfamily of cellular and viral proteins - activation, costimulation, and death. *Cell*, **76,** 959-962.

Solic, N., Collins, J.E., Richter, A., Holt, S.J., Campbell, I., Alexander, P. & Davies, D.E. (1995). Two newly established cell lines derived from the same colonic adenocarcinoma exhibit differences in EGF-receptor ligand and adhesion molecule expression. *International Journal of Cancer*, **62,** 48-57.

Solomon, E., Voss, R., Hall, V., Bodmer, W.F., Jass, J.R., Jeffreys, A.J., Lucibello, F.C., Patel, I. & Rider, S.H. (1987). Chromosome 5 allele loss in human colorectal carcinomas. *Nature*, **328,** 616-619.

Sorger, P.K., Dobles, M., Tournebize, R. & Hyman, A.A. (1997). Coupling cell division and cell death to microtubule dynamics. *Current Opinion in Cell Biology*, **9,** 807-814.

Soulie, P., Poupon, M.F., Remvikos, Y., Dutrillaux, B. & Muleris, M. (1999). Distinct chromosomal alterations associated with *TP53* status of LoVo cells under PALA selective pressure: a parallel with cytogenetic pathways of colorectal cancers. *Oncogene*, **18,** 775-781.

Souza, R.F., Appel, R., Yin, J., Wang, S., Smolinski, K.N., Abraham, J.M., Zou, T.T., Shi, Y.Q., Lei, J., Cottrell, J., Cymes, K., Biden, K., Simms, L., Leggett, B., Lynch, P.M., Frazier, M., Powell, S.M., Harpaz, N., Sugimura, H., Young, J. & Meltzer, S.J. (1996). Microsatellite instability in the insulin-like growth factor II receptor gene in gastrointestinal tumours [published erratum appears in Nat Genet 1996 Dec;14(4):488]. *Nature Genetics*, **14,** 255-257.

Souza, R.F., Yin, J., Smolinski, K.N., Zou, T.T., Wang, S., Shi, Y.Q., Rhyu, M.G., Cottrell, J., Abraham, J.M., Biden, K., Simms, L., Leggett, B., Bova, G.S., Frank, T., Powell, S.M., Sugimura, H., Young, J., Harpaz, N., Shimizu, K., Matsubara, N. & Meltzer, S.J. (1997). Frequent mutation of the *E2F4* cell cycle gene in primary human gastrointestinal tumors. *Cancer Res*, **57,** 2350-2353.

Sprung, C.N., Afshar, G., Chavez, E.A., Lansdorp, P., Sabatier, L. & Murnane, J.P. (1999). Telomere instability in a human cancer cell line. *Mutation Research*, **429,** 209-223.

Stark, G.R. (1993). Regulation and mechanisms of mammalian gene amplification. *Advances in Cancer Research*, **61,** 87-113.

Stark, G.R., Debatisse, M., Giulotto, E. & Wahl, G.M. (1989). Recent progress in understanding mechanisms of mammalian DNA amplification. *Cell*, **57,** 901-908.

Stark, G.R. & Wahl, G.M. (1984). Gene amplification. *Annu Rev Biochem*, **53,** 447-91.

Steinmeyer, K. & Deppert, W. (1988). DNA binding properties of murine p53. *Oncogene*, **3,** 501-507.

Stevens, M.F.G., Hickman, J.A., Langdon, S.P., Chubb, D., Vickers, L., Stone, R., Baig, G., Goddard, C., Gibson, N.W., Slack, J.A., Newton, C., Lunt, E., Fizames, C. & Lavelle, F. (1987). Antitumor activity and pharmacokinetics in mice of 8-carbamoyl-3-methyl-imidazo[5,1-*d*]- 1,2,3,5-tetrazin-4(*3H*)-one (CCRG 81045; M & B-39831), a novel drug with potential as an alternative to dacarbazine. *Cancer Research*, **47,** 5846-5852.

Stewart, Z.A., Leach, S.D. & Pietenpol, J.A. (1999). p21(Waf1/Cip1) inhibition of cyclin E/Cdk2 activity prevents endoreduplication after mitotic spindle disruption. *Molecular Cell Biology*, **19,** 205-15.

Stoler, D.L., Anderson, G.R., Russo, C.A., Spina, A.M. & Beerman, T.A. (1992). Anoxia-inducible endonuclease activity as a potential basis of the genomic instability of cancer cells. *Cancer Research*, **52,** 4372-4378.

Stone, J.G., Tomlinson, I.P.M. & Houlston, R.S. (2000). Optimising methods for determining RER status in colorectal cancers. *Cancer Letters*, **149,** 15-20.

Suardet, L., Gaide, A.C., Calmes, J.M., Sordat, B., Givel, J.C., Eliason, J.F. & Odartchenko, N. (1992). Responsiveness of three newly established human colorectal cancer cell lines to transforming growth factors-beta-1 and factor-beta-2. *Cancer Research*, **52,** 3705-3712.

Suda, T. & Nagata, S. (1994). Purification and characterization of the Fas-Ligand that induces apoptosis. *Journal of Experimental Medicine*, **179,** 873-879.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. & Kroemer, G. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*, **397,** 441-446.

Sutherland, G.R., Baker, E. & Richards, R.I. (1998). Fragile sites still breaking. *Trends in Genetics*, **14,** 501-506.

Sutherland, G.R. & Simmers, R.N. (1988). No statistical association between common fragile sites and nonrandom chromosome breakpoints in cancer cells. *Cancer Genetics and Cytogenetics*, **31,** 9-15.

Suzuki, A., Ito, T., Kawano, H., Hayashida, M., Hayasaki, Y., Tsutomi, Y., Akahane, K., Nakano, T., Miura, M. & Shiraki, K. (2000). Survivin initiates procaspase 3/p21 complex formation as a result of interaction with Cdk4 to resist Fas-mediated cell death. *Oncogene*, **19,** 1346-1353.

Takahashi, T., Tanaka, M., Inazawa, J., Abe, T., Suda, T. & Nagata, S. (1994). Human Fas-Ligand - gene structure, chromosomal location and species-specificity. *International Immunology*, **6,** 1567-1574.

Tamm, I., Kornblau, S.M., Segall, H., Krajewski, S., Welsh, K., Kitada, S., Scudiero, D.A., Tudor, G., Qui, Y.H., Monks, A., Andreeff, M. & Reed, J.C. (2000). Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. *Clinical Cancer Research*, **6,** 1796-1803.

Tanaka, H., Arakawa, H., Yamaguchi, T., Shiraishi, K., Fukuda, S., Matsui, K., Takei, Y. & Nakamura, Y. (2000). A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature*, **404,** 42-49.

Tanaka, K., Yanoshita, R., Konishi, M., Oshimura, M., Maeda, Y., Mori, T. & Miyaki, M. (1993). Suppression of tumourigenicity in human colon carcinoma cells by introduction of normal chromosome 1p36 region. *Oncogene*, **8,** 2253-2258.

Taylor, S.S. & McKeon, F. (1997). Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell*, **89,** 727-735.

Tentori, L., Lacal, P.M., Benincasa, E., Franco, D., Faraoni, I., Bonmassar, E. & Graziani, G. (1998). Role of wild-type p53 on the antineoplastic activity of temozolomide alone or combined with inhibitors of poly(ADP-ribose) polymerase. *Journal of Pharmacology and Experimental Therapeutics*, **285,** 884-893.

Tetsu, O. & McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature*, **398,** 422-426.

Thiagalingam, S., Lengauer, C., Leach, F.S., Schutte, M., Hahn, S.A., Overhauser, J., Willson, J.K., Markowitz, S., Hamilton, S.R., Kern, S.E., Kinzler, K.W. & Vogelstein, B. (1996). Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. *Nature Genetics*, **13,** 343-346.

Thibodeau, S.N., Bren, G. & Schaid, D. (1993). Microsatellite instability in cancer of the proximal colon. *Science*, **260,** 816-819.

Tibbetts, L.M., Chu, M.Y., Hager, J.C., Dexter, D.L. & Calabresi, P. (1977). Chemotherapy of cell-line-derived human colon carcinomas in mice immunosuppressed with antithymocyte serum. *Cancer*, **40,** 2651-2659.

Tlsty, T.D. (1990). Normal diploid human and rodent cells lack a detectable frequency of gene amplification. *Proceedings of the National Academy of Sciences of the United States of America*, **87,** 3132-31326.

Tlsty, T.D. & Adams, P. (1990). Replication of the dihydrofolate reductase genes on double minute chromosomes in a murine cell line. *Experimental Cell Research*, **188,** 164-168.

Tlsty, T.D., White, A. & Sanchez, J. (1992). Suppression of gene amplification in human cell hybrids. *Science*, **255,** 1425-1427.

Toft, N.J., Winton, D.J., Kelly, J., Howard, L.A., Dekker, M., Riele, H.T., Arends, M.J., Wyllie, A.H., Margison, G.P. & Clarke, A.R. (1999). MSH2 status modulates both apoptosis and mutation frequency in the murine small intestine. *Proceedings of the National Academy of Sciences of the United States of America*, **96,** 3911-3915.

Tomita, N., Wei, J., Hibshoosh, H., Warburton, D., Kahn, S.M. & Weinstein, I.B. (1992). Isolation and characterization of a highly malignant variant of the SW480 human colon cancer cell line. *Cancer Research*, **52,** 6840-6847.

Tomlinson, I. & Bodmer, W. (1999). Selection, the mutation rate and cancer: Ensuring that the tail does not wag the dog. *Nature Medicine*, **5,** 11-12.

Troelstra, C., Odijk, H., Dewit, J., Westerveld, A., Thompson, L.H., Bootsma, D. & Hoeijmakers, J.H.J. (1990). Molecular-Cloning of the Human DNA Excision Repair Gene Ercc-6. *Molecular and Cellular Biology*, **10,** 5806-5813.

Tsujii, M., Kawano, S., Tsuji, S., Sawaoka, H., Hori, M. & DuBois, R.N. (1998). Cyclooxygenase regulates angiogenesis induced by colon cancer cells [published erratum appears in Cell 1998 Jul 24;94(2):following 271]. *Cell*, **93,** 705-716.

Uren, A.G., Pakusch, M., Hawkins, C.J., Puls, K.L. & Vaux, D.L. (1996). Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. *Proceedings of the National Academy of Sciences of the United States of America*, **93,** 4974-4978.

Urquidi, V., Tarin, D. & Goodison, S. (2000). Role of telomerase in cell senescence and oncogenesis. *Annual Review of Medicine*, **51,** 65-79.

van Triest, B., Pinedo, H.M., van Hensbergen, Y., Smid, K., Telleman, F., Schoenmakers, P.S., van der Wilt, C.L., van Laar, J.A., Noordhuis, P., Jansen, G. & Peters, G.J. (1999). Thymidylate synthase level as the main predictive parameter for sensitivity to 5-fluorouracil, but not for folate-based thymidylate synthase inhibitors, in 13 nonselected colon cancer cell lines. *Clinical Cancer Research*, **5,** 643-654.

Vasen, H.F. (1994). What is hereditary nonpolyposis colorectal cancer (HNPCC). *Anticancer Research*, **14,** 1613-5.

Vernole, P., Tedeschi, B., Caporossi, D. & Nicoletti, B. (1988). Common fragile sites and human cancer. A study on lymphocytes from neuroblastoma patients. *Cancer Genetics and Cytogenetics*, **36,** 13-23.

Vig, B.K. & Rattner, J.B. (1989). Centromere, kinetochore, and cancer. *Critical Reviews in Oncogenesis*, **1,** 343-371.

Vig, B.K. & Sternes, K.L. (1991). Centromeres without kinetochore proteins. Another mechanism for origin of aneuploidy in neoplasia. *Cancer Genetics and Cytogenetogetics*, **51,** 269-272.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M. & Bos, J.L. (1988). Genetic alterations during colorectal-tumor development. *New EnglandJournal of Medicine*, **319,** 525-532.

Vojtesek, B., Bartek, J., Midgley, C.A. & Lane, D.P. (1992). An immunochemical analysis of the human nuclear phosphoprotein p53. New monoclonal antibodies and epitope mapping using recombinant p53. *Journal of Immunological Methods*, **151,** 237-244.

Wada, R., Matsukuma, S., Abe, H., Kuwabara, N., Suda, K., Arakawa, A. & Kitamura, S. (1996). Histopathological studies of superficial-type early colorectal carcinoma. *Cancer*, **77,** 44-50.

Waga, S., Hannon, G.J., Beach, D. & Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature*, **369,** 574-578.

Wagner, C. (1995). *Biochemical role of folate in cellular metabolism.* Marcel Dekker: New York.

Waldman, T., Kinzler, K.W. & Vogelstein, B. (1995). P21 is necessary for the p53-mediated G_1 arrest in human cancer cells. *Cancer Research*, **55,** 5187-5190.

Waldman, T., Zhang, Y.G., Dillehay, L., Yu, J., Kinzler, K., Vogelstein, B. & Williams, J. (1997). Cell cycle arrest versus cell death in cancer therapy. *Nature Medicine*, **3,** 1034-1036.

Walker, P.R., Saas, P. & Dietrich, P.Y. (1998). Tumor expression of Fas ligand (CD95L) and the consequences. *Current Opinions in Immunology*, **10,** 564-572.

Wallach, D. (1997). Apoptosis - Placing death under control. *Nature*, **388,** 123-130.

Wang, X.W., Vermeulen, W., Coursen, J.D., Gibson, M., Lupold, S.E., Forrester, K., Xu, G.W., Elmore, L., Yeh, H., Hoeijmakers, J.H.J. & Harris, C.C. (1996). The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway. *Genes and Development*, **10,** 1219-1232.

Wang, X.W., Zhan, Q., Coursen, J.D., Khan, M.A., Kontny, H.U., Yu, L., Hollander, M.C., O'Connor, P.M., Fornace, A.J., Jr. & Harris, C.C. (1999). GADD45 induction of a G2/M cell cycle checkpoint. *Proceedings of the National Academy of Sciences of the United States of America*, **96,** 3706-3711.

Wang, Y. & Prives, C. (1995). Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases. *Nature*, **376,** 88-91.

Wattenberg, L.W. (1978). Inhibition of chemical carcinogenesis. *Journal of the National Cancer Institute*, **60,** 11-18.

Weisburger, J.H. (1991). Causes, relevant mechanisms, and prevention of large bowel cancer. *Seminars in Oncology*, **18,** 316-36.

Wheeler, J.M.D., Beck, N.E., Kim, H.C., Tomlinson, I.P.M., Mortensen, N.J.M. & Bodmer, W.F. (1999). Mechanisms of inactivation of mismatch repair genes in human colorectal cancer cell lines: The predominant role of *hMLH1*. *Proceedings of the National Academy of Sciences of the United States of America*, **96,** 10296-10301.

White, E. (1996). Life, death, and the pursuit of apoptosis. *Genes and Development*, **10,** 1-15.

White, M.B., Carvalho, M., Derse, D., Obrien, S.J. & Dean, M. (1992). Detecting single base substitutions as heteroduplex polymorphisms. *Genomics*, **12,** 301-306.

Whitehead, R.H., Jones, J.K., Gabriel, A. & Lukies, R.E. (1987). A new colon carcinoma cell line (LIM1863) that grows as organoids with spontaneous differentiation into crypt-like structures *in vitro*. *Cancer Research*, **47,** 2683-2689.

Winawer, S.J. & Shike, M. (1992). Dietary factors in colorectal cancer and their possible effects on earlier stages of hyperproliferation and adenoma formation. *Journal of the National Cancer Institute*, **84,** 74-75.

Windle, B.E. & Wahl, G.M. (1992). Molecular dissection of mammalian gene amplification: new mechanistic insights revealed by analyses of very early events. *Mutation Research*, **276,** 199-224.

Wrana, J.L., Attisano, L., Wieser, R., Ventura, F. & Massague, J. (1994). Mechanism of activation of the TGF-Beta receptor. *Nature*, **370,** 341-347.

Wu, G.S., Burns, T.F., McDonald, E.R., 3rd, Jiang, W., Meng, R., Krantz, I.D., Kao, G., Gan, D.D., Zhou, J.Y., Muschel, R., Hamilton, S.R., Spinner, N.B., Markowitz, S., Wu, G. & el Deiry, W.S. (1997). KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nature Genetics*, **17,** 141-143.

Wu, L. & Levine, A.J. (1997). Differential regulation of the p21/WAF-1 and mdm2 genes after high-dose UV irradiation: p53-dependent and p53-independent regulation of the mdm2 gene. *Molecular Medicine*, **3,** 441-451.

Wyllie, A.H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature*, **284,** 555-556.

Wyllie, A.H. (1997). Apoptosis: an overview. *British Medical Bulletin*, **53,** 451-465.

Wyllie, A.H., Beattie, G.J. & Hargreaves, A.D. (1981). Chromatin changes in apoptosis. *Histochemistry Journal*, **13,** 681-692.

Yagi, O.K., Akiyama, Y., Nomizu, T., Iwama, T., Endo, M. & Yuasa, Y. (1998). Proapoptotic gene *BAX* is frequently mutated in hereditary nonpolyposis colorectal cancers but not in adenomas. *Gastroenterology*, **114,** 268-274.

Yamamoto, H., Sawai, H., Weber, T.K., RodriguezBigas, M.A. & Perucho, M. (1998). Somatic frameshift mutations in DNA mismatch repair and proapoptosis genes in hereditary nonpolyposis colorectal cancer. *Cancer Research*, **58,** 997-1003.

Yamashita, N., Minamoto, T., Ochiai, A., Onda, M. & Esumi, H. (1995). Frequent and characteristic K-ras activation in aberrant crypt foci of colon. Is there preference among K-ras mutants for malignant progression? *Cancer*, **75,** 1527-1533.

Yanagida, M. (1998). Fission yeast cut mutations revisited: control of anaphase. *Trends in Cell Biology*, **8,** 144-149.

Yang, B., Eshleman, J.R., Berger, N.A. & Markowitz, S.D. (1996). Wild-type p53 protein potentiates cytotoxicity of therapeutic agents in human colon cancer cells. *Clinical Cancer Research*, **2,** 1649-1657.

Yaseen, N.Y., Watmore, A.E., Potter, A.M., Potter, C.W., Jacob, G. & Rees, R.C. (1990). Chromosome studies in eleven colorectal tumors. *Cancer Genetics and Cytogenetics*, **44,** 83-97.

Yin, C.Y., Knudson, C.M., Korsmeyer, S.J. & VanDyke, T. (1997). Bax suppresses tumorigenesis and stimulates apoptosis *in vivo*. *Nature*, **385,** 637-640.

Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A. & Oren, M. (1991). Wild-type P53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. *Nature*, **352,** 345-347.

Yoshikawa, H., Nagashima, M., Khan, M.A., McMenamin, M.G., Hagiwara, K. & Harris, C.C. (1999). Mutational analysis of p73 and p53 in human cancer cell lines. *Oncogene*, **18,** 3415-3421.

Yoshioka, A., Tanaka, S., Hiraoka, O., Koyama, Y., Hirota, Y., Ayusawa, D., Seno, T., Garrett, C. & Wataya, Y. (1987). Deoxyribonucleoside triphosphate imbalance. 5-Fluorodeoxyuridine-induced DNA double strand breaks in mouse FM3A cells and the mechanism of cell death. *Journal of Biological Chemistry*, **262,** 8235-8241.

Yuan, J.Y. (1997). Transducing signals of life and death. *Current Opinion in Cell Biology*, **9,** 247-251.

Yunis, J.J. & Soreng, A.L. (1984). Constitutive fragile sites and cancer. *Science*, **226,** 1199-204.

Zhao, N., Lai, F., Fernald, A.A., Eisenbart, J.D., Espinosa, R., Wang, P.W. & Le Beau, M.M. (1998). Human CDC23: cDNA cloning, mapping to 5q31, genomic structure, and evaluation as a candidate tumor suppressor gene in myeloid leukemias. *Genomics*, **53,** 184-190.

APPENDIX 1
REAGENTS AND SUPPLIERS

Reagents are listed in alphabetical order against the name of their supplier(s). All suppliers are based in the UK unless otherwise specified.

α^{35}SdATP; Amersham International plc
ABC kit; Dako Ltd
Acridine Orange: Sigma Aldrich Chemical Company
Agarose; Sigma Aldrich Chemical Company
Ammonium acetate; Fisher Scientific
Ammonium persulphate; Severn Biotech Ltd
Bench alcohol; Genta Medical
Biotin-16 dUTP; Boehringer-Mannheim Ltd
Biotinylated anti-avidin; Vector Laboratories
Biotinylated rabbit anti-mouse immunoglobulins; Dako Ltd
Blocking agent; Amersham International plc
Boric acid; Sigma Aldrich Chemical Company
Bromophenol blue; Fisher Scientific
BSA fraction V; Fisher Scientific
Calcium chloride; Fisher Scientific
Chloroform; Fisher Scientific
Colcemid; Life Technologies Ltd
Cot1 DNA, human and mouse; Life Technologies Ltd
Coverslips (No.0 & No.1, Chance Propper); Fisher Scientific
'Decon' detergent; Fisher Scientific
Deionised distilled water (DDW); Elga Ltd
Diaminobenzidine (DAB); Sigma Aldrich Chemical Company
Diaminoethanetetra-acetic acid (EDTA), Sigma Aldrich Chemical Company
Diaminophenolindole (DAPI); Boehringer-Mannheim Ltd
Digoxygenin-11-dUTP; Boehringer-Mannheim Ltd
Dimethyl sulfoxide (DMSO); Sigma Aldrich Chemical Company
Dithiothreitol (DTT); Sigma Aldrich Chemical Company
DNA molecular weight marker 1 kilobase DNA ladder; Life Technologies Ltd
dNTPs; Pharmacia Biotechnologies Ltd
Do7 antibody; Dako Ltd
Ethanol; Hayman Ltd
Ethidium bromide; Sigma Aldrich Chemical Company
5-Fluorouracil, Sigma Aldrich Chemical Company
Foetal Calf Serum; Life Technologies Ltd
Formaldehyde solution; Fisher Scientific
Formamide: Fisher Scientific
[γ^{33}P]ATP; Amersham International plc
'Gel Slick'; AT Biochem
Giemsa; Sigma Aldrich Chemical Company
Glacial acetic acid; Fisher Scientific
Glasgow Minimal Essential Medium (GMEM) with L-Glutamine; Life Technologies Ltd
Glycerol; Sigma Aldrich Chemical Company
Glucose; Fisher Scientific
Hydrochloric acid; Fisher Scientific
Hydrogen peroxide; Sigma Aldrich Chemical Company

Ion-exchange resin beads; Bio-Rad Laboratories Ltd
Imidazole; Sigma Aldrich Chemical Company
'Instagel' 40% 19:1 acrylamide:bis acrylamide solution; Severn Biotech Ltd
Iscovo's Modified Dulbecco's Medium; Life Technologies Ltd
Iso-amyl alcohol; Fisher Scientific
Kodak X-OMAT autoradiography film; Amersham International plc
L-glutamine; Life Technologies Ltd
Magnesium chloride; Sigma Aldrich Chemical Company
Magnesium Sulphate; Sigma Aldrich Chemical Company
MDE gel; AT Biochem
Methanol; Fisher Scientific
Microscope slides 'Select' (Chance Propper); Fisher Scientific
Microscope slides (pre-washed); Beriner Glass KG, UK supplier: H.V. Skan LTD.
N,N,N',N',-tetramethylethylenediamine (TEMED); Severn Biotech Ltd
Normal rabbit serum; Life Technologies Ltd
Oligonucleotides;, Cruachem and VHBio
Penicillin-Streptomycin; Life Technologies Ltd
Phosphate buffered saline (PBS); Life Technologies Ltd
Pepsin; Sigma Aldrich Chemical Company
Potassium chloride; Fisher Scientific
Prolong Antifade (mounting medium); Molecular Probes
Propidium iodide; Sigma Aldrich Chemical Company
Proteinase K; ICN Biomedicals Ltd
Sequenase version 2.0 DNA polymerase and all other sequencing reagents; Amersham International plc
Sequencing apparatus; Bio-Rad Ltd
Silver nitrate; Fisher Scientific
SKY^{TM} probe kit; Applied Spectral Imaging, Migdal Ha'Emek, Israel
Sodium carbonate (decahydrate); Fisher Scientific
Sodium chloride; Fisher Scientific
Sodium citrate; Fisher Scientific
Sodium dodecyl sulphate (SDS); ICN Biomedicals Ltd
Sodium hydroxide; Fisher Scientific
Sodium dihydrogen orthophosphate; Fisher Scientific
Sodium thiosulphate; Fisher Scientific
Streptavidin alkaline phosphatase; Boehringer-Mannheim Ltd
Streptavidin alkaline phosphatase anti-digoxygenin; Boehringer-Mannheim Ltd
T4 polynucleotide kinase + buffer; Life Technologies Ltd
ThermoSequenase radio labelled terminator cycle sequencing kit, including all enzymes, termination master mixes and reaction buffer; Amersham International plc
Thermostable DNA polymerase + buffer IV; Advanced Biotechnologies Ltd
Tissue culture plasticware; Iawa Ltd
Trypsin; Life Technologies Ltd
Tris; Sigma Aldrich Chemical Company
Trisodium citrate; Sigma Aldrich Chemical Company
Tween 20 (Polyoxyethylenesorbitan monolaurate); Sigma Aldrich Chemical Company, Fisher Scientific
Urea; Fisher Scientific
Versene; Life Technologies Ltd
Whatman paper; Whatman Ltd
Xylene; Fisher Scientific
Xylene cyanol FF; Sigma Aldrich Chemical Company

APPENDIX 2

Karyotypic details of constituent metaphases of each cell line

Constituent metaphases of each cell line are described in vertical columns, and where relevant, were grouped into clones. 'Marker' is used to denote abnormal chromosomes, and those observed in only one metaphase are still recorded under the category 'rare markers'. Abnormal chromosomes (markers) are described according to the standard ISCN (International System for Human Cytogenetic Nomenclature) (Mitelman, 1995).

m (lower case): metaphase; md: mode; mn: mean; sd: standard deviation; M: marker; chr: chromosome; meta: metaphase (used in the footnotes).

Appedix 2

Cell line: C70

		Metaphase	m2	m4	m5	m6	m7	m8	m9	m10	m15	md	mn	sd
Normal chr	**1**		3	4	5	4	4	3	5	4	5	**4**	**4.11**	**0.78**
	2		1	1	4	1	1	1	3	2	3	**1**	**1.89**	**1.17**
	3		6	6	6	7	7	6	6	7	6	**6**	**6.33**	**0.50**
	4		2	2	2	1	2	2	3	1	3	**2**	**2.00**	**0.71**
	5		4	5	3	4	7	5	4	6	4	**4**	**4.67**	**1.22**
	6		3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	7		8	9	6	7	9	9	9	9	9	**9**	**8.33**	**1.12**
	8		5	5	5	4	5	5	5	4	5	**5**	**4.78**	**0.44**
	9		5	5	4	5	5	5	5	5	5	**5**	**4.89**	**0.33**
	10		4	3	3	3	6	3	4	2	4	**3**	**3.56**	**1.13**
	11		2	3	2	3	4	3	3	3	3	**3**	**2.89**	**0.60**
	12		6	6	6	6	5	6	5	4	5	**6**	**5.44**	**0.73**
	13		5	8	6	6	5	8	5	6	5	**5**	**6.00**	**1.22**
	14		4	4	4	4	4	4	4	4	4	**4**	**4.00**	**0.00**
	15		5	6	6	5	6	6	5	6	5	**6**	**5.56**	**0.53**
	16		3	3	3	2	3	3	3	3	3	**3**	**2.89**	**0.33**
	17		2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	18		4	4	4	4	4	4	4	4	4	**4**	**4.00**	**0.00**
	19		4	4	4	4	5	4	4	2	4	**4**	**3.89**	**0.78**
	20		9	8	8	9	10	8	9	9	9	**9**	**8.78**	**0.67**
	21		2	1	2	2	2	1	2	1	2	**2**	**1.67**	**0.50**
	22		3	3	3	3	4	3	3	4	3	**3**	**3.22**	**0.44**
	X		5	5	5	5	4	5	5	5	5	**5**	**4.89**	**0.33**
	Y		0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr			**95**	**100**	**96**	**94**	**107**	**99**	**101**	**96**	**101**	**96**	**98.78**	**4.06**

Cell line: C70

	Metaphase	m2	m4	m5	m6	m7	m8	m9	m10	m15	md	mn	sd
Common marker chr													
M1	der(1)t(1;5)(p12;p13)	1	2	2	1	2	2	2	2	2	**2**	**1.78**	**0.44**
M2	del(2)(p21)	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M3	del(2)(?)*	4	4	2	5	5	4	3	4	3	**4**	**3.78**	**0.97**
M4	der(4)del(4)(q31q35)t(4;18)(?p15;?)	2	2	2	3	2	2	2	2	2	**2**	**2.11**	**0.33**
M5	der(8)t(5;8)	0	0	0	0	0	0	1	0	1	**0**	**0.22**	**0.44**
M6	der(10)t(3;10)(?;q23-24)	2	2	2	1	1	2	2	1	2	**2**	**1.67**	**0.50**
M7	del(11)(?q23)	1	1	1	1	0	1	0	0	0	**1**	**0.56**	**0.53**
M8	dup(11)(?)	3	3	2	1	2	3	3	3	3	**3**	**2.56**	**0.73**
M9	der(12;22)t(12;22)(?p13;p11)t(12;22)(?q24;q11	1	1	1	0	1	1	1	1	1	**1**	**0.89**	**0.33**
M10	dup(13)(?)**	2	2	2	1	2	2	2	3	2	**2**	**2.00**	**0.50**
M11	der(16)t(10;16)(q23-24;q24)	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
M12	der(17)t(6;17)(?q23;q24-25)	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
M13	der(17;21)(q10;q10)	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
M14	der(19)t(19;22)(?p13.1;?q11.2)	2	2	2	1	1	2	2	1	2	**2**	**1.67**	**0.50**
Rare markers													
M15	der(1)t(1;2)	0	0	0	0	0	1	0	0	0	**0**	**0.11**	**0.33**
M16	der(2)t(1;2)	0	0	0	0	0	1	0	0	0	**0**	**0.11**	**0.33**
M17	der(4)del(4)(p10)	0	0	0	0	0	0	0	1	0	**0**	**0.11**	**0.33**
M18	der(?)t(12;15)t(12;22)	0	0	0	0	0	0	0	0	1	**0**	**0.11**	**0.33**
Total marker chr		**25**	**26**	**23**	**21**	**23**	**28**	**25**	**25**	**26**	**25**	**24.67**	**2.06**
Total normal and markers		**120**	**126**	**119**	**115**	**130**	**127**	**126**	**121**	**127**	**127**	**123.44**	**4.88**

* deletion of 2p at different points with possibility of dup(2)(q?)

**may be der(13;13)(q10;q10)del(q14q34)t(13;13)(?q14;?q34)

Cell line: HT29

	Metaphase	m4	m6	m7	m10	m11	m13	m14	m16	m17	m18	md	mn	sd
Normal chr **1**		3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
2		3	3	3	2	3	3	3	4	3	2	**3**	**2.90**	**0.57**
3		3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
4		2	3	2	2	2	2	2	2	2	2	**2**	**2.10**	**0.32**
5		2	2	3	3	3	3	2	3	2	3	**3**	**2.60**	**0.52**
6		2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
7		4	3	4	4	3	4	3	2	4	4	**4**	**3.50**	**0.71**
8		2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
9		3	3	2	2	3	2	2	2	2	2	**2**	**2.30**	**0.48**
10		3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
11		4	4	4	3	2	4	3	3	4	3	**4**	**3.40**	**0.70**
12		3	3	3	3	3	3	3	2	3	3	**3**	**2.90**	**0.32**
13		1	1	1	0	0	1	0	0	1	0	**1**	**0.50**	**0.53**
14		2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
15		6	4	4	4	3	3	3	3	4	4	**4**	**3.80**	**0.92**
16		3	3	3	3	2	3	2	2	3	3	**3**	**2.70**	**0.48**
17		2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
18		1	2	1	2	2	2	2	2	2	2	**2**	**1.80**	**0.42**
19		2	3	3	1	3	3	3	4	4	1	**3**	**2.70**	**1.06**
20		4	3	3	3	3	4	3	4	4	3	**3**	**3.40**	**0.52**
21		2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
22		3	2	2	3	3	2	3	2	2	3	**3**	**2.50**	**0.53**
X		2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
Y		0	0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr		**62**	**60**	**59**	**56**	**56**	**60**	**55**	**56**	**61**	**56**	**56**	**58.10**	**2.56**

Cell line: HT29

Metaphase		m4	m6	m7	m10	m11	m13	m14	m16	m17	m18	md	mn	sd
Common marker chr														
M1	der(2)t(1;2)(q32;q11-13)	1	1	1	1	1	2	1	1	1	1	**1**	**1.10**	**0.32**
M2	del(4)(?q31)	1	0	1	1	1	1	1	1	0	1	**1**	**0.80**	**0.42**
M3	der(5)t(5;6)(p10;?)	1	1	1	1	1	1	1	1	2	1	**1**	**1.10**	**0.32**
M4	hsr(8)(p22-23)	1	1	1	1	1	2	1	1	1	1	**1**	**1.10**	**0.32**
M5	der(6;9)(p10;q10)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M6	der(9;11)	0	0	0	1	0	0	0	0	0	1	**0**	**0.20**	**0.42**
M7	der(11;13)	0	0	0	0	1	0	0	1	0	0	**0**	**0.20**	**0.42**
M8	i(13)(q10)	1	1	1	2	1	1	1	1	1	2	**1**	**1.20**	**0.42**
M9	der(13)t(7;13)(?;q10)	0	0	0	0	0	0	1	1	0	0	**0**	**0.20**	**0.42**
M10	der(11;16)(q10;?p10)	0	0	0	0	1	0	1	1	0	0	**0**	**0.30**	**0.48**
M11	der(17;19)(q10;?p10)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M12	del(18)(q12)	2	1	1	1	1	1	1	1	1	1	**1**	**1.10**	**0.32**
M13	del(20)	0	1	1	1	1	0	1	0	0	1	**1**	**0.60**	**0.52**
M14	der(22)t(17;22)(?;q12)	1	1	1	2	1	1	1	2	2	2	**1**	**1.40**	**0.52**
M15	del(X)(?p21)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
Rare markers														
M16	der(4)t(4;8)	0	0	0	0	0	0	0	0	1	0	**0**	**0.10**	**0.32**
M17	der(5)t(5;20)	0	0	0	0	0	0	1	0	0	0	**0**	**0.10**	**0.32**
M18	dup(19)	0	0	0	0	0	0	0	0	0	1	**0**	**0.10**	**0.32**
Total marker chr		**11**	**10**	**11**	**14**	**13**	**12**	**14**	**14**	**12**	**15**	**14**	**12.60**	**1.65**
Total normal and markers		**73**	**70**	**70**	**70**	**69**	**72**	**69**	**70**	**73**	**71**	**70**	**70.70**	**1.49**

Cell line: LIM1863

	Metaphase	m1	m2	m3	m4	m5	m7	m8	m9	m10	m11	md	mn	sd
Normal chr	**1**	3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	2	3	3	3	3	3	2	3	3	3	3	**3**	**2.90**	**0.32**
	3	3	3	2	3	3	3	3	3	3	3	**3**	**2.90**	**0.32**
	4	3	3	2	3	3	3	3	3	3	3	**3**	**2.90**	**0.32**
	5	3	3	3	3	3	1	3	3	3	3	**3**	**2.80**	**0.63**
	6	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	7	4	4	4	4	4	4	4	4	4	4	**4**	**4.00**	**0.00**
	8	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	9	3	3	3	3	3	2	3	3	3	3	**3**	**2.90**	**0.32**
	10	3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	11	3	3	2	3	3	3	3	3	3	3	**3**	**2.90**	**0.32**
	12	3	3	3	3	3	2	3	3	3	3	**3**	**2.90**	**0.32**
	13	0	0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
	14	3	3	2	3	3	1	3	3	3	3	**3**	**2.70**	**0.67**
	15	4	4	4	4	4	4	4	4	4	4	**4**	**4.00**	**0.00**
	16	3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	17	0	0	1	0	0	1	0	0	0	0	**0**	**0.20**	**0.42**
	18	3	3	3	3	3	2	3	3	3	3	**3**	**2.90**	**0.32**
	19	3	3	2	3	3	2	3	3	3	2	**3**	**2.70**	**0.48**
	20	7	7	5	7	7	6	7	7	7	7	**7**	**6.70**	**0.67**
	21	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	22	2	2	2	2	2	2	2	2	2	3	**2**	**2.10**	**0.32**
	X	4	4	4	4	4	3	4	4	4	4	**4**	**3.90**	**0.32**
	Y	0	0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr		**66**	**66**	**60**	**66**	**66**	**56**	**66**	**66**	**66**	**66**	**66**	**64.40**	**3.50**

Cell line: LIM1863

Metaphase	m1	m2	m3	m4	m5	m7	m8	m9	m10	m11	md	mn	sd
Marker chr													
M1* der(2;3;8)t(2;3)t(3;8)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M2 del(8)(p?)	4	3	4	4	3	1	3	3	3	4	**3**	**3.20**	**0.92**
M3 der(8;17)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M4 der(1;11)	1	1	1	1	1	0	1	1	1	1	**1**	**0.90**	**0.32**
M5 der(12)t(11;12)	1	1	1	1	1	0	1	1	1	1	**1**	**0.90**	**0.32**
M6 i(13)(q10)	2	4	4	4	4	4	4	4	4	4	**4**	**3.80**	**0.63**
M7 der(9;13)	2	1	2	1	1	2	1	1	1	2	**1**	**1.40**	**0.52**
M8 der(17)t(3;17)	0	0	1	0	0	0	0	0	0	1	**0**	**0.20**	**0.42**
M9 der(17)t(X;17)	2	2	0	2	2	1	2	2	2	1	**2**	**1.60**	**0.70**
Total marker chr	**14**	**14**	**15**	**15**	**14**	**10**	**14**	**14**	**14**	**16**	**14**	**14.00**	**1.56**
Total normal and markers	**80**	**80**	**75**	**81**	**80**	**66**	**80**	**80**	**80**	**82**	**80**	**78.40**	**4.72**

*In m7 this marker is different, it is t(3;8) without involvement of chr 2

Cell line: SW1417

		Metaphase	m3	m4	M5	m8	m9	m10	m11	m13	m14	md	mn	sd
Normal chr	**1**		1	1	1	1	1	1	2	1	1	**1**	**1.11**	**0.33**
	2		3	2	3	3	3	4	2	3	4	**3**	**3.00**	**0.71**
	3		4	4	4	3	3	4	2	3	4	**4**	**3.44**	**0.73**
	4		2	2	2	1	2	2	2	2	2	**2**	**1.89**	**0.33**
	5		1	1	2	1	2	1	1	1	1	**1**	**1.22**	**0.44**
	6		2	2	2	2	2	2	1	2	2	**2**	**1.89**	**0.33**
	7		1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	8		1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	9		2	2	1	2	1	1	1	2	1	**1**	**1.44**	**0.53**
	10		2	2	2	2	3	3	2	3	2	**2**	**2.33**	**0.50**
	11		4	4	3	4	4	3	4	4	3	**4**	**3.67**	**0.50**
	12		2	3	3	1	3	3	3	2	2	**3**	**2.44**	**0.73**
	13		3	2	2	2	2	3	2	3	3	**2**	**2.44**	**0.53**
	14		3	3	2	3	3	2	3	3	3	**3**	**2.78**	**0.44**
	15		1	1	1	0	1	1	1	1	1	**1**	**0.89**	**0.33**
	16		3	2	3	3	3	2	3	3	2	**3**	**2.67**	**0.50**
	17		2	2	2	2	2	2	2	1	1	**2**	**1.78**	**0.44**
	18		0	2	1	0	1	1	1	1	1	**1**	**0.89**	**0.60**
	19		1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	20		3	2	2	2	2	2	2	2	2	**2**	**2.11**	**0.33**
	21		2	2	2	2	2	3	2	2	2	**2**	**2.11**	**0.33**
	22		4	4	4	4	4	4	4	4	4	**4**	**4.00**	**0.00**
	X		1	2	2	2	2	2	2	2	2	**2**	**1.89**	**0.33**
	Y		0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr			**48**	**48**	**47**	**43**	**49**	**49**	**45**	**48**	**46**	**48**	**47.00**	**2.00**
Common Marker Chr:														
M1	del(1)(?)		2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
M2	del(2)(?)		0	1	0	0	0	0	2	0	0	**0**	**0.33**	**0.71**
M3	der(2)t(2;3)		0	1	0	0	1	0	0	0	0	**0**	**0.22**	**0.44**
M4	der(2)t(2;20)		1	0	1	0	0	0	0	1	0	**0**	**0.33**	**0.50**
M5	del(3)(?)		0	0	0	0	0	0	2	1	0	**0**	**0.33**	**0.71**
M6	del(5)(?)		2	2	2	1	1	2	2	2	2	**2**	**1.78**	**0.44**

Cell line: SW1417

Metaphase		m3	m4	M5	m8	m9	m10	m11	m13	m14	md	mn	sd
M7	der(5)t(5;17)	1	1	0	1	1	1	1	1	1	**1**	**0.89**	**0.33**
M8	der(5;18)	0	0	1	0	0	0	0	1	0	**0**	**0.22**	**0.44**
M9	der(5)t(5;20)	0	1	0	1	1	1	1	0	1	**1**	**0.67**	**0.50**
M10	del(6)(q?)	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M11	dup(7)(q?)	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M12	der(7)t(1;7)t(1;8)	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
M13	der(8)t(1;8)	1	1	1	1	1	1	1	0	1	**1**	**0.89**	**0.33**
M14	del(9)(?)	0	1	2	1	2	2	2	1	2	**2**	**1.44**	**0.73**
M15	del(10)(?)	0	1	0	1	0	0	0	0	1	**0**	**0.33**	**0.50**
M16	del(12)(q?)	1	0	0	0	0	0	0	1	1	**0**	**0.33**	**0.50**
M17	i(15)(q10)	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M18	der(17)t(16;17)	0	0	0	0	0	0	0	1	1	**0**	**0.22**	**0.44**
M19	del(18)(?)	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M20	dup(18)(?)	2	0	0	2	1	1	1	1	1	**1**	**1.00**	**0.71**
M21	der(19)t(9;19)	2	2	3	2	1	2	2	2	2	**2**	**2.00**	**0.50**
M22	dup(20)(?)	1	2	2	2	2	2	2	2	2	**2**	**1.89**	**0.33**
Rare Markers:													
M23	der(1;2)	0	0	0	0	0	0	1	0	0	**0**	**0.11**	**0.33**
M24	der(2)t(2;4)	0	0	0	1	0	0	0	0	0	**0**	**0.11**	**0.33**
M25	der(3)t(2;3)	0	0	0	0	1	0	0	0	0	**0**	**0.11**	**0.33**
M26	der(3;4)	0	0	0	1	0	0	0	0	0	**0**	**0.11**	**0.33**
M27	der(1;3;8)t(1;8)t(1:3)	0	0	0	0	0	0	0	1	0	**0**	**0.11**	**0.33**
M28	der(10)t(8;10)	0	0	1	0	0	0	0	0	0	**0**	**0.11**	**0.33**
M29	der(10;17)	0	0	1	0	0	0	0	0	0	**0**	**0.11**	**0.33**
M30	der(11)t(X;11)	0	0	0	0	0	0	0	0	1	**0**	**0.11**	**0.33**
M31	der(12)t(10;12)	0	0	0	1	0	0	0	0	0	**0**	**0.11**	**0.33**
M32	del(16)(?)	0	1	0	0	0	0	0	0	0	**0**	**0.11**	**0.33**
M34	del(X)(?)	1	0	0	0	0	0	0	0	0	**0**	**0.11**	**0.33**
Total marker chr		**20**	**22**	**22**	**23**	**20**	**20**	**25**	**23**	**24**	**20**	**22.11**	**1.83**
Total normal and markers		**68**	**70**	**69**	**66**	**69**	**69**	**70**	**71**	**70**	**70**	**69.11**	**1.45**

Cell line: SW403

	Metaphase	m1	m3	m5	m7	m8	m9	m10	m12	m13	m14	md	mn	sd
Normal chr	**1**	2	2	2	2	2	2	2	2	3	2	**2**	**2.10**	**0.32**
	2	3	3	3	3	2	3	4	3	2	3	**3**	**2.90**	**0.57**
	3	3	3	2	3	3	3	2	3	3	3	**3**	**2.80**	**0.42**
	4	2	2	2	2	1	2	2	2	2	2	**2**	**1.90**	**0.32**
	5	3	3	3	2	3	3	3	3	3	3	**3**	**2.90**	**0.32**
	6	2	2	3	2	2	2	2	2	3	2	**2**	**2.20**	**0.42**
	7	4	4	5	4	3	4	4	4	4	5	**4**	**4.10**	**0.57**
	8	0	1	1	1	1	1	1	2	2	1	**1**	**1.10**	**0.57**
	9	3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	10	3	3	2	3	3	3	3	3	3	2	**3**	**2.80**	**0.42**
	11	3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	12	2	2	2	2	2	2	2	2	1	1	**2**	**1.80**	**0.42**
	13	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	14	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	15	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	16	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	17	1	1	2	1	1	2	2	2	1	2	**1**	**1.50**	**0.53**
	18	1	1	2	1	1	1	1	1	2	1	**1**	**1.20**	**0.42**
	19	3	3	3	2	2	2	3	2	3	3	**3**	**2.60**	**0.52**
	20	4	4	4	2	3	3	3	3	2	4	**4**	**3.20**	**0.79**
	21	3	2	3	3	3	3	3	3	3	3	**3**	**2.90**	**0.32**
	22	1	1	1	1	0	0	1	1	1	1	**1**	**0.80**	**0.42**
	X	3	3	3	3	3	2	3	2	2	2	**3**	**2.60**	**0.52**
	Y	0	0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr		**53**	**53**	**56**	**50**	**48**	**51**	**54**	**53**	**53**	**53**	**53**	**52.40**	**2.22**

Cell line: SW403

Metaphase		m1	m3	m5	m7	m8	m9	m10	m12	m13	m14	md	mn	sd
Common marker chr														
M1	del(1)(?p)	0	1	1	1	0	1	1	1	0	1	**1**	**0.70**	**0.48**
M2	dup(8)(?q)	1	1	1	1	1	1	0	1	0	0	**1**	**0.70**	**0.48**
M3	i(8)(q10)	1	1	0	1	1	1	1	0	0	1	**1**	**0.70**	**0.48**
M4	der(2;8)	0	1	1	1	1	1	1	1	1	1	**1**	**0.90**	**0.32**
M5	der(12)t(12;15)	1	1	1	1	1	1	1	1	1	0	**1**	**0.90**	**0.32**
M6	dup(13)(q?)	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
M7	del(17)(?)	1	1	1	2	0	0	1	0	1	0	**1**	**0.70**	**0.67**
M8	der(17)t(17;22)	1	1	0	0	1	1	0	1	1	1	**1**	**0.70**	**0.48**
M9	del(18)(?)	1	1	1	0	1	0	0	0	0	0	**0**	**0.40**	**0.52**
M10	dup(18)(q?)	1	1	0	1	0	1	1	1	0	1	**1**	**0.70**	**0.48**
M11	der(18;22)*	1	0	0	1	1	1	1	1	0	1	**1**	**0.70**	**0.48**
M12	der(20)t(X;20)	2	1	1	1	1	1	1	1	1	1	**1**	**1.10**	**0.32**
M13	der(22)t(7;22)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M14	der(22)t(18;22)*	0	0	0	0	1	1	0	0	0	0	**0**	**0.20**	**0.42**
Rare marker														
M15	del(12)(p?)	0	0	0	0	0	0	0	0	0	1	**0**	**0.10**	**0.32**
Total marker chr		**13**	**13**	**10**	**13**	**12**	**13**	**11**	**11**	**8**	**11**	**13**	**11.50**	**1.65**
Total normal and markers		**66**	**66**	**66**	**63**	**60**	**64**	**65**	**64**	**61**	**64**	**66**	**63.90**	**2.08**

* M11 is different from M14 and both seems to constitute a reciprocal translocation.

Cell Line SW480

	Clone:	1	1	1	1	1		2	2	2	2	2	2			
	Metaphase	m3	m7	m9	m2	m8	md:1	m5	m10	m17	m19	m20	m22	md:2	mn:1+2	sd1+2
Normal chr 1		2	1	1	1	1	1	4	4	4	4	4	4	4	2.73	1.49
2		2	2	2	1	3	2	4	1	1	3	3	3	3	2.27	1.01
3		3	2	3	2	2	2	3	4	4	3	3	3	3	2.91	0.70
4		2	2	2	2	2	2	3	3	3	3	3	3	3	2.55	0.52
5		1	1	1	1	1	1	2	2	2	2	2	2	2	1.55	0.52
6		1	2	2	2	2	2	3	2	2	3	3	3	3	2.27	0.65
7		2	2	2	2	2	2	3	4	5	3	4	3	3	2.91	1.04
8		1	1	1	1	1	1	4	4	4	4	4	4	4	2.64	1.57
9		1	1	1	1	1	1	2	3	3	3	4	4	3	2.18	1.25
10		2	1	1	1	1	1	2	1	2	2	2	3	2	1.64	0.67
11		3	3	3	3	3	3	3	2	2	3	3	3	3	2.82	0.40
12		1	1	1	1	2	1	2	2	2	3	2	3	2	1.82	0.75
13		3	3	2	2	3	3	4	5	5	3	5	4	5	3.55	1.13
14		2	2	1	2	2	2	3	1	1	3	1	1	1	1.73	0.79
15		2	2	2	2	3	2	3	3	3	3	3	3	3	2.64	0.50
16		2	2	2	2	2	2	3	3	3	3	4	4	3	2.73	0.79
17		2	2	2	3	2	2	4	4	4	3	4	4	4	3.09	0.94
18		1	1	1	1	1	1	1	1	1	1	2	2	1	1.18	0.40
19		2	1	1	1	1	1	2	2	3	2	3	2	2	1.82	0.75
20		2	2	2	2	2	2	4	3	3	4	4	4	4	2.91	0.94
21		3	3	3	3	3	3	5	3	3	5	5	5	5	3.73	1.01
22		2	2	2	2	2	2	3	4	4	3	4	4	4	2.91	0.94
X		2	2	2	2	2	2	2	2	2	2	2	2	2	2.00	0.00
Y		0	0	0	0	0	0	0	0	0	0	0	0	0	0.00	0.00
Total normal chr		44	41	40	40	44	44	69	63	66	68	74	73	NA	56.55	14.48

Cell Line SW480

	Clone:	1	1	1	1	1		2	2	2	2	2	2			
	Metaphase	m3	m7	m9	m2	m8	md:1	m5	m10	m17	m19	m20	m22	md:2	mn:1+2	sd1+2
Marker chr shared by clone-1 and clone-2																
M1	der(2)t(2;12)	1	1	1	1	0	**1**	0	1	0	0	1	1	**0**	**0.64**	**0.50**
M2	der(5)t(5;20)*	1	1	1	1	1	**1**	2	1	2	2	2	1	**2**	**1.36**	**0.50**
M3	der(7)t(7;14)	0	1	1	1	1	**1**	2	2	2	2	2	2	**2**	**1.45**	**0.69**
M4	del(12)(?)	1	1	1	1	1	**1**	2	1	3	2	1	1	**1**	**1.36**	**0.67**
M5	del(18)(q?)	2	2	1	2	1	**2**	3	3	3	3	3	2	**3**	**2.27**	**0.79**
M6	der(5;8;19)t(5;19)t(8,19)	1	1	0	1	1	**1**	4	4	3	4	3	3	**4**	**2.27**	**1.49**
M7	der(20)t(5;20)*	2	2	2	2	2	**2**	3	3	2	3	3	3	**3**	**2.45**	**0.52**
Clone-1 markers																
M8	der(1)t(1;9)*	1	1	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.45**	**0.52**
M9	del(3)(?)	1	1	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.45**	**0.52**
M10	der(7)t(7;13)	1	1	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.45**	**0.52**
M11	der(8;19)	1	1	0	1	1	**1**	0	0	0	0	0	0	**0**	**0.36**	**0.50**
M12	der(8;9)	1	1	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.45**	**0.52**
M13	der(9)t(1;9)*	1	1	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.45**	**0.52**
M14	der(10)t(10;12)(3;12)	0	1	0	1	1	**1**	0	0	0	0	0	0	**0**	**0.27**	**0.47**
M15	del(17)(q?)	1	1	0	0	0	**0**	0	0	0	0	0	0	**0**	**0.18**	**0.40**
Clone-2 markers																
M16	del(2)(?)	0	0	0	0	0	**0**	1	1	1	0	0	0	**1**	**0.27**	**0.47**
M17	der(2)t(2;18)	0	0	0	0	0	**0**	1	1	1	1	1	2	**1**	**0.64**	**0.67**
M18	der(5;12)	0	0	0	0	0	**0**	0	1	1	2	1	1	**1**	**0.55**	**0.69**
M19	der(7)t(1;7)	0	0	0	0	0	**0**	0	1	0	0	0	1	**0**	**0.18**	**0.40**
M20	der(10;12)	0	0	0	0	0	**0**	1	2	1	2	2	2	**2**	**0.91**	**0.94**
M21	der(10)t(10;15)	0	0	0	0	0	**0**	0	0	1	0	0	1	**0**	**0.18**	**0.40**
M22	der(11)t(11;15)	0	0	0	0	0	**0**	2	2	2	1	1	1	**2**	**0.82**	**0.87**
M23	der(12)t(12;14)*	0	0	0	0	0	**0**	0	0	0	0	1	1	**0**	**0.18**	**0.40**
M24	der(14)t(12;14)*	0	0	0	0	0	**0**	0	2	2	0	2	2	**2**	**0.73**	**1.01**
Total marker chr		**15**	**17**	**12**	**16**	**14**	**#N/A**	**21**	**25**	**24**	**22**	**23**	**24**	**24**	**19.36**	**4.65**
Total normal and markers		**59**	**58**	**52**	**56**	**58**	**58**	**90**	**88**	**90**	**90**	**97**	**97**	**90**	**75.91**	**18.78**

* apparently balanced translocation

Twenty two metaphases were initially examined. 9/22 were clone-1, while 13/22 were clone-2. Only 11 metas are recorded here.

Cell line SW620

	Metaphase	m1	m4	m5	m7	m8	m9	m11	m12	m13	m14	md	mn	sd
Normal chr	**1**	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	2	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	3	1	1	1	1	1	1	0	1	1	1	**1**	**0.90**	**0.32**
	4	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	5	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	6	2	1	1	1	1	1	1	1	1	1	**1**	**1.10**	**0.32**
	7	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	8	0	0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
	9	2	2	2	2	2	2	2	2	1	2	**2**	**1.90**	**0.32**
	10	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	11	3	2	3	2	2	2	3	2	3	3	**3**	**2.50**	**0.53**
	12	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	13	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	14	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	15	2	2	2	2	1	2	2	2	1	2	**2**	**1.80**	**0.42**
	16	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	17	2	2	2	2	2	2	1	2	2	1	**2**	**1.80**	**0.42**
	18	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	19	2	2	2	1	2	2	2	2	1	2	**2**	**1.80**	**0.42**
	20	2	1	2	2	2	2	2	2	2	2	**2**	**1.90**	**0.32**
	21	2	2	2	2	2	2	2	2	1	2	**2**	**1.90**	**0.32**
	22	2	1	2	2	2	2	2	2	2	2	**2**	**1.90**	**0.32**
	X	2	2	2	1	1	2	1	2	1	2	**2**	**1.60**	**0.52**
	Y	0	0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr		**37**	**33**	**36**	**33**	**33**	**35**	**33**	**35**	**31**	**35**	**33**	**34.10**	**1.79**

Cell line SW620

Metaphase	m1	m4	m5	m7	m8	m9	m11	m12	m13	m14	md	mn	sd
Common marker chr:													
M1 der(2)t(2;12)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M2 del(3)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M3 del(4)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M4 del(5)	0	0	1	2	0	0	0	0	0	0	**0**	**0.30**	**0.67**
M5* der(5)t(5;20)	1	1	1	0	1	1	1	1	2	1	**1**	**1.00**	**0.47**
M6* der(6)t(6;7)	0	0	1	1	1	1	1	1	1	1	**1**	**0.80**	**0.42**
M7 del(7)	1	1	1	1	0	1	1	1	1	1	**1**	**0.90**	**0.32**
M8* der(7)t(6;7)	1	1	1	0	1	1	1	1	0	1	**1**	**0.80**	**0.42**
M9 der(8)t(8;13)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M10 der(8)t(8;17)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M11 der(10)t(10;13)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M12**der(16)dup(16)t(3;16)t(6;16)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M13 der(5;18)	1	1	0	0	1	1	0	1	1	1	**1**	**0.70**	**0.48**
M14* der(20)t(5;20)	1	1	1	1	2	1	1	1	1	1	**1**	**1.10**	**0.32**
M15 der(X;18)	0	0	0	1	0	0	1	0	0	0	**0**	**0.20**	**0.42**
Rare markers:													
M16 del(1)	0	1	0	0	0	0	0	0	0	0	**0**	**0.10**	**0.32**
M17 der(3)t(3;10)	0	0	0	0	0	0	1	0	0	0	**0**	**0.10**	**0.32**
M18 der(X;15)	0	0	0	0	0	0	0	0	1	0	**0**	**0.10**	**0.32**
Total marker chr	12	13	13	13	13	13	14	13	14	13	**13**	**13.10**	**0.57**
Total normal and markers	**49**	**46**	**49**	**46**	**46**	**48**	**47**	**48**	**45**	**48**	**48**	**47.20**	**1.40**

* reciprocal translocation

** der(16)dup(16)t(10;16)t(10;16) in m 11

Cell line:SW837

	clone	1	1	1	1	1		2	2	2	2			
	Metaphase	m4	m7	m8	m9	m10	md1	m1	m2	m3	m11	md2	mn1+2	sd1+2
Normal chr	1	0	1	0	0	0	**0**	1	1	1	1	**1**	**0.56**	**0.53**
	2	2	2	1	2	2	**2**	1	1	1	1	**1**	**1.44**	**0.53**
	3	1	1	1	1	1	**1**	1	1	1	0	**1**	**0.89**	**0.33**
	4	2	2	2	2	2	**2**	2	2	1	2	**2**	**1.89**	**0.33**
	5	2	2	2	2	2	**2**	1	2	2	2	**2**	**1.89**	**0.33**
	6	2	1	1	1	1	**1**	1	1	1	1	**1**	**1.11**	**0.33**
	7	1	1	1	1	1	**1**	1	1	1	1	**1**	**1.00**	**0.00**
	8	1	1	1	1	1	**1**	1	1	1	1	**1**	**1.00**	**0.00**
	9	2	1	2	2	2	**2**	2	2	2	2	**2**	**1.89**	**0.33**
	10	2	2	2	2	2	**2**	2	2	2	2	**2**	**2.00**	**0.00**
	11	1	1	1	1	1	**1**	1	1	1	1	**1**	**1.00**	**0.00**
	12	2	2	2	2	2	**2**	2	2	2	2	**2**	**2.00**	**0.00**
	13	0	0	0	0	0	**0**	0	0	0	0	**0**	**0.00**	**0.00**
	14	2	2	2	2	2	**2**	2	2	2	2	**2**	**2.00**	**0.00**
	15	1	1	1	1	1	**1**	1	1	1	1	**1**	**1.00**	**0.00**
	16	2	2	2	2	2	**2**	1	1	1	0	**1**	**1.44**	**0.73**
	17	1	1	1	1	1	**1**	1	1	1	1	**1**	**1.00**	**0.00**
	18	1	1	1	1	1	**1**	1	1	1	1	**1**	**1.00**	**0.00**
	19	1	1	1	1	1	**1**	1	1	1	1	**1**	**1.00**	**0.00**
	20	2	2	2	2	2	**2**	1	1	1	1	**1**	**1.56**	**0.53**
	21	1	2	2	1	2	**2**	2	2	2	2	**2**	**1.78**	**0.44**
	22	2	1	2	2	2	**2**	2	2	2	2	**2**	**1.89**	**0.33**
	X	0	0	0	0	0	**0**	0	0	0	0	**0**	**0.00**	**0.00**
	Y	0	0	0	0	0	**0**	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr		**31**	**30**	**30**	**30**	**31**	**30**	**28**	**29**	**28**	**27**	**28**	**29.33**	**1.41**

Cell line:SW837

	clone	1	1	1	1	1		2	2	2	2			
	Metaphase	m4	m7	m8	m9	m10	md1	m1	m2	m3	m11	md2	mn1+2	sd1+2
Common marker chr														
M1	del(1)(?)	0	0	0	0	1	**0**	1	0	0	0	**0**	**0.22**	**0.44**
M2*	der(1)t(1;8)	2	0	2	2	1	**2**	1	1	1	1	**1**	**1.22**	**0.67**
M3	der(2)t(2;17)	0	0	0	0	0	**0**	1	1	1	1	**1**	**0.44**	**0.53**
M4	der(3)t(3;11)	1	1	1	1	1	**1**	1	1	1	1	**1**	**1.00**	**0.00**
M5	del(6)(?)	0	1	1	1	1	**1**	0	1	1	1	**1**	**0.78**	**0.44**
M6	der(7)t(2;7)	0	0	0	0	0	**0**	1	0	1	1	**1**	**0.33**	**0.50**
M7**	der(7)t(7;19)	1	1	1	1	1	**1**	0	0	0	0	**0**	**0.56**	**0.53**
M8	der(8)t(8;17)	1	1	0	1	1	**1**	1	1	0	1	**1**	**0.78**	**0.44**
M9	der(11)t(1;11)	1	1	1	1	1	**1**	1	0	1	1	**1**	**0.89**	**0.33**
M10	der(13;15)	1	1	1	1	1	**1**	1	1	1	1	**1**	**1.00**	**0.00**
M11#	der(16)t(16;20)	0	0	0	0	0	**0**	1	1	1	1	**1**	**0.44**	**0.53**
M12#	der(20)t(16;20)	0	0	0	0	0	**0**	1	1	1	1	**1**	**0.44**	**0.53**
M13	der(X)t(X;5)	1	1	1	1	1	**1**	1	1	1	1	**1**	**1.00**	**0.00**
Rare markers:														
M15	del(4)(?)	0	0	0	0	0	**0**	0	0	1	0	**0**	**0.11**	**0.33**
M16	der(4;8;17)t(8;17)t(4;17)	0	0	0	0	0	**0**	0	0	1	0	**0**	**0.11**	**0.33**
M14	der(5)t(2;5)	0	0	0	0	0	**0**	1	0	0	0	**0**	**0.11**	**0.33**
M17	der(9)t(8;9)	0	1	0	0	0	**0**	0	0	0	0	**0**	**0.11**	**0.33**
Total marker chr		**8**	**8**	**8**	**9**	**9**	**8**	**12**	**9**	**12**	**11**	**12**	**9.56**	**1.67**
Total normal and markers		**39**	**38**	**38**	**39**	**40**	**39**	**40**	**38**	**40**	**38**	**40**	**38.89**	**0.93**

* 2 forms of M2 one of them has deleted chr 1 in addition to
the translocation (when both the 2 forms are there that means there is one of each form)
= reciprocal translocation
** t(2+7+19) in meta08
12 metas were examined 7/12 wree clone1 while 5/12 were clone2

Cell line:VACO4A

		Metaphase	m1	m2	m3	m4	m5	m6	m8	m10	md	mn	sd
Normal chr	**1**		1	2	2	1	2	2	2	2	**2**	**1.75**	**0.46**
	2		3	2	3	3	3	3	3	3	**3**	**2.88**	**0.35**
	3		2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	4		2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	5		3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	6		2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	7		4	4	4	4	4	4	4	4	**4**	**4.00**	**0.00**
	8		1	2	2	2	2	2	2	2	**2**	**1.88**	**0.35**
	9		3	2	4	2	3	3	3	3	**3**	**2.88**	**0.64**
	10		2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	11		3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	12		3	2	3	3	3	3	3	3	**3**	**2.88**	**0.35**
	13		2	2	2	1	2	2	2	2	**2**	**1.88**	**0.35**
	14		4	4	4	4	3	4	4	4	**4**	**3.88**	**0.35**
	15		1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	16		2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	17		2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	18		2	2	2	3	2	2	2	2	**2**	**2.13**	**0.35**
	19		2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	20		2	2	2	2	1	2	1	2	**2**	**1.75**	**0.46**
	21		0	2	2	2	2	2	2	2	**2**	**1.75**	**0.71**
	22		2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	X		2	1	2	2	1	1	2	2	**2**	**1.63**	**0.52**
	Y		0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr			**50**	**50**	**55**	**52**	**51**	**53**	**53**	**54**	**50**	**52.25**	**1.83**

Cell line:VACO4A

Metaphase		m1	m2	m3	m4	m5	m6	m8	m10	md	mn	sd
Common marker chr												
M1	i(1)(q10)	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M2*	der(3)t(3;10)(q10;q10)	1	1	1	1	1	1	0	1	**1**	**0.88**	**0.35**
M3	i(8)(q10)	2	1	2	1	2	1	2	1	**2**	**1.50**	**0.53**
M4	der(8;20)(q10;?)	2	3	2	2	1	2	1	2	**2**	**1.88**	**0.64**
M5	del(9)(q?)	0	1	0	1	0	0	0	0	**0**	**0.25**	**0.46**
M6	der(13)dup(13)(q?)t(13;15)	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
M7	der(4;17)	0	0	1	1	1	0	1	0	**0**	**0.50**	**0.53**
M8	der(10;17)	1	1	0	0	0	1	0	1	**1**	**0.50**	**0.53**
M9	dup(20)(?)	0	0	0	0	1	0	1	0	**0**	**0.25**	**0.46**
M10	der(X)t(X;2)	0	1	0	0	1	1	0	0	**0**	**0.38**	**0.52**
Rare markers												
M11	der(1)t(1;21)(q10;?)	1	0	0	0	0	0	0	0	**0**	**0.13**	**0.35**
M12	der(2)t(2;3)(q10;?)	0	1	0	0	0	0	0	0	**0**	**0.13**	**0.35**
M13	del(3)(q10)	0	0	0	0	0	0	1	0	**0**	**0.13**	**0.35**
M14	der(3;7)	0	0	1	0	0	0	0	0	**0**	**0.13**	**0.35**
M15	der(20;21)	1	0	0	0	0	0	0	0	**0**	**0.13**	**0.35**
Total marker chr		**11**	**12**	**10**	**9**	**10**	**9**	**9**	**8**	**9**	**9.75**	**1.28**
Total normal and markers		**61**	**62**	**65**	**61**	**61**	**62**	**62**	**62**	**62**	**62.00**	**1.31**

* this marker takes different shape in m10

Cell line: DLD1

	Metaphase	m2	m3	m4	m5	m6	m7	m8	m9	m10	m11	md	mn	sd
Normal chr	**1**	2	2	1	2	1	2	2	2	2	1	**2**	**1.70**	**0.48**
	2	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	3	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	4	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	5	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	6	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	7	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	8	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	9	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	10	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	11	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	12	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	13	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	14	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	15	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	16	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	17	2	2	2	2	2	2	2	2	1	2	**2**	**1.90**	**0.32**
	18	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	19	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	20	2	2	2	2	1	2	2	2	2	2	**2**	**1.90**	**0.32**
	21	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	22	2	2	2	2	2	2	2	2	1	2	**2**	**1.90**	**0.32**
	X	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	Y	1	1	1	1	1	1	1	1	0	1	**1**	**0.90**	**0.32**
Total normal chr		**44**	**44**	**42**	**44**	**41**	**44**	**44**	**44**	**41**	**42**	**44**	**43.00**	**1.33**
Marker chr														
M1	dup(1)(p?)	0	0	1	0	1	0	0	0	0	1	**0**	**0.30**	**0.48**
M2	dup(2)(p13p23)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M3	der(6)t(6;11)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
Total marker chr		**2**	**2**	**3**	**2**	**3**	**2**	**2**	**2**	**2**	**3**	**2**	**2.30**	**0.48**
Total normal and markers		**46**	**46**	**45**	**46**	**44**	**46**	**46**	**46**	**43**	**45**	**46**	**45.30**	**1.06**

Cell line: GP2d

	Metaphase	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10	md	mn	sd
Normal chr	**1**	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	2	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	3	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	4	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	5	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	6	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	7	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	8	1	2	2	2	2	2	2	2	2	2	**2**	**1.90**	**0.32**
	9	2	2	2	2	2	2	2	1	2	2	**2**	**1.90**	**0.32**
	10	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	11	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	12	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	13	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	14	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	15	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	16	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	17	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	18	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	19	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	20	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	21	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	22	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	X	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	Y	0	0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr		**41**	**42**	**42**	**42**	**42**	**42**	**42**	**41**	**42**	**42**	**42**	**41.80**	**0.42**
Marker chr														
M1*	del(3)(?)	1	1	1	1	1	1	1	1	2	1	**1**	**1.10**	**0.32**
M2	del(5)(q?)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M3	der(6)t(3;6)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M4	dup(10)(q?)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
Total marker chr		**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**5**	**4**	**4**	**4.10**	**0.32**
Total normal and markers		**45**	**46**	**46**	**46**	**46**	**46**	**46**	**45**	**47**	**46**	**46**	**45.90**	**0.57**

* The two M1 in m9 are more deleted than in other metaphases

Cell line: HCT116

	Metaphase	m2	m4	m5	m6	m7	m8	m9	m10	m11	m12	md	mn	sd
Normal chr	**1**	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	2	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	3	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	4	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	5	2	2	2	2	2	2	2	1	2	2	**2**	**1.90**	**0.32**
	6	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	7	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	8	1	2	2	2	2	2	2	2	2	2	**2**	**1.90**	**0.32**
	9	2	1	2	2	2	2	2	2	2	2	**2**	**1.90**	**0.32**
	10	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	11	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	12	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	13	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	14	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	15	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	16	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	17	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	18	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	19	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	20	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	21	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	22	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	X	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	Y	0	0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr		**41**	**41**	**42**	**42**	**42**	**42**	**42**	**41**	**42**	**42**	**42**	**41.70**	**0.48**
Marker chr														
M1 der(10)dup(10)(q?)t(10;16)		1	0	1	1	1	1	1	1	1	1	**1**	**0.90**	**0.32**
M2 der(16)t(8;16)		1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M3 der(18)t(17;18)		1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
Total marker chr		**3**	**2**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**3**	**2.90**	**0.32**
Total normal and markers		**44**	**43**	**45**	**45**	**45**	**45**	**45**	**44**	**45**	**45**	**45**	**44.60**	**0.70**

Cell line: LS174T

	Metaphase	m1	m2	m3	m5	m6	m7	m8	m9	m11	md	mn	sd
Normal chr	**1**	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	3	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	4	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	5	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	6	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	7	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	8	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	9	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	10	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	11	2	1	2	2	2	2	2	2	2	**2**	**1.89**	**0.33**
	12	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	13	2	2	2	2	2	2	1	2	2	**2**	**1.89**	**0.33**
	14	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	15	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	16	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	17	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	18	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	19	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	20	2	2	2	2	2	2	3	2	2	**2**	**2.11**	**0.33**
	21	2	2	2	2	1	2	2	2	2	**2**	**1.89**	**0.33**
	22	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	X	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	Y	0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr		**47**	**46**	**47**	**47**	**46**	**47**	**47**	**47**	**47**	**47**	**46.78**	**0.44**

NO Markers

Cell line: VACO5

	Metaphase	m1	m2	m6	m7	m8	m12	m13	m16	m17	m18	md	mn	sd
Normal chr	**1**	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	2	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	3	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	4	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	5	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	6	2	2	2	2	2	2	2	1	2	2	**2**	**1.90**	**0.32**
	7	2	2	2	2	2	2	2	1	2	2	**2**	**1.90**	**0.32**
	8	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	9	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	10	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	11	2	2	2	2	2	2	2	1	2	2	**2**	**1.90**	**0.32**
	12	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	13	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	14	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	15	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	16	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	17	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	18	2	2	2	2	2	2	2	1	2	2	**2**	**1.90**	**0.32**
	19	1	2	2	2	2	2	2	2	2	2	**2**	**1.90**	**0.32**
	20	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	21	1	2	2	0	2	1	1	2	2	2	**2**	**1.50**	**0.71**
	22	2	2	1	2	2	2	2	2	2	2	**2**	**1.90**	**0.32**
	X	2	2	2	1	2	2	2	2	2	2	**2**	**1.90**	**0.32**
	Y	0	0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr		**44**	**46**	**45**	**43**	**46**	**45**	**45**	**42**	**46**	**46**	**46**	**44.80**	**1.40**
Marker chr														
M1	del(7)(?)	0	0	1	0	0	0	0	0	0	1	**0**	**0.20**	**0.42**
M2*	del(21)(p?)	1	0	0	1	0	1	1	0	0	0	**0**	**0.40**	**0.52**
Total marker chr		**1**	**0**	**1**	**1**	**0**	**1**	**1**	**0**	**0**	**1**	**1**	**0.60**	**0.52**
Total normal and markers		**45**	**46**	**46**	**44**	**46**	**46**	**46**	**42**	**46**	**47**	**46**	**45.40**	**1.43**

*In some metaphases there is a possibility of t(5;21) in addittion to the deletion though remained difficult to confirm even after painting with 2 chr colours due to the non spesific binding at the satellite piece.

Cell line: LoVo

	Metaphase	m1	m2	m3	m4	m5	m6	m7	m8	m9	m10	md	mn	sd
Normal chr	**1**	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	2	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	3	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	4	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	5	3	3	3	3	3	3	3	3	2	2	**3**	**2.80**	**0.42**
	6	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	7	3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	8	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	9	2	2	2	2	2	2	2	1	2	2	**2**	**1.90**	**0.32**
	10	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	11	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	12	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	13	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	14	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	15	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	16	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	17	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	18	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	19	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	20	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	21	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	22	2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	X	1	1	1	0	1	1	1	1	1	1	**1**	**0.90**	**0.32**
	Y	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
Total normal chr		**46**	**46**	**46**	**45**	**46**	**46**	**46**	**45**	**45**	**45**	**46**	**45.60**	**0.52**
Marker chr														
M1 der(2)t(2;12)		1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M2 der(12)t(2;12)		1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M3 i(15)(q10)		1	1	1	1	1	1	2	1	1	1	**1**	**1.10**	**0.32**
Total marker chr		**3**	**3**	**3**	**3**	**3**	**3**	**4**	**3**	**3**	**3**	**3**	**3.10**	**0.32**
Total normal and markers		**49**	**49**	**49**	**48**	**49**	**49**	**50**	**48**	**48**	**48**	**49**	**48.70**	**0.67**

M1&M2 are reciprocal translocations

Cell line: HCA7

		Clone	1	1	1	1	1	1	1	1		2	2	2		3	3	3	3	3	3		mn	sd
		Metaphase	m1	m3	m5	m8	m11	m20	m21	m22	md1	m4	m6	m16	md2	m7	m9	m10	m15	m18	m19	md3	1+2+3	1+2+3
Normal chr	**1**		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	2		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	2	2	2	2	2	2	**2**	**1.35**	**0.49**
	3		0	1	0	0	0	0	0	0	**0**	0	0	0	**0**	1	0	0	0	0	0	**0**	**0.12**	**0.33**
	4		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	5		0	2	2	2	2	2	2	2	**2**	2	2	2	**2**	2	2	2	2	2	2	**2**	**1.88**	**0.49**
	6		0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	1	1	1	1	1	1	**1**	**0.35**	**0.49**
	7		1	0	1	1	1	1	1	1	**1**	1	1	1	**1**	2	1	2	1	1	2	**2**	**1.12**	**0.49**
	8		2	2	2	2	2	1	2	2	**2**	2	2	2	**2**	2	2	2	2	2	2	**2**	**1.94**	**0.24**
	9		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	1	1	2	1	1	**1**	**1.06**	**0.24**
	10		2	2	2	2	2	2	2	2	**2**	2	2	2	**2**	2	1	1	1	1	1	**1**	**1.71**	**0.47**
	11		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	2	2	2	2	2	**2**	**1.29**	**0.47**
	12		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	2	1	1	1	1	1	**1**	**1.06**	**0.24**
	13		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	0	0	1	1	1	1	**1**	**0.88**	**0.33**
	14		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	2	2	2	2	2	2	**2**	**1.35**	**0.49**
	15		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	16		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	0	1	1	0	1	**1**	**0.88**	**0.33**
	17		2	2	2	2	2	2	2	2	**2**	4	2	4	**4**	2	1	2	2	2	2	**2**	**2.18**	**0.73**
	18		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	0	1	1	1	1	**1**	**0.94**	**0.24**
	19		1	1	2	2	2	2	2	2	**2**	2	2	2	**2**	2	2	2	2	2	2	**2**	**1.88**	**0.33**
	20		2	2	2	2	2	2	2	2	**2**	1	2	1	**1**	2	2	2	2	2	2	**2**	**1.88**	**0.33**
	21		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	2	1	1	2	1	1	**1**	**1.12**	**0.33**
	22		2	2	2	2	2	2	2	2	**2**	3	2	3	**3**	2	1	2	2	2	2	**2**	**2.06**	**0.43**
	X		0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	0	0	0	0	0	0	**0**	**0.00**	**0.00**
	Y		0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr			**24**	**26**	**27**	**27**	**27**	**26**	**27**	**27**	**27**	**29**	**27**	**29**	**29**	**33**	**25**	**31**	**32**	**29**	**31**	**31**	**28.06**	**2.51**
Common Marker chr																								
M1*	der(1)del(q)t(1;16)(p13;p11.2)		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M2	der(2)t(2;11)(q14-21;q21)		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.65**	**0.49**
M3	del(3)(p13p21)		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	1	2	1	1	2	**1**	**1.12**	**0.33**
M4	der(3)t(1;3)(?p36;q27-29)		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.65**	**0.49**
M5	der(3;14)		0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	0	1	1	1	1	0	**1**	**0.24**	**0.44**
M6	der(4)t(X;4)(p22;q25-26)		1	1	1	1	1	1	1	1	**1**	2	1	2	**2**	1	1	1	1	1	2	**1**	**1.18**	**0.39**
M7	der(6)t(6;7)(q21-22;q31)		1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.65**	**0.49**

Cell line: HCA7

Clone	1	1	1	1	1	1	1	1		2	2	2		3	3	3	3	3	3		mn	sd
Metaphase	m1	m3	m5	m8	m11	m20	m21	m22	md1	m4	m6	m16	md2	m7	m9	m10	m15	m18	m19	md3	1+2+3	1+2+3
M8 der(6)t(6;18)(q13-15;q11.2)	1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M9 del(7)(q31?)	0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	0	1	0	1	1	0	**0**	**0.18**	**0.39**
M10** der(7)t(6;7)(q21-22;q31)	1	1	1	1	1	1	1	1	**1**	2	1	2	**2**	0	0	0	0	0	0	**0**	**0.76**	**0.66**
M11 der(9)t(9;21)(p12-13;q11.2)	1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	0	1	1	0	1	1	**1**	**0.88**	**0.33**
M12 der(10)t(3;10)	0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	0	1	1	1	1	1	**1**	**0.29**	**0.47**
M13 der(11)t(2;11)(q14-21;q21)	1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.65**	**0.49**
M14 dup(12)(q?)	1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	0	1	1	1	1	1	**1**	**0.94**	**0.24**
M15 der(14)t(14;14)(p11.2;q13-21)	1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.65**	**0.49**
M16 del(15)(?q12q15)	1	1	1	1	1	1	1	1	**1**	0	0	0	**0**	1	1	1	1	1	1	**1**	**0.82**	**0.39**
M17 der(15;20)t(15;20)	0	0	0	0	0	0	0	0	**0**	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.18**	**0.39**
M18 der(16)t(1;16)(p13;p11.2)	0	0	0	0	0	0	0	0	**0**	1	0	1	**1**	1	1	1	1	1	1	**1**	**0.47**	**0.51**
M19 der(16)t(1;16)(p13;p11.2)t(1;3)(p36;q27-29	1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	0	0	0	0	0	0	**0**	**0.65**	**0.49**
M20 der(18)t(6;18)(q13-15;q11.2)	1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M21 der(X)t(X;4)(p22;q25-26)	1	1	1	1	1	1	1	1	**1**	1	1	1	**1**	1	1	1	1	1	1	**1**	**1.00**	**0.00**
Rare Markers:																						
M22 der(3;13)t(3;13)	0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	1	0	0	0	0	0	**0**	**0.06**	**0.24**
M23 der(5)t(5;19)	1	0	0	0	0	0	0	0	**0**	0	0	0	**0**	0	0	0	0	0	0	**0**	**0.06**	**0.24**
M24 der(9)t(9;11)	0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	1	0	0	0	0	0	**0**	**0.06**	**0.24**
M25 der(11)t(3;11)	0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	1	0	0	0	0	0	**0**	**0.06**	**0.24**
M26 der(13)t(1;13)	0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	0	1	0	0	0	0	**0**	**0.06**	**0.24**
M27 der(12;13)t(12;13)	0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	1	0	0	0	0	0	**0**	**0.06**	**0.24**
M28 der(16)t(16;18)	0	0	0	0	0	0	0	0	**0**	0	0	0	**0**	0	1	0	0	0	0	**0**	**0.06**	**0.24**
M29 der(19)t(5;19)	1	0	0	0	0	0	0	0	**0**	0	0	0	**0**	0	0	0	0	0	0	**0**	**0.06**	**0.24**
Total marker chr	**18**	**16**	**16**	**16**	**16**	**16**	**16**	**16**	**16**	**19**	**16**	**19**	**19**	**12**	**15**	**13**	**12**	**13**	**13**	**13**	**15.41**	**0.36**
Total normal and markers	**42**	**42**	**43**	**43**	**43**	**42**	**43**	**43**	**43**	**48**	**43**	**48**	**48**	**45**	**40**	**44**	**44**	**42**	**44**	**44**	**43.47**	**0.37**

* der(1)t(1;16)t(1;13) in meta 9 which will form reciprocal t with M26

**Two differrent versions, one of them with deleted 7q in addittion

The followings are reciprocal translocations: (M1&M18/M19); (M2&M13); (M4&M19); (M6&M21); (M7&M10); (M8&M20); (M23&M29).

Cell line:LS411

		Clone	1	1	1	1	1	1	2	2	3	3	md	mn	sd
		Metaphase	m1	m4	m6	m8	m10	m12	m5	m14	m13	m9	1+2+3	1+2+3	1+2+3
Normal chr	**1**		3	4	3	3	3	3	3	3	2	3	**3**	**3.00**	**0.47**
	2		3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	3		3	3	3	3	3	3	2	3	3	3	**3**	**2.90**	**0.32**
	4		3	3	3	2	3	3	3	3	3	3	**3**	**2.90**	**0.32**
	5		3	3	2	2	3	3	3	3	3	3	**3**	**2.80**	**0.42**
	6		2	2	1	3	2	3	3	2	1	2	**2**	**2.10**	**0.74**
	7		2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	8		3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	9		3	3	3	3	2	3	3	3	3	3	**3**	**2.90**	**0.32**
	10		3	4	3	4	3	3	4	4	4	4	**4**	**3.60**	**0.52**
	11		3	3	3	3	3	3	3	3	3	1	**3**	**2.80**	**0.63**
	12		3	3	3	2	3	3	3	3	3	3	**3**	**2.90**	**0.32**
	13		2	2	2	2	2	2	2	2	1	2	**2**	**1.90**	**0.32**
	14		1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	15		4	3	2	3	3	3	3	3	3	3	**3**	**3.00**	**0.47**
	16		3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	17		2	3	3	3	3	3	2	3	2	3	**3**	**2.70**	**0.48**
	18		2	2	2	2	2	2	2	2	2	2	**2**	**2.00**	**0.00**
	19		2	3	3	1	2	3	3	3	3	2	**3**	**2.50**	**0.71**
	20		3	3	3	3	3	3	3	3	3	3	**3**	**3.00**	**0.00**
	21		1	1	1	1	1	1	1	1	0	1	**1**	**0.90**	**0.32**
	22		2	2	3	4	2	2	2	2	2	2	**2**	**2.30**	**0.67**
	X		1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
	Y		0	0	0	0	0	0	0	0	0	0	**0**	**0.00**	**0.00**
Total normal chr			**57**	**60**	**56**	**57**	**56**	**59**	**58**	**59**	**54**	**56**	**56**	**57.20**	**1.81**
Common marker chr															
M1	del(1)		0	0	2	1	0	0	0	0	0	0	**0**	**0.30**	**0.67**
M2	del(3)		1	1	1	1	1	1	2	1	1	1	**1**	**1.10**	**0.32**
M3	del(5)		1	1	2	2	1	1	1	1	1	1	**1**	**1.20**	**0.42**
M4	del(6)		1	0	0	0	0	0	1	1	0	0	**0**	**0.30**	**0.48**
M5	dup(6)		0	0	0	0	1	0	0	0	1	1	**0**	**0.30**	**0.48**
M6	der(6)t(5;6)		1	0	1	0	0	0	0	0	1	1	**0**	**0.40**	**0.52**
M7	dup(7)		1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**

Cell line:LS411

	Clone	1	1	1	1	1	1	2	2	3	3	md	mn	sd
	Metaphase	m1	m4	m6	m8	m10	m12	m5	m14	m13	m9	1+2+3	1+2+3	1+2+3
M8	der(7)dup(7)t(7;12)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M9	der(8;22)	1	1	1	1*	1	1	1	1	1	0	**1**	**0.89**	**0.42**
M10	del(9)	1	1	0	1	2	1	1	1	1	1	**1**	**1.00**	**0.47**
M11	del(11)(q?)	0	0	0	0	0	0	0	0	1	2	**0**	**0.30**	**0.67**
M12	del(12)	1	1	2	2	1	1	0	1	1	1	**1**	**1.10**	**0.57**
M13	i(13)(q10)	1	1	1	1	1	1	2	1	1	1	**1**	**1.10**	**0.32**
M14	i(14)(q10)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M15	del(17)	1	1	1	1	1	1	1	0	1	1	**1**	**0.90**	**0.32**
M16	der(10;17)	0	0	0	0	0	0	1	1	0	0	**0**	**0.20**	**0.42**
M17	del(18)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
M18	dup(19)(p?)	1	1	0	1	1	0	0	0	0	1	**1**	**0.50**	**0.53**
M19**	der(12;21)	1	1	1	1	1	1	2	1	1	1	**1**	**1.10**	**0.32**
M20	der(6;22)	0	1	0	0	1	1	0	1	0	0	**0**	**0.40**	**0.52**
M21***	der(X)dup(X)t(X;5)	1	1	1	1	1	1	1	1	1	1	**1**	**1.00**	**0.00**
Rare markers														
M22	der(3;6)t(3;6)	0	0	0	0	0	0	1	0	0	0	**0**	**0.10**	**0.32**
M23	der(10)t(9;10)	0	0	0	0	1	0	0	0	0	0	**0**	**0.10**	**0.32**
Total marker chr		**16**	**15**	**15**	**15**	**18**	**14**	**18**	**15**	**16**	**17**	**15**	**15.90**	**1.37**
Total normal and markers		**73**	**75**	**71**	**72**	**74**	**73**	**76**	**74**	**70**	**73**	**73**	**73.10**	**1.79**

*t(6;8) rather than t(8;22) in meta 8

** 2 forms of M19 somtimes exist together and seems to be reciprocal translocation

*** t(X;15) in meta 1 and just del(X) without translocation in meta 13

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

Abdel-Rahman W.M., Katsura K., Rens W., Gorman P.A., Sheer D., Bicknell D., Bodmer W.F., Arends M.J., Wyllie A.H., Edwards P.A.W. (2001). Spectral karyotyping suggests additional subsets of colorectal cancers characterized by pattern of chromosome rearrangement. *Proceedings of the National Academy of Sciences of the United States of America* **(in press).**

Abdel-Rahman, W.M., Georgiades, I.B., Curtis, L.J., Arends, M.J. & Wyllie, A.H. (1999). Role of *BAX* mutations in mismatch repair-deficient colorectal carcinogenesis. *Oncogene*, **18,** 2139-2142.

Abdel-Rahman, W.M., Arends, M.J., Wyllie, A.H., Morris, R.G. & Ramadan, M.E. (1999). Death pathway genes *Fas (Apo-1/CD95)* and *Bik (Nbk)* show no mutations in colorectal carcinomas. *Cell Death and Differentiation*, **6,** 387-388.

Abdel-Rahman, W.M., Arends, M.J., Morris, R.G., Ramadan, M.E., Georgiades, I.B., Curtis, L.J. & Wyllie, A.H. (1999). Mutations in death pathway genes in colorectal carcinogenesis. (Oral presentation in *Pathological Society of Great Britain and Ireland, 179th meeting*, Dundee, UK). Published in *Journal of Pathology*, **189,** A2.

Abdel-Rahman, W.M., Katsura, K., Rens, W., Gorman, P.A., Sheer, D., Bicknell, D., Bodmer, W.F., Arends, M.J., Edwards, P.A.E. & Wyllie, A.H. (2000). Spectral Karyotyping reveals a new subset of colorectal cancers characterized by balanced translocations. Poster presentation in *7th European Workshop on Cytogenetics and Molecular Genetics of Human Solid Tumours*: Edinburgh, U.K.

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Role of *BAX* mutations in mismatch repair-deficient colorectal carcinogenesis

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***BAX* gene mutations occur in approximately 50% of RER+ colorectal cancers. To determine the role of these mutations in tumour progression we analysed multiple different tumour sites from RER+ colorectal cancers for *BAX* mutations. Sixty colorectal carcinomas were analysed for microsatellite instability at loci BAT-26, L-myc, TGFβRII, D13S160 and D2S123. Twelve out of 60 tumours (20%) were RER+. Forty-five different tumour sites from the 12 RER+ carcinomas were analysed for *BAX* mutations at the [(G)8] tract in exon 3. Six out of 12 (50%) RER+ tumours showed *BAX* mutations, four of which showed a homogenous pattern of such mutations detected in all tumour sites. In the other two cases, *BAX* mutations were present in some but not all tumour sites sampled from the same patient. In contrast, *TGFβRII* mutations were found in 9/12 cases (75%) and in each of these were present in all the sampled sites. Two cases showed neither *BAX* nor *TGFβRII* mutation. These data suggest that mutations in *TGFβRII* may occur at a very early stage in tumour progression, perhaps in the founder clone. *BAX* mutations, however, are clearly not necessary for formation of the founder clone and can occur for the first time later in tumour progression.**

Keywords: *BAX*; colorectal cancer; RER; mismatch repair

Introduction

The BCL2 family of proteins control an important checkpoint prior to activation of the caspase family of proteases in apoptosis (Brown, 1997; White, 1996). One prominent, widely expressed member of this family is BAX, a 21 kDa protein with the capacity to homodimerize or heterodimerize with other members of the BCL2 family (Oltvai *et al.*, 1993). Whereas BAX-BAX homodimers are potent death inducers, the BAX-BCL2 heterodimers and BCL2-BCL2 homodimers appear to promote survival (Kroemer, 1997). Moreover, the tumour suppressor protein p53, a major element in the response to lethal stimuli arising from DNA damage or hypoxia, can transactivate *BAX* (Miyashita and Reed, 1995) whilst down-regulating *BCL2* (Miyashita *et al.*, 1994). BAX is thus a significant effector in the initiation of apoptosis.

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Recently, clonally expanded, inactivating mutations in *BAX* have been observed in a proportion of colorectal cancers, together with evidence for under-expression of the BAX protein in these tumours (Rampino *et al.*, 1997; Yamamoto *et al.*, 1998; Yagi *et al.*, 1998; Ouyang *et al.*, 1998). This provides some circumstantial evidence for the hypothesis that the founder cells of cancers may arise through selective loss of a death pathway and the resultant inappropriate survival of cells that have sustained DNA damage or other severe intracellular injury. This hypothesis carries the significant implication that cancer cells that arise in this way are liable to be resistant to many cytotoxic agents to which more normal cells would be sensitive, because of constitutional loss of a critical death pathway. Alternative explanations for the *BAX* mutations exist, however. These mutations are restricted to tumours with mismatch repair (MMR) deficiency, and usually occur in a tract of eight consecutive deoxyguanosines [(G)8] in the third coding exon (Rampino *et al.*, 1997). Since this is a classical target site for nucleotide mismatch (Aaltonen *et al.*, 1993; Thibodeau *et al.*, 1993), it is possible that mutations within [(G)8] simply reflect the well-recognized effect of MMR deficiency on mutation incidence in tandem repeat microsatellite sequences.

In this paper we seek to distinguish between these possibilities by studying the homogeneity of *BAX* mutation within colorectal cancers. We argued that genetic changes that are critical for carcinogenesis are likely to be shared by all cells in the expanding tumour and should therefore be detectable at all sites throughout the tumour. In contrast, changes that reflect the genomic instability of malignant cells, but are not essential for the transition to malignancy might be expected to occur in some but not all of the divergent subclones within the given tumour. Accordingly, in this paper we studied *BAX* mutations in the [(G)8] tract in a series of tumours sampled at multiple sites.

Results

RER characterization

Twelve out of 60 patients (20%) demonstrated microsatellite instability (Table 1). In some cases (nos. 17, 27, 52, 53 and 55) individual tumour sites from the same carcinoma demonstrated different sets of mutations at the five microsatellite loci tested. In some tumours, biopsies from different sites each exhibited different mutations at the same microsatellite locus

Table 1 Mismatch repair deficient carcinomas analysed for RER and *BAX* status

No	*Age*	*Sex*	*Site*	*Histology*	*Sample site*	*RER status* *BAT-26*	*L-Myc*	*TGFβRII*	*D13S160*	*D2S123*	Bax *mutation*
17	81	F	cae	mucoid	A	+	+	+	–	+	+(loss)
					B	+	+	+	–	+	+(loss)
					C	–	+	+	–	+	–
18	67	F	sig	ac md	A	+	–	–	–	–	–
					B	+	–	–	–	–	–
					C	+	–	–	–	–	–
19	70	M	asc	ac pd	A	+	+	+	–	+	+(loss)
					B	+	+	+	–	+	+(loss)
					C	+	+	+	–	+	+(loss)
22	43	F	cae	ac/mu	A	+	–	–	–	–	+(loss)
					B	+	–	–	–	–	+(loss)
25	65	F	cae	ac/mu	A	+	+	+	+	–	+(loss)
					B	+	+	+	+	–	+(loss)
					C	+	+	+	+	–	–
					D	+	+	+	+	–	+(loss)
27	88	F	asc	ac/mu	A	+	+	+	–	+	–
					B	+	+	+	+	+	–
					C	+	+	+	+	+	–
					D	+	+	+	+	+	–
					E	+	+	+	+	+	–
					F	+	+	+	+	+	–
					G	–	+	+	–	+	–
28	75	F	cae	ac/mu	A	+	+	+	+	–	–
					B	+	+	+	+	–	–
38	64	M	asc	ac md	A	+	+	+	–	+	+(loss)
					B	+	+	+	–	+	+(loss)
					C	+	+	+	–	+	+(loss)
					D	+	+	+	–	+	+(loss)
47	60	M	asc	ac pd	A	+	–	+	+	+	–
					B	+	–	+	+	+	–
					C	+	–	+	+	+	–
					D	+	–	+	+	+	–
52	76	M	cae	ac pd	A	+	+	+	+	–	–
					B	+	+	+	+	–	–
					C	+	+	+	+	+	–
					D	+	+	+	+	+	–
53	77	F	asc	ac pd	A	+	+	–	–	–	–
					B	+	+	–	+	–	–
					C	+	+	–	–	+	–
					D	+	+	–	–	–	–
55	70	F	asc	ac pd	A	+	+	+	+	–	+(gain)
					B	+	+	+	–	–	+(gain)
					C	+	+	+	+	–	+(gain)
					D	+	+	+	–	–	+(gain)

Cae = caecum, asc = ascending colon, sig = sigmoid colon, mucoid = mucoid carcinoma, ac md = moderately differentiated adenocarcinoma, ac pd = poorly differentiated adenocarcinoma, ac/mu = mixed adenocarcinoma glandular/mucoid pattern, +(loss) = deletion of one G from the [(G)8] tract, +(gain) = insertion of one G in the [(G)8] tract

(Figure 1). However, in no case was microsatellite instability present at one tumour site, and completely absent at all tested loci in others. Hence it was possible to classify all sampled sites of all tumours as RER+, although in two tumours (nos. 18 and 22) the instability was evident in only one of the five tested loci.

BAX *mutation analysis*

Mutation in the *BAX* [(G)8] mononucleotide repeat tract occurred in six out of 12 (50%) RER+ tumours. In five, this involved loss of a repeated nucleotide and in one a gain. Four showed a homogenous pattern, with identical changes in [(G)8] detected in all sites sampled from each carcinoma (Figure 2a and Table 1). In the other two cases, however, [(G)8] *BAX* mutations were present in some but absent in other sites from the same cancers. Thus, case number 17 showed mutation in tumour sites A and B, but not C and case number 25 showed mutations in A, B and D but not C (Figure 2b and Table 1). In both these cases, the tumour site in which *BAX* [(G)8] was unchanged showed unequivocal evidence of instability at most of the other tested microsatellite loci.

Discussion

It is clear that deficiency in mismatch repair permits the generation of large numbers of mutations throughout the genome, mainly in microsatellite loci and repetitive polynucleotide tracts (Aaltonen *et al*., 1993; Thibodeau *et al*., 1993). In sporadic colorectal tumours, the microsatellite instability is generally found in carcinomas but not adenomas, whereas both adenomas and carcinomas from HNPCC patients may show a high proportion of such instability (Samowitz and Slattery, 1997; Bubb *et al*., 1996; Jacoby *et al*., 1995; Thibodeau *et al*., 1993). These observations strongly suggest that microsatellite instability is acquired at the adenoma-carcinoma interface in the evolution of sporadic tumours, but can appear at an

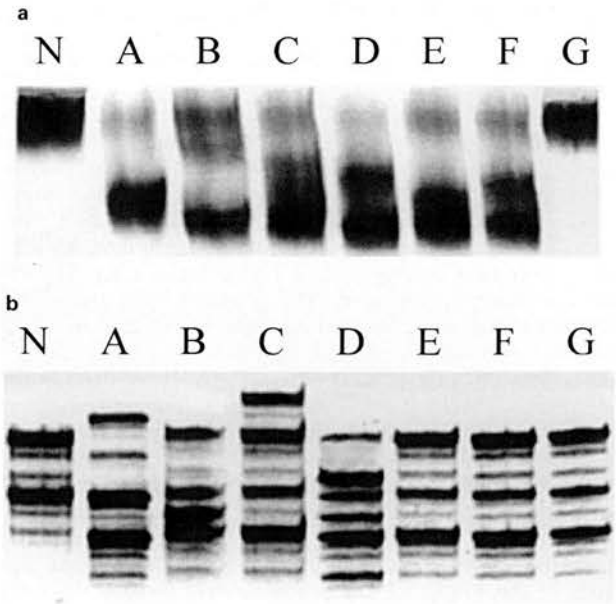

Figure 1 The band shift pattern of case no. 27 at the BAT-26 (**a**) and L-myc (**b**) microsatellite loci (N = normal mucosa; A-G = 7 different tumour sites from the same carcinoma)

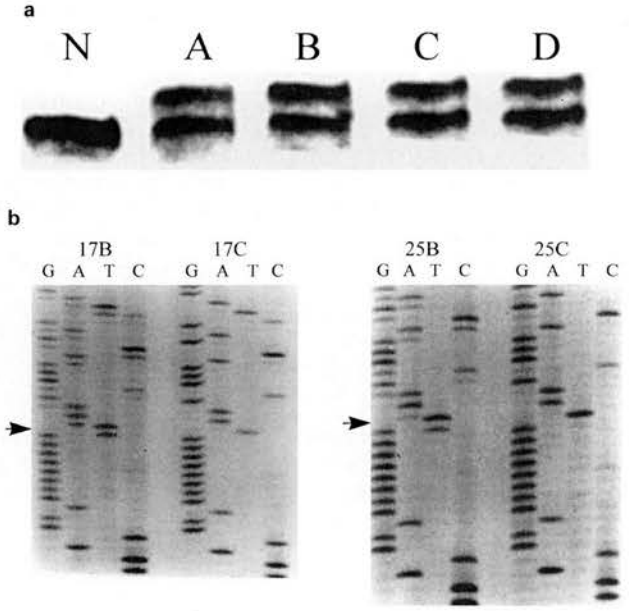

Figure 2 (**a**) *BAX* gene analysis of case no. 55 showing the band shift pattern on a polyacrylamide gel demonstrating one nucleotide insertion in the [(G)8] tract from all tumour sites (A–D), compared to normal mucosa (N). (**b**) Sequence analysis of case 17 (B and C) and 25 (B and C). In both cases, site C does not show the one nucleotide deletion in the [(G)8] tract observed in site B (indicated by arrow)

earlier stage in patients who carry germline mutations in MMR genes. Previous studies have shown clonal expansion of shifts of different amplitude at the same microsatellite locus sampled from different sites in the same tumour (Chung *et al*., 1997), as observed in the present work also. This indicates that a proportion of these microsatellite mutations are acquired as clonal variants throughout the process of tumour formation and reflect but do not cause the evolution of such tumours. In contrast, mutations at some genetic loci have been found with great consistency in RER+ tumours. An outstanding example is transforming growth factor beta-type 2 receptor (TGFβRII), which is mutated in upwards of 90% of all tested RER+ colorectal cancers (Markowitz *et al*., 1995; Parsons *et al*., 1995). In this series, more than 75% of RER+ cancers showed a mutation in the one site within the TGFβRII gene which we tested. Together with independent evidence that TGFβ exerts a suppressive effect on colorectal epithelial growth (Wrana *et al*., 1994), these observations have been interpreted as indicative of a causal role for TGFβRII inactivation in colorectal carcinogenesis.

Frameshift mutations have been detected in the [(G)8] tract of exon 3 of the *BAX* gene in 48% of 63 RER+ sporadic colorectal cancers in a total of two separate studies (Rampino *et al*., 1997; Ouyang *et al*., 1998), and in a similar proportion of RER+ colorectal cancers from HNPCC patients (Yamamoto *et al*., 1998; Yagi *et al*., 1998). Our own observations are entirely concordant with these results: we detected *BAX* mutations in six out of 12 (50%) RER+ carcinomas. In the presence of *BAX* and TGFβRII mutations, others have shown second allele mutations in pure cultures of cell lines (Rampino *et al*., 1997; Markowitz *et al*., 1995), but this is much more difficult to demonstrate in primary tumours due to contamination by stromal and lymphoid cells. Hence some uncertainty remains over the functional status of BAX in primary human tumours, even when there is evidence for mutation in one allele. However, we report here, we believe for the first time, two patterns for such mutations. In four of the six tumours bearing *BAX* mutations, identical alterations in the [(G)8] tract were found in all sites sampled within each cancer, supporting the hypothesis that *BAX* mutation was present in the founder malignant clone. This pattern is also consistent with that described for *BAX* mutations in gastric cancers (Chung *et al*., 1997). However, in two of six cases we demonstrated a second pattern in which *BAX* mutation is not shared by all the tumour sites of the same cancer. We can not completely exclude the possibility that the apparently unaltered [(G)8] tract found in subclones of these tumours represents a reversion, through a second mutation, of the [(G)8] mutation present elsewhere. This possibility can in the future be tested in appropriate cell culture models. A more obvious explanation, however, is that in these tumours the *BAX* [(G)8] mutation was not present in the founder malignant clone but was acquired later in cancer progression. In these tumours, it is difficult to sustain the view that mutational inactivation of *BAX* could have been a critical event early in carcinogenesis. These data therefore raise some doubt as to the significance of loss of BAX-dependent apoptosis pathways in colorectal carcinogenesis. Rather than indicating that failure of apoptosis exerts a critical role in carcinogenesis, some *BAX* mutations in colorectal tumours may merely aid tumour progression, or indeed may simply reflect the consequences of mismatch repair deficiency without a functional connotation.

Materials and methods

Tissue samples

Fresh tumour samples were collected from patients with colorectal carcinoma undergoing surgery in the Royal Infirmary of Edinburgh NHS Trust. Samples were collected from the operating theatre within 30 min of resection and transported to the Pathology Department. Two to seven small tumour pieces and the matched normal tissues from each case were frozen in liquid nitrogen, then stored at −70°C. DNA was extracted from frozen tissues by the method of Goelz *et al.* (1985).

RER status analysis

Sixty tumours were originally screened for microsatellite instability and only RER+ cases were analysed for *BAX* gene mutation. All the samples were tested at five microsatellite loci including the highly unstable BAT-26 locus, claimed to be sufficient alone for identifying the RER status (Zhou *et al.*, 1998; Hoang *et al.*, 1997). The other 4 loci included L-myc, TGFβRII, D13S160, and D2S123 using the primers and conditions described elsewhere (Young *et al.*, 1995; Huang *et al.*, 1996).

BAX *gene analysis*

Twelve RER+ cases with a total of 45 different tumour sites and the matched normal tissue constituted in the material for *BAX* gene mutational analysis.

A DNA segment of 94-base pairs encompassing the [(G)8] tract in *BAX* was amplified by PCR using the primers described by Rampino *et al.* (1997). PCR was carried out for 30 cycles, each consisting of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, extension for 30 s at 72°C. Reactions consisted of 50 ul volume containing 100 ng genomic DNA, 10 pmols of each primer, 200 uM of each dNTP (Advanced Biotechnologies Ltd), 1.5 mM $MgCl_2$, 1.25 U of thermostable Taq DNA polymerase and buffer consisting of 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 0.05% non-ionic detergent (Life Technologies UK). The PCR hot start method was used. The products were analysed by electrophoresis on 2% agarose gels for detection of the amplified product.

The forward primer was end labelled with γ-^{33}P-ATP using T4 polynucleotide kinase according to the manufacturer's instructions (Life Technologies UK). This primer was then used to label the original PCR product by performing one PCR cycle using the same conditions as described above. The radio-labelled products were electrophoretically separated in denaturing 6% polyacrylamide gel and subjected to autoradiography for detection of band shifts. At the same time, all the original PCR products were directly sequenced with Thermosequenase radiolabelled terminator cycle sequencing kits (Amersham Life Science).

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References

Aaltonen LA, Peltomaki P, Leach FS, Sistonen P, Pylkkanen L, Mecklin JP, Jarvinen H, Powell SM, Jen J, Hamilton SR, Petersen GM, Kinzler KW, Vogelstein B and Delachapelle A. (1993). *Science*, **260**, 812–816.

Brown R. (1997). *Br. Med. Bull.*, **53**, 466–477.

Bubb VJ, Curtis LJ, Cunningham C, Dunlop MG, Carothers AD, Morris RG, White S, Bird CC and Wyllie AH. (1996). *Oncogene*, **12**, 2641–2649.

Chung YJ, Park SW, Song JM, Lee KY, Seo EJ, Choi SW and Rhyu MG. (1997). *Oncogene*, **15**, 1719–1726.

Goelz SE, Hamilton SR and Vogelstein B. (1985). *Biochem. Biophys. Res. Commun.*, **130**, 118–126.

Hoang JM, Cottu PH, Thuille B, Salmon RJ, Thomas G and Hamelin R. (1997). *Cancer Res.*, **57**, 300–303.

Huang J, Papadopoulos N, Mckinley AJ, Farrington SM, Curtis LJ, Wyllie AH, Zheng S, Willson JKV, Markowitz SD, Morin P, Kinzler KW, Vogelstein B and Dunlop MG. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 9049–9054.

Jacoby RF, Marshall DJ, Kailas S, Schlack S, Harms B and Love R. (1995). *Gastroenterology*, **109**, 73–82.

Kroemer G. (1997). *Nature Med.*, **3**, 614–620.

Markowitz S, Wang J, Myeroff L, Parsons R, Sun LZ, Lutterbaugh J, Fan RS, Zborowska E, Kinzler KW, Vogelstein B, Brattain M and Willson JKV. (1995). *Science*, **268**, 1336–1338.

Miyashita T, Krajewski S, Krajewska M, Wang HG, Lin HK, Liebermann DA, Hoffman B and Reed JC. (1994). *Oncogene*, **9**, 1799–1805.

Miyashita T and Reed JC. (1995). *Cell*, **80**, 293–299.

Oltvai ZN, Milliman CL and Korsmeyer SJ. (1993). *Cell*, **74**, 609–619.

Ouyang H, Furukawa T, Abe T, Kato Y and Horii A. (1998). *Clin. Cancer Res.*, **4**, 1071–1074.

Parsons R, Myeroff LL, Liu B, Willson JKV, Markowitz SD, Kinzler KW and Vogelstein B. (1995). *Cancer Res.*, **55**, 5548–5550.

Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC and Perucho M. (1997). *Science*, **275**, 967–969.

Samowitz WS and Slattery ML. (1997). *Am. J. Pathol.*, **151**, 33–35.

Thibodeau SN, Donovan KA, Tester DJ, Bren GD, Halling KC, Cunningham J, Morris AV and Roche PC. (1993). *Am. J. Hum. Genet.*, (Suppl.), **53**, 1.

White E. (1996). *Genes Dev.*, **10**, 1–15.

Wrana JL, Attisano L, Wieser R, Ventura F and Massague J. (1994). *Nature*, **370**, 341–347.

Yagi OK, Akiyama Y, Nomizu T, Iwama T, Endo M and Yuasa Y. (1998). *Gastroenterology*, **114**, 268–274.

Yamamoto H, Sawai H, Weber TK, RodriguezBigas MA and Perucho M. (1998). *Cancer Res.*, **58**, 997–1003.

Young J, Searle J, Buttenshaw R, Thomas L, Ward M, Chenevixtrench G and Leggett B. (1995). *Genes Chrom. Cancer*, **12**, 251–254.

Zhou XP, Hoang JM, Li YJ, Seruca R, Carneiro F, SobrinhoSimoes M, Lothe RA, Gleeson CM, Russell SEH, Muzeau F, Flejou JF, HoangXuan K, Lidereau R, Thomas G and Hamelin R. (1998). *Genes Chrom. Cancer*, **21**, 101–107.

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Letter to the Editor

Death pathway genes *Fas (Apo-1/CD95)* and *Bik (Nbk)* show no mutations in colorectal carcinomas

Dear Editor,

Many proteins with an established role in carcinogenesis and cancer progression (e.g. p53, RB, C-MYC, BCL2, RAS & E1B 19K) can regulate apoptosis and altered susceptibility to apoptosis may contribute to carcinogenesis.[1] Data from genetically manipulated animals show that oncosuppressor deficiency may abrogate the apoptosis of cells that have sustained DNA damage, thus permitting survival and proliferation of cells bearing mutations.[2] Apoptosis is widely observed in tumours, however, indicating that loss of ability to induce apoptosis – if important in carcinogenesis – must be restricted to particular pathways. Clear identification of these pathways in human tumours becomes an important aim, as it would help define the molecular basis not only of carcinogenesis but also of tumour resistance to various therapeutic measures. The study of colorectal cancers affords a particular advantage in this respect because a proportion have an underlying deficiency in DNA nucleotide mismatch repair (MMR), alternatively known as 'replication error positive' (RER+) phenotype. A high proportion of RER+ colorectal carcinomas do indeed bear clonally expanded mutations in microsatellite loci or repetitive sequences within cancer-related genes emphasising the importance of such genes in the process of carcinogenesis and cancer progression. Such mutated target genes include *APC*, *TGFβ-RII*,[3] and *Bax*,[4,5] all of which have also been implicated in pathways initiating apoptosis. Here, we provide information on two more death pathway genes: *Fas (Apo-1/CD95)* and *Bik-1 (Nbk)*.

Active FAS receptor triggers apoptosis in many cell types and appears to be necessary for apoptosis initiated by C-MYC and certain DNA-damaging chemotherapeutic agents. Binding of the FAS ligand induces trimerization of the receptor and this stimulates the intracytoplasmic FAS death domain to recruit a protein complex that includes caspase 8, thus activating the caspase cascade and hence the terminal effector events of apoptosis.[6] *Fas* is an attractive potential target for carcinogenic mutations. Individuals with the rare Canal-Smith syndrome, in which *Fas* is constitutively abnormal, have a high cancer incidence.[7] Moreover, FAS protein is constitutively expressed in normal colorectal epithelium and most colorectal adenomas, but expression is frequently less and sometimes undetectable in carcinomas.[8] The mechanism and significance of these changes, however, has not been established. BIK is a potent death-inducing protein sharing the BH3 domain of the BCL2 protein family. It has capacity to interact with the endogenous survival promoting proteins, BCL2 and BCL_{XL}, and their functional viral homologues BHRF1 and E1B-19K. BIK can induce apoptosis independently of p53 and BAX in some cell types[9] but its role in colorectal epithelium is unknown. We therefore studied the genes encoding FAS and BIK proteins in a series of primary colorectal carcinomas, to test the hypothesis that mutation in these apoptosis-promoting genes may be implicated in the process of human colorectal carcinogenesis.

DNA was extracted from frozen samples of 24 selected colorectal carcinomas (and normal mucosa) including 12 RER− and 12 RER+ after testing for microsatellite instability at 5–11 microsatellite loci (Figure 1a). Of the 12 RER+ tumours ten were right sided, ten had poor or mucinous differentiation, and six were immunohistochemically positive for p53 accumulation. The 12 RER tumours included three right-sided tumours, three with mucinous or poor differentiation and nine that were p53 positive by immunohistochemistry.

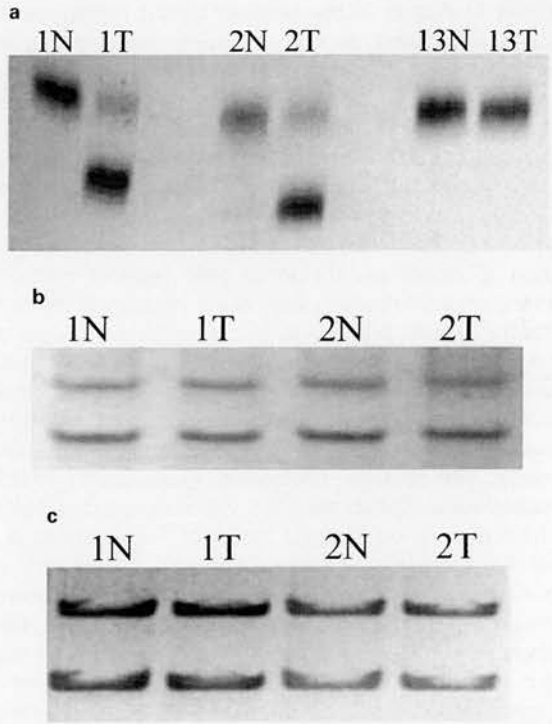

Figure 1 (**a**) Denaturing polyacrylamide gel showing RER characterisation at the BAT26 microsatellite locus: samples 1 and 2 show band shifts in the tumour lane (T) compared to the normal (N), consistent with derivation from RER+ cancers, whereas sample 13 does not show any band shifts and is derived from a RER− cancer. (**b**) SSCP for *Fas* exon 9I for samples 1 and 2 from RER+ tumours, showing no band shifts in the tumour (T) compared to the normal (N) lanes. (**c**) SSCP for *Bik* for samples 1 and 2 from RER+ tumours, showing no band shifts in the tumour (T) compared to the normal (N) lanes

Ten primer pairs were designed to PCR-amplify the nine *Fas* exons. *Bik* was examined less comprehensively, focusing on a potential target site for MMR deficiency mutations: a repetitive sequence containing CTG triplets [(CTG)2 GCG (CTG)5 GCG (CTG)3 CCG CTG] occupying positions 409–450, included within the Bik coding sequence. The PCR products were analyzed for mutations using single strand conformation polymorphism (SSCP).[10] Heteroduplex analysis[11] was performed on the PCR products from *Fas* exon 5 because these were difficult to resolve by SSCP. Where abnormal banding patterns were observed DNA was directly sequenced. By these criteria there was no evidence of mutation in any of the 24 cancer or normal mucosa samples examined, either in all the *Fas* gene exons (Figure 1b), or in the target repeat sequence in the *Bik* gene (Figure 1c).

On searching the coding sequence of *Fas* for nucleotide repeats we found a [(T)7] tract in exon 4, an [(A)6] tract in exon 9 and a [(T)5] tract in exon 3 in addition to some shorter repeat tracts. Other studies have shown that mononucleotide repeats of similar length are susceptible to MMR deficiency mutation and there is thus little doubt that *Fas* provides several intra-exonic targets for mutation in RER+ cells. The mutation screening method (SSCP) detects 80–90% of mutations when applied to single strand DNA between 150–250 nucleotides in length. We designed the PCR primers to generate products to fit this range as far as possible. Hence it is improbable that mutations in *Fas* or in the tandem repeat region identified in *Bik* are frequent in human colorectal cancers, even when these bear the mutation-susceptible RER+ phenotype.

The importance of clonal selection in carcinogenesis and tumour progression has recently been re-emphasised.[12] Our results contrast with the high incidence of mutation in RER+ colorectal cancers in other genes, notably *TGFβ-RII* (75%),[3] and *Bax* (50%).[4,5] This failure to identify any instance of clonal expansion of cells bearing *Fas* or *Bik* mutations thus suggests that such mutations confer no substantial growth advantage in colorectal carcinogenesis.

The question remains why FAS protein expression should be subnormal in a high proportion of colorectal carcinomas. The present results and those of others using Southern blot technology[13] exclude structural alterations in the gene. An obvious alternative mechanism would be depressed transcription secondary to reduced availability of wild type p53, since the first intron of *Fas* includes a p53 responsive element.[14] Of our 24 cases, 15 showed clear abnormality in p53, exhibited by immunohistochemical stabilisation affecting a high proportion of the nuclei. Although *Fas* down-regulation has the capacity to reduce cellular responses to lethal stimuli, it is significant that mutational inactivation of the gene is not observed in cancer tissues in which mutational events in other genes are common and clonally expanded. We conclude that *Fas* inactivation is unlikely to be a critical early event in colorectal carcinogenesis.

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1. Arends MJ and Wyllie AH (1991) Apoptosis–mechanisms and roles in pathology. Int. Rev. Exp. Pathol. 32: 223–254
2. Griffiths SD, Clarke AR, Healy LE, Ross G, Ford AM, Hooper ML, Wyllie AH and Greaves M (1997) Absence of p53 permits propagation of mutant cells following genotoxic damage. Oncogene 14: 523–531
3. Parsons R, Myeroff LL, Liu B, Willson JKV, Markowitz SD, Kinzler KW and Vogelstein B (1995) Microsatellite instability and mutations of the transforming growth factor beta type-II receptor gene in colorectal cancer. Cancer Res. 55: 5548–5550
4. Rampino N, Yamamoto H, Ionov Y, Li Y, Sawai H, Reed JC and Perucho M (1997) Somatic frameshift mutations in the Bax gene in colon cancers of the microsatellite mutator phenotype. Science 275: 967–969
5. Abdel-Rahman WM, Georgiades IB, Curtis LJ, Arends MJ and Wyllie AH (1999) Role of Bax mutations in mismatch repair-deficient colorectal carcinogenesis. Oncogene (in press)
6. Yuan YJ (1997) Transducing signals of life and death. Curr. Opin. Cell Biol. 9: 247–251
7. Drappa J, Vaishnaw AK, Sullivan KE, Chu JL and Elkon KB (1996) Fas gene mutations in the Canale-Smith syndrome, an inherited lymphoproliferative disorder associated with autoimmunity. N. Engl. J. Med. 335: 1643–1649
8. Moller P, Koretz K, Leithauser F, Bruderlein S, Henne C, Quentmeier A and Krammer PH (1994) Expression of APO-1 (CD95), a member of the NGF/TNF-receptor superfamily, in normal and neoplastic colon epithelium. Int. J. Cancer 57: 371–377
9. Boyd JM, Gallo GJ, Elangovan B, Houghton AB, Malstrom S, Avery BJ, Ebb RG, Subramanian T, Chittenden T, Lutz RJ and Chinnadurai G (1995) Bik, a novel death inducing protein shares a distinct sequence motif with BCL-2 family proteins and interacts with viral and cellular survival promoting proteins. Oncogene 11: 1921–1928
10. Orita M, Suzuki Y, Sekiya T and Hayashi K (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5: 874–879
11. White MB, Carvalho M, Derse D, O'Brien SJ and Dean M (1992) Detecting single base substitutions as heteroduplex polymorphisms. Genomics 12: 301–306
12. Tomlinson I and Bodmer W (1999) Selection, the mutation rate and cancer: ensuring that the tail does not wag the dog. Nat. Med. 5: 11–12
13. Butler LM, Hewett PJ, Butler WJ and Cowled PA (1998) Down regulation of Fas gene expression in colon cancer is not a result of allelic loss or gene rearrangement. Br. J. Cancer 77: 1454–1459
14. Muller M, Wilder S, Bannasch D, Israeli D, Lehlbach K, Li-weber M, Friedman SL, Galle PR, Stremmel W, Oren M and Krammer PH (1998) p53 activates the CD95 (Apo-1/Fas) gene in response to DNA damage by anticancer drugs. J. Exp. Med. 188: 2033–2045