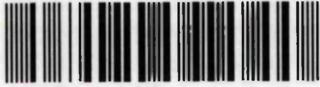

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STUDY OF TUMOUR SUPPRESSOR GENES IN MALIGNANT TUMOURS OF LIVER, PANCREAS AND BILIARY SYSTEM

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A thesis submitted to University of London
in fulfilment of the conditions governing
candidates for the degree of PhD.

October 1993 4

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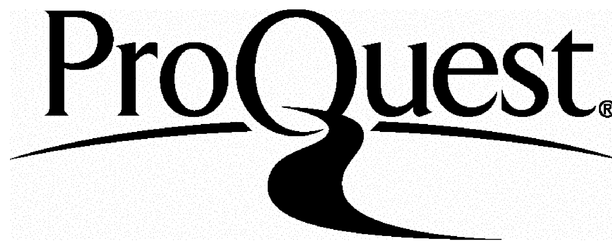

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Abstract

The importance of tumour suppressor gene inactivation by gene deletion and/or mutation in tumour development has been established. Loss of heterozygosity (LOH), or allele loss, in tumour DNA is thought to represent the loss of such genes. Detection of LOH in particular tumours has been widely used for searching for the loci of tumour suppressor genes in the tumours.

Few studies on tumour suppressor genes in tumours of liver, pancreas and biliary system have been performed. The work in this thesis was undertaken to test the hypothesis that loss of tumour suppressor genes by LOH occurred in these tumours. Eighty-two such tumours (liver tumours: 53; carcinoma of the pancreas: 15 and cholangiocarcinoma: 14) were investigated to search for the consistent LOH. Paired normal and tumour DNA were analyzed by Southern hybridisation using a panel of restriction fragment length polymorphism (RFLP) DNA probes, previously assigned to different chromosomal regions. In informative patients, band losses in tumour DNA compared with bands of normal DNA on autoradiographs were scored as LOH or allele loss.

In hepatocellular carcinoma (HCC) without liver cirrhosis, LOH in the chromosome region 5q35-qter was found in 6 out of 6 informative patients detected by the probe λMS8, and the loss was distinct from the adenomatous polyposis coli (APC) locus in the region of 5q21-22. The results strongly suggested that a putative tumour suppressor gene for HCC without liver cirrhosis

was located in the region 5q35-qter. A high frequency of loss was also found in 17p13, the chromosome region encompassing the p53 tumour suppressor gene. HCC with liver cirrhosis had no loss in the region of 5q35-qter, but in 1q42-43, 5p and 17p13. LOH occurred at a low frequency in fibrolamellar carcinomas and in none of the hepatocellular adenomas studied. In contrast, multiple allelic losses were found in two sarcomatoid liver carcinomas. Cholangiocarcinoma had a similar LOH pattern to that in HCC without liver cirrhosis, ie, losses in 5q35-qter and 17p13. Liver metastases from colorectal cancers showed a high incidence of allele loss on chromosomes 5q21-22, 17p13 and 18q. In carcinoma of the pancreas, LOH was detected in 1p33-35, 6q27 and 11q13.

An extensive pilot study was performed to isolate the putative tumour suppressor gene in chromosomal region 5q35-qter for HCC without liver cirrhosis and cholangiocarcinoma. Effort was made to isolate DNA sequences from chromosome 5 that were expressed in normal human liver tissue, on the assumption that tumour suppressor genes are transcribed in normal tissues. This was performed by cross-screening a human chromosome 5 genomic DNA library in phage Charon 21A with a normal human liver cDNA library. Twenty-one positive clones in phages were obtained after the third round of screening. These clones were recloned to plasmid Bluescript KS+ for further use since clones would be easier to use in plasmid vectors than in phages. A proposal to identify the putative tumour suppressor gene from these clones was made.

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Abbreviation

APC	adenomatous polyposis coli
bp	basepair(s)
BSA	bovine serum albumin
CaP	carcinoma of the pancreas
CIAP	calf intestinal alkaline phosphatase
cpm	counts per minutes
DCC	deleted in colorectal carcinoma
ddH_2O	distilled deionized water
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylene diamine tetraacetic acid, disodium salt
EtBr	ethidium bromide
FAL	fractional allele loss
FAP	familial adenomatous polyposis coli
FLC	fibrolamellar carcinoma
HCC	hepatocellular carcinoma
kb	kilobase
kd	kilodalton
LB	Luria-Bertani (medium)
LOH	loss of heterozygosity
MCC	mutated in colorectal cancer
OD	optical density

PCR	polymerase chain reaction
pfu	plaque forming units
RB	retinoblastoma gene
RFLP	restriction fragment length polymorphism
rpm	rotations per minute
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
Tris	2-amino 2-hydroxymethyl propane-1,3-diol
WT	Wilms' tumour
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

Chapter 1 Introduction

1.1 Cancer is a genetic disease

Carcinogenesis is a multifactorial and multistep process. In the process genetic factors play a pivotal role. In fact there is a wealth of evidence that cancer is a set of fundamentally genetic diseases (Bishop, 1986, 1987; Lasko et al, 1991). While about 1% of total cancer cases have their tumours inherited (Mulvihill, 1977), genetic abnormalities have been demonstrated in all types of cancers, either inherited or non-inherited, common or uncommon tumours. These changes include chromosomal aberrations (Solomon et al, 1991), oncogene activation (Bishop, 1983), abnormalities in growth factors and their receptors (Aaronson, 1991), tumour suppressor gene inactivation (Weinberg, 1991; Lasko et al, 1991) and other yet to be identified abnormalities in genetic make-up (Volpe, 1990; Peltomäki et al, 1993). Among these changes activation of oncogenes and /or inactivation of tumour suppressor genes are crucial. Other alterations can be the cause and/or consequences of changes of these two classes of cancer genes.

1.1.1 Chromosomal abnormalities in cancer

In 1960, Nowell and Hungerford discovered the first consistent chromosome abnormality in a human cancer - the Philadelphia (Ph') chromosome in patients with chronic myeloid leukaemia. After the introduction of chromosome banding

techniques by Caspersson et al in 1970, cancer cytogenetics has been developing. It has become apparent from the cytogenetic data that the chromosomal changes of human tumours are nonrandom (Heim & Mitelman, 1987). These changes have been found in every tumour studied and an extensive database of chromosomal aberrations has been compiled (Sandberg, 1990; Mitelman, 1991).

In cytogenetics, tumours are broadly classified as haematological, including leukaemia and lymphoma, and solid, including carcinoma and sarcoma. In haematological tumours, the nonrandom chromosomal changes have led to the isolation of unique translocation breakpoints, both diagnostic for the specific tumours and useful in guiding the molecular genetic approaches (Nowell & Croce, 1990; Solomon et al, 1991). It is more technically difficult to perform cytogenetic studies in solid tumours. Although these tumours account for more than 90% human cancers, less than 20% cytogenetic data in human neoplasia are based on the solid tumours (Sandberg, 1990). Nevertheless cytogenetic analysis has also been valuable in the study of solid tumours. As in the case of haematological tumours, the cytogenetic data in solid tumours are not only useful in the diagnosis and prognosis of these cancers (Sandberg, 1990), but also imply the changes of oncogenes and tumour suppressor genes (Solomon et al, 1991).

Chromosomal abnormalities in cancer can be seen as structural and numerical. Structural changes include translocation, inversion, deletion, insertion and amplification. Numerical alterations are losses and duplication of whole

chromosomes, hence the modal number, ie, the most common chromosome number in a tumour cell population, may not be the normal 46 (Sandberg, 1990). The chromosome structural and numerical aberrations in cancers of liver, pancreas and biliary system will be reviewed later in this chapter.

1.1.2 Activation of oncogenes

By extensive investigation into the action of oncogenic retroviruses, "oncogenes" and their normal guise, "proto-oncogenes" were discovered in the human genome (Bishop, 1983). Proto-oncogenes are normal cellular genes, homologous to oncogenic nucleotide sequences of retroviruses. Their products stimulate cell growth. More than 40 proto-oncogenes have been identified in the human genome (Marshall, 1989). These cellular genes can be activated into cancer-causing oncogenes.

About 20 different oncogenes are reproducibly activated in human malignancies (Cooper, 1992). The mechanisms by which proto-oncogenes are activated into oncogenes include gene mutation, amplification and chromosome translocation. The ras proto-oncogene family (N-, H-, K-ras) may become oncogenic by a single point mutation, usually at codon 12, 13 or 61, resulting in an amino acid substitution in the gene product (Barbacid, 1987). Such a single alteration can change the phenotype of the cell (Weinberg, 1989). Introduction of a mutated ras gene into a normal cell line, NIH 3T3, can transform the cells. Ras activation

has been found in a variety of human cancers, with the highest frequency in carcinoma of the pancreas (reviewed in Lemoine, 1990a, 1990b).

Many tumour cells contain amplified oncogenes. Gene amplification may lead to overexpression of the gene product, which may perpetuate cell growth. An example of oncogene activation by gene amplification is myc oncogenes (Tronick & Aaronson, 1990). Myc amplification has been implicated in the development and progression of both leukaemia and solid tumours. Other proto-oncogenes have been found amplified as well. They include abl, ets-1, myb, K- and N-ras (Tronick & Aaronson, 1990).

Chromosome translocation can also activate oncogenes. The Ph' chromosome, a translocation from chromosome 9 to chromosome 22, involves activating the abl oncogene at the chromosome translocation site (reviewed in Solomon et al, 1991).

1.1.3 Growth factors and cancer

Growth factors cause cells in the resting or G_0 phase to enter and proceed through cell cycle. The growth control of normal cells is carefully regulated by the normal function of growth factors. In contrast to that, the growth control of cancer cells is unregulated, that is, their growth rate is higher than their death rate. This imbalance arises as a result of one of the genetic events: unregulated expression of growth factors and/or their receptors (Aaronson, 1991).

There is much evidence for genetic aberrations affecting growth factors or their receptors in human malignancies. Many growth factors and their receptors are often overexpressed in cancers (Lemoine et al, 1992a). It has been recognised that some of oncogenes encode proteins with significant homology to cellular growth factors or growth factor receptors (Leonard & Sharon, 1990). The first evidence came from the identification that the amino acid sequence of one of the two major polypeptide chains of human platelet-derived growth factor (PDGF) is highly similar to that predicted for the transforming protein encoded by the v-sis oncogene present in the simian sarcoma virus. A number of cancer cell lines, including those of lung carcinomas, hepatocellular carcinomas, hepatoblastomas, breast carcinomas, and different types of sarcomas, produce PDGF. The growth factor may have an autocrine interaction with PDGF receptors on those cancer cells (reviewed in Tronick & Aaronson, 1990).

Subsequently, some growth factor receptors were found to have close similarity with some oncogene encoded proteins, such as the epidermal growth factor (EGF) receptors with portions of proteins encoded by v-erb B oncogenes (Downward et al, 1984). EGF receptors are often overexpressed in human cancers (Lemoine et al, 1992a). EGF receptors, together with c-erb B-2 and c-erb B-3, belong to the type 1 growth factor receptor family (Lemoine et al, 1992a). Hepatocyte growth factor (HGF) receptor was identified as the product of c-met proto-oncogene (Bottaro et al, 1991; Naldini et al, 1991). The changes of HGF and its receptor in hepatocellular carcinomas will be reviewed later in this chapter.

1.1.4 Inactivation of tumour suppressor genes

While activation of oncogenes can induce transformation, inactivation of tumour suppressor genes is at least as important in the multistep process of carcinogenesis (Weinberg, 1991). Tumour suppressor genes are normal cellular genes whose products regulate cellular growth and differentiation. Their action has an important role in inhibiting the uncontrolled cellular proliferation characteristic of cancer. When tumour suppressor genes are missing from cell genomes, the cell may become malignant. There exist several lines of evidence supporting this. These will be discussed in detail in the next section.

1.2 Tumour suppressor genes

1.2.1 Tumour suppression by cell fusion

Although the research has been recently focused on the tumour suppressor genes and has lagged 10 years behind that focused on oncogenes (Weinberg, 1991), the evidence of tumour suppression appeared as early as in 1960s. It came from experiments in which somatic cells were fused in vitro. Harris, Klein and colleagues reported in 1969 that fusion of a non-malignant mouse fibroblast line with either of the three highly malignant tumours of the mouse: the Ehrlich, the SEWA and the MSWBS tumours, led to the suppression of malignancy (Harris et al, 1969). The parent malignant tumours were transplantable in mouse. A few Ehrlich cells injected into the peritoneal cavity killed most mice within 3 weeks. However, the cell hybrids of this tumour with the non-malignant fibroblasts were unable to form tumours in the animals injected. The chromosomal constitution of these hybrid cells was highly unstable and chromosomes were rapidly lost from the hybrids (Bregula et al, 1970). When some of the chromosomes from the non-malignant parent cells were lost in the hybrids, the reversion to tumour-forming ability occurred (Harris et al, 1969).

Harris and Klein's observations gave the first evidence that something, ie the later known tumour suppressor genes, might locate on the chromosomes of normal cells, which suppressed the malignancy in the hybrids. After the hybrids lost the

chromosomes containing tumour suppressor genes, they reverted to malignancy. Similar observations were made later by themselves and other groups in both rodents and human (reviewed in Harris, 1988; Sager, 1989; Stanbridge, 1990a; Marshall, 1991).

Owing to the rapid chromosome loss in hybrids, it was difficult to test the genetic basis for the above hypothesis. The parental origin of chromosomes in the hybrids was not easy to determine. Nevertheless, in "stable" hybrids between malignant HeLa cervical carcinoma cells x normal human fibroblasts, loss of normal human chromosome 11 was implicated in re-expression of malignancy (Stanbridge et al, 1981). Support for involvement of chromosome 11 has come from studies where a single chromosome 11 introduced into HeLa cells by microcell fusion caused suppression of malignancy (Saxon et al, 1986). Microcell fusion for introduction of normal cells into tumour cells will be discussed more in Section 1.2.5.

1.2.2 Two-hit hypothesis

In 1971, by analyzing the heritable childhood cancer, retinoblastoma, Knudson proposed a "two-hit" hypothesis, which indirectly predicted the existence of tumour suppressor genes in cell genome. In proposing this, he addressed the two forms of this disease - familial and sporadic. While bilateral retinoblastoma is characteristic of the familial disease, unilateral retinoblastoma is characteristic of the sporadic disease. Based on detailed analysis of the age of diagnosis of familial

and sporadic retinoblastoma, and the counting of the number of tumours per eye in bilateral cases, Knudson suggested the two-hit hypothesis. The theory suggests that two separate mutational events at the same gene locus are required for carcinogenesis. In the familial form the first mutation is dominantly inherited via the germline and the second arises spontaneously at the somatic level. In the sporadic form both events occur somatically. These allelic mutational events act recessively at the cellular level, and cancer develops after both copies of the gene are lost (Knudson, 1971, 1986).

The Knudson hypothesis leads indirectly to the idea of a tumour suppressor gene. In some familial retinoblastoma pedigrees, actual tumour formation can skip a generation, thus a person unaffected by retinoblastoma has an affected parent and an affected child (Knudson, 1986). The fortunate escapees may inherit a mutant gene from a parent and pass it to their children, but without themselves incurring the second somatic event. It follows that a single copy of a normal gene is sufficient to suppress tumour formation (Brock, 1993).

The supportive evidence for the two-hit model came when the nature of the "mutational events" was discovered. Cytogenetic data showed that deletions of human chromosome 13 band q14 were associated with retinoblastoma (Knudson et al, 1976; Yunis & Ramsay, 1978; Balaban et al, 1982). Subsequently Cavenee et al (1983) reported an RFLP analysis on the paired DNA from normal tissue and retinoblastoma and revealed loss of heterozygosity (LOH) in chromosome

13q14 in retinoblastoma. This was the first study revealing LOH in human tumours (discussed later). The final proof for the two-hit model in genesis of retinoblastoma is the cloning of the retinoblastoma gene (RB) from chromosome 13q14 and introduction of a normal RB gene into tumour cells to suppress the malignancy (Sections 1.2.4 and 1.2.5). There is now little doubt that removal of two copies of the normal retinoblastoma gene in retinal cells at an early development stage is responsible for retinoblastoma, thus Knudson's two-hit model is well fit for the development of retinoblastoma.

1.2.3 Loss of heterozygosity in tumours

In 1985 Knudson suggested that every form of cancer has a rare inherited counterpart and that the two-hit model could also be applied to other inherited form of cancer susceptibility. This suggestion has been supported by the demonstration of chromosome allele loss in a variety of solid human tumours, not only in inherited childhood tumours but also in sporadic common adult cancers, hence the two-hit model can be extended to tumour types other than retinoblastoma (Lasko et al, 1991).

In the two-hit model, one of the mutational events can be the loss of an allele at a tumour suppressor gene locus through mitotic recombination, gene conversion, chromosomal loss after non-disjunction, or other mechanisms (Cavenee et al, 1983; Lasko et al, 1991). The other allele can be also lost or mutated, hence both

copies of the gene are inactivated and tumour may develop. The mechanisms that lead to loss of one copy of tumour suppressor gene can involve the flanking chromosomal regions as well. Thus, anonymous DNA probes (markers) mapping to nearby chromosomal sites, which may have shown heterozygosity prior to tumour progression, will suffer a parallel loss of heterozygosity. Therefore, the allele loss in tumours can be detected by DNA probes mapping around or at the gene locus and shown as LOH in tumour DNA.

The DNA probes used in LOH analysis are those detecting DNA polymorphism at restriction endonuclease sites. Differences in the sizes of fragments resulting from the digestion of the corresponding region of DNA from homologous chromosomes have been termed restriction fragment length polymorphisms (RFLPs) (Gusella, 1986). Such RFLPs are inherited as Mendelian traits in a codominant fashion. Therefore, when a person is informative (heterozygous) for a given RFLP, one allele is inherited from the father and the other from the mother (Lasko et al, 1991). Loss of an allele (LOH) can be seen as a band loss on a Southern blot.

Detection of LOH has been widely used for searching for loci of tumour suppressor genes in the genome (Weinberg, 1991). A battery of RFLP probes can be used to analyze DNA from any paired samples of control and tumour DNAs. The consistent LOH in the particular region of chromosomes in a tumour would indicate the possible location of genes of interest. It has been shown that LOH

is limited to a few specific regions of the genome (Lasko et al, 1991). The locations of several cloned tumour suppressor genes were predicted by or consistent with the data of LOH (next section).

After the first LOH study in retinoblastoma (Cavenee et al, 1983), such loss has also been found on chromosome 11 in Wilms' tumour (Fearon et al, 1984), chromosome 22 in familial acoustic neuroma (Seizinger et al, 1986), chromosome 11 in familial multiple endocrine neoplasia type 1 (Bale et al, 1991), chromosomes 5 and 22 in colon carcinoma from familial polyposis coli (Okamoto et al, 1988) and on other chromosomes in other inherited tumours (reviewed in Lasko et al, 1991). Fearon et al (1985) first investigated LOH in a common adult tumour, bladder cancer, and found allele loss on 11p in 42% of tumours studied. Similar loss has subsequently been seen in other common cancers (summarized in Table 1.1). Indeed, LOH occurs in all types of solid tumours (Lasko et al, 1991).

Several studies have shown that the frequency of LOH in a certain tumour type may be associated with patients' clinical features (Vogelstein et al, 1989; Kern et al, 1989). Usually, the patients with more LOH in their tumours will have a poorer prognosis (Vogelstein et al, 1989). Apart from the possibility for mapping putative tumour suppressor genes and the association with tumour prognosis, detection of loss of heterozygosity may become a useful means for determining tumour origins (Tsuda et al,1992).

TABLE 1.1 EXAMPLES OF LOSS OF HETEROZYGOSITY IN COMMON HUMAN TUMOURS

Tumour	Chromosome Arm	Reference
Bladder Cancer	9q	Cairns et al, 1993
	11p	Fearon et al, 1985
Breast Carcinoma	1q, 3p, 11p, 13q, 17p, 17q	Ali et al, 1987
		Mackay et al, 1988a,b
		Chen et al, 1989a
		Devilee et al, 1989
		Andersen et al, 1992
		Cornelis et al, 1993
	6q	Devilee et al, 1991a
Colorectal Tumour	5q	Solomon et al, 1987
		Rees et al, 1989
	5q,17p,18q	Vogelstein et al, 1988
	8p	Emi et al, 1992
	14q	Young et al, 1993
Cervical Cancer	3p	Yokota et al, 1989
Gastric Carcinoma	1p, 5q, 17p	Sano et al, 1991
Hepatocellular Carcinoma	8p	Emi et al, 1992
	16q	Tsuda et al, 1990
Lung Cancer	3p, 13q, 17p	Kok et al, 1987
		Mori et al, 1989
		Tsuchiya et al, 1992
	11p	Skinner et al, 1990
Ovarian Cancer	6q, 11p, 17p	Lee et al, 1990
		Saito et al, 1992
	17q	Eccles et al, 1992
Prostate Cancer	10q, 16q	Carter et al, 1990
Renal Cell Carcinoma	3p	Bergerheim et al, 1989
	6p	Rukstalis et al, 1989

1.2.4 Cloned tumour suppressor genes

All the evidence discussed above strongly indicates the presence of tumour suppressor genes in the cell genome. The direct proof is the cloning of these genes. So far several such genes or candidates have been cloned (Table 1.2). Among these, RB and p53 are the first two examples of tumour suppressor genes. While RB is considered a prototype tumour suppressor gene, p53 has become an important tumour suppressor gene since it has been implicated in most, if not all, human tumours.

1.2.4.1 RB

As discussed in Section 1.2.2, both cytogenetic and RFLP analyses showed the retinoblastoma gene to be localised on chromosome 13q14. Sparks et al (1980) assigned the esterase D gene, a marker tightly linked with retinoblastoma gene, to chromosome 13q14. The result further pinpointed the retinoblastoma locus to 13q14. A DNA probe, H3-8, selected from a human chromosome lambda phage library, was assigned to 13q14 (Lalande et al, 1984). This probe could detect chromosome deletion in 3 out of 37 retinoblastoma (Dryja et al, 1986). By chromosome walking starting from H3-8 and esterase D gene locus, the retinoblastoma (RB) gene, was cloned by Friend et al (1986) and Lee et al (1987).

TABLE 1.2 CLONED TUMOUR SUPPRESSOR GENES OR THE CANDIDATES

Gene	Full Name	Chromosome Location	Reference
RB	Retinoblastoma gene	13q14	Friend et al, 1986 Lee et al, 1987
p53		17p13	Oren et al, 1981 Oren & Levine, 1983 Finlay et al, 1989
WT1	Wilms' tumour gene	11p13	Call et al, 1990 Gessler et al, 1990
NF1	Neurofibromatosis type 1 gene	17q11.2	Cawthon et al, 1990 Wallace et al, 1990
DCC	Deleted in colorectal carcinoma gene	18q21.3	Fearon et al, 1990
MCC	Mutated in colorectal cancer gene	5q21	Kinzler et al, 1991a
APC	Familial adenomatous polyposis coli gene	5q21	Kinzler et al, 1991b Groden et al, 1991

RB is a large gene, spanning some 200 kb of genomic DNA. Twenty-seven exons have been identified, accounting for the total 4.7 kb cDNA (Hong et al, 1989). The gene is expressed in all tissues and highly conserved among a wide range of species, including mouse and human. The 4.7 kb transcript encodes a 105 kd nuclear phosphoprotein. The protein undergoes cell-cycle-specific phosphorylation (Ludlow et al, 1990). This phosphorylation apparently inactivates its function as a repressor of cell growth (reviewed in Friend, 1990). A growth inhibitor, transforming growth factor ß1 (TGF-ß1), has shown to inhibit the phosphorylation of RB protein, thus suppress cell growth (Laiho et al, 1990).

Abnormalities of RB gene are frequently found in retinoblastoma and osteosarcoma (Friend et al, 1986, 1987; Fung et al, 1987; Horowitz et al, 1990). In some patients with familial form of retinoblastoma germline structure deletion of the gene in fibroblasts can be also detected (Fung et al, 1987). The result provides a strong support for the Knudson two-hit model. Besides retinoblastoma and osteosarcoma, RB gene is frequently inactivated in some common human cancers including breast cancer (T'Ang et al, 1988; Hong et al, 1989), small cell lung carcinoma (Harbour et al, 1988; Horowitz et al, 1990), bladder carcinoma (Horowitz et al, 1990) and prostate carcinoma (Bookstein et al, 1990a). Other tumours, such as Wilms' tumour, cervical carcinoma, hepatocellular carcinoma, colorectal carcinoma and melanoma demonstrate only infrequent inactivation of RB gene (T'Ang et al 1988; Horowitz et al, 1990).

The changes of RB gene in above human cancers include homozygous internal deletion or total deletion of the gene (Fung et al, 1987; T'Ang et al, 1988). Among the 27 exons of the gene, deletion of exons 13-17 is frequently observed in retinoblastoma (Hong et al, 1989). Duplication of exons of the gene can be another structural change of the gene. All these aberrations in gene structure result in absence or truncation of the RB transcript, hence the absence of the RB protein (T'Ang et al, 1988; Horwitz et al, 1990). Among those retinoblastoma without identifiable gene structural changes there is either absence of an RB transcript or abnormal expression of the RB transcript (Fung et al, 1987). In addition to the aberrations in gene structure and expression, loss of RB gene function can occur at the protein level. RB protein can be the target of oncogene products or oncoprotein from viruses (reviewed in Weinberg, 1991).

1.2.4.2 p53

The p53 gene product was initially discovered as 53 kd cellular protein that binds to the large T antigen of simian virus 40 (SV40) (Lane & Crawford, 1979; Linzer & Levine, 1979). The gene was subsequently cloned (Oren et al, 1981; Oren & Levine, 1983). p53 was classified an oncogene, because the concentration of p53 protein was increased in tumour cell lines and SV40 transformed cells (Crawford et al, 1981; McCormick et al, 1981) and p53 cDNA could collaborate with cotransfected ras oncogene to transform normal cells (Eliyahu et al, 1984; Parada et al, 1984). However, these p53 clones were not wild-type. They were isolated

from tumour cells and were mutants (reviewed in Marshall, 1991). The wild-type, finally, was found to suppress cell transformation and tumour cell growth and identified as a tumour suppressor gene (Finlay et al, 1989; Baker et al, 1990).

The human p53 gene lies on the short arm of chromosome 17 (17p13) and comprises 393 codons (Levine et al, 1991). It has turned out that aberrations in the gene seem to be the most common genetic changes in human cancers (Levine et al, 1991; Hollstein et al, 1991). Abnormalities of p53 gene in hepatocellular, colorectal and pancreatic carcinomas will be discussed in Section 1.3, this chapter. As the rule for inactivation of a tumour suppressor gene (the two-hit model, Section 1.2.2), two separate mutational events are required for loss of function of both wild type p53 alleles. In most tumours, one p53 allele has been lost via a deletion and the other changed by a point mutation (Nigro et al, 1989). Loss of heterozygosity at 17p13 has been found in most human cancers (Lasko et al, 1991), usually representing loss of a copy of p53 gene (Baker et al, 1989).

The point mutations usually occur in one of the four hotspots along p53 gene (Vogelstein & Kinzler, 1992a). The majority of the mutations are missense, resulting in mutant forms of the protein with a dominant transforming activity (gain-of-function mutation), as shown in the early work (Eliyahu et al, 1984; Parade et al, 1984). Thus, while wild type p53 is a tumour suppressor gene, some of the mutants can indeed act as a transforming oncogene (Marshall, 1991). Wild type p53 protein has a lifetime of only 6 to 20 minutes (Levine, 1990) and the

mutant forms can accumulate in transformed or tumour cells, resulting in a steady-state concentration in such cells, detectable by immunohistochemical techniques.

Inactivation of tumour suppressor activity of the wild type p53 is an almost universal step in the development of human cancers. This has in turn stimulated an intense search for the biochemical and biological functions of p53 and the effects of mutation on these properties and a model for p53 growth control has been proposed (Vogelstein & Kinzler, 1992a): The biochemical function of p53 has been identified as a specific transcription factor. p53 protein binds DNA specifically and activates the expression of genes adjacent to a p53-binding site. The p53 biological function as an inhibitor of cell cycle progression may be mediated by the products of these activated genes. However these genes are yet to be identified (Vogelstein & Kinzler, 1992a). The transcription activity can be measured in vitro using purified p53 protein (Farmer et al, 1992). The transcriptional activity of p53 can be disrupted by gene loss, mutation or binding of the p53 protein with oncoproteins, such as large T antigen of SV40, or with products of altered cellular genes (Oliner et al, 1992).

1.2.4.3 WT1

Wilms' tumour is an embryonal malignancy of the kidney in young children, in which the Knudson's two-hit model seems to apply (Knudson & Strong, 1972). The tumour is similar to retinoblastoma in some aspects. It can occur in familial

and sporadic forms. The familial cases are associated with the WAGR syndrome (for, Wilms' tumour, aniridia, genitourinary abnormalities and mental retardation). Tumours of familial cases are most frequently bilateral. These observations correlate well with the situation in retinoblastoma. However, while in retinoblastoma the loss of the RB gene is the only consistent genetic change associated with the tumour, there are at least three putative genes implicated in the development of Wilms' tumour. Therefore the two-hit model needs to be expanded to fit the Wilms' tumour.

The three putative tumour suppressor genes for Wilms' tumour are those on chromosome 11p13 (Rose et al, 1990), 11p15 (Dowdy et al, 1991), and possibly 16q (Maw et al, 1992). The gene on 11p13 has been cloned and termed WT1 gene (Call et al, 1990; Gessler et al, 1990).

Cytogenetic data showed deletions of a region of human chromosome 11p13 in the Wilms' tumour (Franke, 1983). Loss of heterozygosity on 11p13 was also found in Wilms' tumour (Fearon et al, 1984; Koufos et al, 1984). These data suggested the rough location of WT1 gene. Rose et al (1990) further localised the candidate gene to a region of 345 kb on 11p13. The gene was then cloned from this region by Call et al (1990). Another group simultaneously cloned the same gene (Gessler et al, 1990).

The WT1 gene is expressed in a limited range of cell types, predominantly in the

kidney and a subset of haematopoietic cells. The gene product is a zinc-finger protein which is likely to be a transcription factor (Call et al, 1990). The gene is also specifically expressed in human developing embryonal kidney, genital ridge, fetal gonad and mesothelium, indicating a role of the gene in genitourinary development (Pritchard-Jones et al, 1990). In some of the Wilms' tumour cell lines the gene is missing from both copies of chromosome 11 and no mRNA expression can be demonstrated (Vile, 1992). Germline mutations in the gene have also been identified in the Wilms' tumour patients (Pelletier et al, 1991). All the evidence above confirms that the WT1 is a tumour suppressor gene, whose inactivation is involved, separately or jointly with abnormalities in other two putative Wilms' tumour genes, in the development of Wilms' tumour (Vile, 1992).

1.2.4.4 NF1

Neurofibromatosis type 1 (NF1) is an autosomal inherited disorder that affects about 1 in 3500 newborns of all ethnic groups (Stumpf et al, 1987) and is associated with an increased risk of malignancies (Riccardi & Eichner, 1986). The NF1 gene was mapped on the long arm of chromosome 17 (Barker et al, 1987; Seizinger et al, 1987), and further localised to 17q11.2 (Schmidt et al, 1987; Ledbetter et al, 1989; Yagle et al, 1990). In 1990 two groups isolated the same NF1 gene simultaneously (Wallace et al, 1990; Viskochil et al, 1990; Cawthon et al, 1990). Inactivation of this gene is the predisposing lesion for NF1. Deletions, point mutations and insertion have been found in NF1 patients, which suggests

loss of function of the tumour suppressor gene (Wallace et al, 1990; Cawthon et al, 1990). The product of this gene is related to GTPase-activating proteins or GAP (Xu et al, 1990; Ballester et al, 1990). GAPs activate the GTPase activity of the product of the cellular proto-oncogene, ras (Lemoine, 1990b). The exact mechanisms of the NF1 protein's action with ras protein are not known.

1.2.4.5 DCC

Colorectal carcinoma is the most extensively characterized among common human cancers in terms of the molecular-genetic changes (Section 1.3.2.1, this chapter). Among the seven examples of cloned tumour suppressor genes in Table 1.2, three (DCC, MCC and APC) are primarily involved in the development of colorectal carcinoma, and the role of p53 as a human tumour suppressor gene was also first identified in colorectal carcinoma (Baker et al, 1989; 1990).

Besides other genetic changes, loss of heterozygosity on the long arm of chromosome 18 occurs in more than 70 percent of colorectal carcinomas, with the common region of deletion localised to 18q21-qter (Vogelstein et al, 1988, 1989). A polymorphic DNA marker, p15-65, mapped to 18q21.3, detected a homozygous (complete) deletion of the DNA sequences in one colorectal carcinoma, and a gain of heterozygosity in another tumour (Fearon et al, 1990). These results suggested that a tumour suppressor gene for colorectal carcinoma might locate on 18q21.3. By chromosome walking starting from the marker p15-65, and

polymerase chain reaction in a novel exon-connection strategy, Fearon et al (1990) cloned the expressed sequence in the region 18q21.3. This is the DCC (deleted in colorectal carcinoma) gene.

DCC is a very large gene, encompassing more than one million base pairs (see Weinberg, 1991), with mRNA transcript size of 10 to 12 kb (Fearon et al, 1990). DCC is expressed in brain, normal colonic mucosa and several tumour cell lines, with the highest concentration in the brain. The expression in colorectal carcinoma is reduced or absent. In the study of Fearon et al (1990), only two out of 17 tested colorectal tumour cell lines expressed DCC mRNA amounts in excess of 5 percent of that produced in normal colonic mucosa. Somatic mutations within the DCC gene have been observed in a number of colorectal carcinomas, including homozygous deletion (hence the gene name, deleted in colorectal carcinoma) (Fearon et al, 1990). Loss of heterozygosity and expression of DCC gene is implicated in the progression from early to advanced stages of colorectal carcinomas from both familial adenomatous polyposis (FAP) and non-FAP patients (Kikuchi-Yanoshita et al, 1992).

Besides in colorectal carcinoma, loss of expression of DCC has been found in pancreatic adenocarcinoma (Hohne et al, 1992) and in tumorigenic papillomavirus type 18-immortalized human keratinocyte cell line transformed by carcinogen nitrosomethylurea (Klingelhutz et al, 1993). LOH at this gene locus in breast carcinoma (Devilee et al, 1991b), in gastric carcinoma (Uchino et al, 1992), and

in the tumorigenic keratinocyte cell line (Klingelhutz et al, 1993) has also been detected. These findings suggest a role of DCC inactivation in development of other tumour types.

The deduced protein from DCC shows significant homology to neural cell adhesion molecules (CAMs) (Fearon et al, 1990). CAMs bind to extracellular matrix or basement membrane components and are involved in cell-surface interactions and cell differentiation (Edelman, 1988). The homology of DCC protein to CAMs suggests that disruptions of DCC are involved in tumour metastasis and dedifferentiation (Fearon et al, 1990). LOH on 18q (deletion of DCC), along with deletion of p53 and high fractional allele loss, has been associated with distant metastases and poorer prognosis in patients with colorectal carcinoma (Kern et al, 1989; Hamilton, 1992).

1.2.4.6 MCC and APC

Most colorectal carcinomas, if not all, develop from the adenomatous polyps. Most polyps arise sporadically. The rare dominantly inherited condition, FAP, imposes a striking phenotype of multiple adenomatous polyps, hence predisposing to colorectal carcinoma. First clue of the FAP gene location was provided by Herrera et al (1986), who demonstrated a constitutional interstitial deletion on chromosome 5q13-15 or 5q15-22 in a FAP patient. Following this discovery, two groups mapped the FAP gene (or APC gene, for adenomatous polyposis coli) to

chromosome 5q21-22 (Bodmer et al, 1987; Leppert et al, 1987). LOH in this region was subsequently demonstrated in both colorectal adenomas and carcinomas from both FAP and sporadic patients (Solomon et al, 1987; Okamoto et al, 1988; Vogelstein et al, 1988, 1989; Rees et al, 1989). Thus one or more putative tumour suppressor genes at 5q21-22 appeared to be involved in the early stages of colorectal tumorigenesis in both familial and sporadic patients. The race was then on to clone tumour suppressor genes from this region. In 1991, Vogelstein and colleagues first cloned MCC (mutated in colorectal cancer) gene (Kinzler et al, 1991a) and then , simultaneously with another group, the proper APC gene (Kinzler et al, 1991b; Groden et al, 1991).

In the process of cloning MCC, Kinzler et al (1991a) found that a probe from cosmid clone 5.71, which recognised restriction fragment length polymorphism in 5q21, detected an extra band in one of the 150 tumours screened. The sequence in this tumour detected by 5.71 probe was rearranged, suggesting that a gene near the sequence in 5.71 was affected. Using exon-connection strategy, they cloned the expressed gene, MCC.

The boundary of rearrangement in the tumour detected by 5.71 was found within MCC after the gene cloned. Other two colorectal carcinomas had point mutations of the gene (Kinzler et al, 1991a). The gene has been implicated in sporadic colorectal carcinomas, and frequent LOH at the gene locus found in such tumours (Ashton-Rickardt et al, 1991; Miki et al, 1991).

MCC is expressed in a variety of tissue types including normal colon and brain. The deduced gene product may be capable of binding to and regulating a G protein. G proteins bind to guanosine nucleotides and are important intermediaries for transmitting signals in cells. The normal MCC product may suppress cell growth by interacting with a G protein (Kinzler et al, 1991a).

Although cloned from 5q21, the chromosome region encompassing the gene responsible for FAP, MCC is not the APC gene, since no germ-line mutations of MCC gene in FAP patients can be detected (Groden et al, 1991). Five months after appearance of MCC gene, APC gene was cloned by Kinzler et al (1991b) and Groden et al (1991). The two genes (MCC and APC) are separated by approximately 150 kb. APC is expressed in normal colon and other tissue types. The gene product has short similarity to MCC gene protein and may bind and regulate G protein. APC protein may also have short similarity to the ral2 gene product of yeast, which is important in the regulation of oncogene ras activity (Kinzler et al, 1991b).

Point mutations and deletions of APC gene have been detected in tumours from both familial and sporadic carcinomas, and germ-line mutations found in FAP patients (Nishisho et al, 1991; Groden et al, 1991; Joslyn et al, 1991; Cottrell et al, 1992; Miyoshi et al, 1992a). Truncated APC proteins have been detected in most of colorectal adenoma and carcinoma cell lines (Smith et al, 1993). These results confirm that APC is a tumour suppressor gene, fitting the two-hit model.

The location of germ-line mutations in the APC gene has been correlated with clinical types of FAP: germ-line mutations in FAP patients with profuse polyps are between codon 1250 and codon 1464, whereas mutation in FAP patients with fewer polyps are in the other regions of APC gene (Nagase et al, 1992). APC mutations appear to occur early during colorectal tumorigenesis and have been detected in tumours as small as 0.5 cm in diameter (Powell et al, 1992). This is in accordance with the chromosome 5 allele loss which often occurs early (Rees et al, 1989; Fearon & Vogelstein, 1990).

As in the case of the DCC gene, both MCC and APC are implicated in other types of cancers apart from the colorectal tumours. LOH at both MCC and APC loci occurs in lung cancer (Ashton-Rickardt et al, 1991; D'Amico et al, 1992), oesophageal cancers (Boynton et al, 1992), and gastric cancers (McKie et al, 1993). The APC gene is mutated in gastric (Horii et al, 1992a) and pancreatic cancers (Horii et al, 1992b).

1.2.5 Suppression of malignancy by introduction of normal chromosomes or cloned tumour suppressor genes into tumour cells

As discussed in Section 1.2.1, first evidence for the existence of tumour suppressor gene came from the cell fusion between malignant and non-malignant cells. After the successful demonstration of tumour suppressor genes' location on

chromosomes and, finally, cloning of these genes, the direct confirmation for the role of tumour suppressor genes has appeared when chromosomes containing normal tumour suppressor genes, or directly, cloned normal tumour suppressor genes are introduced into tumour cells with subsequent suppression of malignancy.

Two of the three putative tumour suppressor genes for Wilms' tumour have been localised on chromosome 11 (Fearon et al, 1984; Koufos et al, 1984; Rose et al, 1990; Dowdy et al, 1991; Section 1.2.4.3). In 1987 Weissman et al introduced a normal human chromosome 11 into G401 Wilms' tumour cell line by microcell fusion. The parental G401 could form large progressive tumours in 100 percent of inoculated nude mice. However the cells transferred with a normal chromosome 11 consistently failed to form any tumour in nude mice, thus the tumorigenicity of the hybrids was completely suppressed (Weissman et al, 1987). This experiment elegantly confirmed the presence of at least one tumour suppressor gene for Wilms' tumour on chromosome 11. WT1 was subsequently cloned from 11p13 (Call et al, 1990; Gessler et al, 1990). The second locus for Wilms' tumour is also on chromosome 11, band p15.5-p14. This is also confirmed by introduction of a chromosome 11 construct into G401. The construct, in which 11p13 is deleted but 11p15.5-p14 is retained, suppresses tumour formation when introduced to G401 (Dowdy et al, 1991). Loss of heterozygosity on chromosomes 5, 17 and 18 in colorectal carcinoma has been well established and tumour suppressor genes MCC, APC, p53 and DCC from these chromosomes, identified (Table 1.3). Introduction of normal chromosome 5 or 18 by microcell fusion into

COKFu colon carcinoma cell line clearly suppresses the tumorigenicity of the tumour cells (Tanaka et al, 1991). The growth of colorectal carcinoma cells is also suppressed by replacement of wild type p53 (Baker et al, 1990).

Inactivation of p53 gene is involved in the development of hepatocellular carcinoma (HCC) as in the other tumour types (Section 1.3.1.4.2, this chapter). Puisieux et al (1993) transfected an HCC cell line, Hep 3B, with wild type or mutant p53 expression vectors. The cells transfected with wild type p53 formed colonies six times fewer than those with mutant type, thus the wild type p53 suppressed the growth of the HCC cells (Puisieux et al, 1993).

RB is the first identified tumour suppressor gene. Its tumour suppressing activity has been extensively studied by introduction of a normal RB gene into different tumour cells, in which RB is inactivated, including retinoblastoma, osteosarcoma, prostate and bladder carcinomas (Huang et al, 1988; Sumegi et al, 1990; Bookstein et al, 1990b; Xu et al, 1991; Madreperla et al, 1991; Takahashi et al, 1991; Templeton et al, 1991; Chen et al, 1992; Muncaster et al, 1992; Goodrich et al, 1992). All these studies but one (Muncaster et al, 1992) showed suppression of tumorigenicity by normal RB. The first such study was done by Huang et al (1988) who introduced a cloned RB gene into retinoblastoma and osteosarcoma by retroviral mediated gene transfer. Expression of the exogenous RB gene changed tumour cell morphology, inhibited growth rate, reduced numbers of colonies formed in soft agar and, most stringently, suppressed tumour formation

in nude mice (Huang et al, 1988). Bookstein et al (1990b) expanded this work by introducing a normal RB gene into a common human cancer - prostate carcinoma. A prostate cell line, DU145, lost normal expression of RB gene. Restoration of normal expression by retrovirus mediated RB gene transfer in the cell line completely suppressed the tumour forming ability in nude mice, although cell growth rate in vitro was not inhibited (Bookstein et al, 1990b).

The suppression of malignancy by introduction of normal tumour suppressor gene via gene transfer requires a suitable construct of a vector and a gene to ensure a certain level of expression of exogenous gene in tumour cells, since low levels of expression of the replaced normal gene may not be sufficient enough to suppress tumorigenicity. This is probably the case of that study in which suppression of tumorigenicity by introduction of normal RB gene was not demonstrated (Muncaster et al, 1992). Artificial promoters in the gene transfer construct may not be able to ensure the required level of expression of exogenous gene, whereas the whole chromosome transfer by microcell fusion may provide a more natural regulation of an introduced gene. Banerjee et al (1992) reported a more marked inhibition of cell growth and tumorigenicity following the transfer of a chromosome 13 containing a normal RB allele than the introduction of RB cDNA by retroviral infection or plasmid transfection. In conclusion, there is little doubt that suppression of the tumorigenicity can be obtained by normal RB gene and other tumour suppressor genes, such as p53, and this may eventually provide a new approach for cancer therapy (Bookstein et al, 1990b; Baker et al, 1990;

Banerjee et al, 1992; Section 1.4, this chapter).

1.3 Molecular-genetic study of liver, pancreas and biliary tumours

1.3.1 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is a major cause of death from malignancies in the world, with a particularly high incidence in sub-Saharan Africa, China and the Far East. In Western countries, the incidence is lower. Environmental factors, including the hepatitis B virus (HBV), or hepatitis C virus (HCV) infection, and aflatoxin B_1 exposure, appear to determine the geographic clustering of the disease. Of all these risk factors, viral hepatitis is by far the most important in the development of HCC.

Recently molecular genetic changes in HCC has also been extensively studied. Hepatocarcinogenesis may be involved with a number of genetic alterations including chromosomal rearrangement, abnormalities in growth factors, oncogene activation and loss or inactivation of tumour suppressor genes. Some of these changes may be associated with HBV.

1.3.1.1 Viral hepatitis

Chronic infection with HBV confers a significant risk of HCC. Epidemiological studies show that in areas with a high incidence of HCC, such as sub-Saharan Africa, China and the Far East, HBV infection is endemic. A prospective study of 22,707 men in Taiwan showed that the incidence of HCC among carriers of the hepatitis B surface antigen was 223 times higher than among non-carriers (Beasley et al, 1981). In most HCCs with HBV infection, viral DNA is integrated into the tumour genome (Sherman & Shafritz, 1990), although the significance of this is unclear (discussed below). More direct evidence for a causal role for hepatitis viruses comes from studies of the primary liver cancers of animals. A group of animal viruses are closely related to human HBV, which affect a number of species, including woodchucks (hence the virus WHV), ground squirrels (GSHV) and ducks (DHBV). Popper et al (1987) found that in woodchucks infected with WHV, more than 90% of the animal died of HCC. This may be associated with WHV tumour integration (Shimoda et al, 1990).

In the early 1980s, HBV integration into DNA was described in nearly all cases of HCC (Bréchot et al, 1982). Later studies showed that the cellular sites of the integration were variable and the pattern of viral integration in each HCC appears to be random (Di Bisceglie, 1989; Lancaster, 1992). Integrations of HBV near the HAP gene (a member of the superfamily of steroid receptor genes) and a cyclin A gene have been reported in a few cases of HCC (de Thé et al, 1987; Wang et

al, 1990). The significance of such integration is unclear but if this results in induction of one or more of these genes, cellular activation might follow since (for example) the superfamily of steroid receptors function as regulatory proteins.

Usually only a portion of the HBV genome can be integrated into the host genomic DNA. One of the fragments commonly integrated is the HBx gene. The HBx protein product may be able to alter host gene expression and lead to the development of HCC. Kim et al (1991) inserted the HBx gene and its enhancer into the germline of mice, resulting in development of HCC.

1.3.1.2 Cytogenetic studies of HCC

Few cytogenetic studies have been carried out on HCC involving several HCC cell lines and only one primary HCC (Simon et al, 1982; Pinto et al, 1985; Simon & Knowles, 1986; Simon et al, 1990; Simon et al, 1991). Although multiple numerical and structural chromosome abnormalities were found, no HCC-specific aberration was identified. None of the cytogenetic studies were able to find a common HBV integration site. The modal chromosome numbers in the HCC cell lines studied were all above 50 (normal number: 46). Many of the abnormalities however could have arisen as secondary adaptive phenomena to in vitro culture. Cytogenetic analysis of the only primary HCC studied, which was unrelated to HBV infection, showed a modal number 46, deletions of one copy of chromosomes 13 and 16, and structural rearrangement at five chromosomal

sites: 1p22; 1p32; 5q34; 6q13-qter and 22q12 (Simon et al, 1990).

The most commonly affected chromosomes from seven HCC cell lines and the primary HCC were 1, 3, 5, 10, 11, 21 and 22 (Table 1.3). Of particular interest is that the aberrations of chromosome 1 (mostly on the short arm) were detected in all the cell lines and the primary HCC. Simon et al (1991) studied one HCC cell line and five primary HCC and found that five out of the six cases had loss of heterozygosity of 1p. Structural rearrangements of chromosome 1 have been observed in a variety of tumours, including those of childhood such as retinoblastoma and Wilms' tumour, and common adult tumours such as adenocarcinomas of colorectum, ovary, uterus, bladder and breast (Solomon et al, 1991). Loss of a gene on chromosome 1 may be involved in tumour progression.

TABLE 1.3 CHROMOSOMES COMMONLY INVOLVED IN ABNORMALITIES IN HCC CELLS[a]

Chromosome	Frequency (%)[b]	Note
1	8 (100)	Most common breakpoint: 1p21-22
3	5 (63)	
5	4 (50)	Most common region rearranged or deleted: 5q31-35
10	5 (63)	
11	7 (88)	11p: 3; 11q:4
21	5 (63)	
22	6 (75)	

a. Modified from Simon et al, 1982, Simon & Knowles, 1986, Simon et al, 1990 and Simon et al, 1991.

b. No. of abnormal cases out of seven HCC cell lines and one primary HCC.

1.3.1.3 Role of the oncogenes in HCC

Research has focused on ras and myc oncogene expression. The frequency of ras activation is low whilst it is high for myc. There also appears to be enhanced expression of erb B-2. A novel oncogene lca (liver cancer) has been reported from a single hepatocellular carcinoma.

RAS

Gu et al (1986) detected activated N-ras in three out of 10 primary HCCs and one HCC cell line using NIH3T3 cell transfection assay. Overexpression of N-ras mRNA was found in six of nine primary HCC. Rearrangement and amplification of N-ras was observed in two primary tumours. The p21 protein product was elevated in transformed 3T3 cells.

Tada et al (1990) did not demonstrate point mutations in Ha-, Ki- and N-ras at codons 12, 13 and 61 in 12 HCCs using a PCR-direct sequencing method. In contrast six of nine cholangiocarcinomas had point mutations of Ki-ras at codons 12 or 61. Other groups have found point mutations of ras genes in HCC, but the frequency is low: two out of 21 cases had a point mutation in H-ras at codon 12 (Ogata et al, 1991) and one out of six cases in Ki-ras also at codon 12 (Stork et al, 1991). In another study, two out of 21 HCCs had a point mutation in Ki-ras, one at codon 12, and the other at codon 61 and three had mutations in N-ras;

one patient had the same N-ras mutation in the surrounding cirrhotic liver tissue (Challen et al, 1991). Takada and Koike (1989) isolated 56 clones from an HCC and found that only one of the clones contained activated N-ras. A recent study showed that none of the 13 HCC studied was mutated in codons 12 or 61 of the c-Ha-ras, while two of them had p53 gene mutations (Kress et al, 1992, next section).

Zhang et al (1990a) observed overexpression of N-ras in 12 cancers and the surrounding non-tumour liver tissues; six patients with liver cirrhosis but without HCC also had slightly increased N-ras expression. Ogata et al (1991) reported a low frequency of point mutation of Ha-ras at codon 12 (2/21), allele loss (1/9), and hypermethylation (2/19); corresponding abnormalities were nearly always found in cirrhotic tissue adjacent to the HCC.

MYC and ERB B-2

Activation of myc genes may also take part in hepatocarcinogenesis. It is notable that myc and ras cooperate in transformation and that both oncogenes are often activated in a variety of tumours (Weinberg, 1989). One study showed that 12 of 14 HCCs had expression of both myc and ras proteins, in addition to that of erb B-2 (Voravud et al, 1989). Zhang et al (1990a) found overexpression in both c-myc and N-ras in all the twelve HCCs studied. Gu et al (1986) showed overexpression of N-ras in six of nine cases of HCC and c-myc in seven out of

eight cases; all the tumours were from patients with HBV infection. They proposed that ras and myc genes might be activated by HBV and chemical carcinogens. Although there is no direct evidence for this as far as HBV infection is concerned WHV integration in woodchuck livers activates both c-myc (Hsu et al, 1988) and N-myc (Fourel et al, 1990).

LCA

A putative oncogene has been isolated from an HBV positive HCC, which is named lca (for liver cancer) (Ochiya et al, 1986). This gene has a size of 10 kb at least and is located on chromosome 2. It is able to transform NIH3T3 cells, but the efficiency is very low. To date, the involvement of this gene has not been reported in any other hepatocellular carcinoma.

1.3.1.4 Tumour suppressor gene in HCC

1.3.1.4.1 Loss of heterozygosity in HCC

In hepatocellular carcinoma, allele losses have been documented on chromosomes 1, 4, 5, 8, 10, 11, 13, 16, and 17 (Table 1.4). None of these losses has yet led to identification of any specific tumour suppressor gene for HCC.

Loci on chromosomes 11 and 13 were the first to be implicated as sites for

tumour suppressor genes in HCC. Rogler et al (1985) found an allele deletion on the short arm of chromosome 11 (11p13-14) in one HCC and the loss was associated with HBV integration. Subsequently allele losses at 11p13-15 were found in 6 out of 14 cases of HCC, and at 13q12-qter in 5 out of 10 cases of HCC, all in patients with associated HBV infection (Wang & Rogler, 1988). The region of 11p13-15 contains the tumour suppressor genes for Wilms' tumour (WT1 and WT2), and 13q14 contains the retinoblastoma gene (RB), implying that these genes might be involved in hepatocarcinogenesis. Some groups (Fujimori et al, 1991; Walker et al, 1991) have also found HCC allele losses on 11p and 13q in both HBV positive and negative patients, but others have not (Zhang et al, 1990b). Kiechle-Schwarz et al (1990) studied eight patients without HBV infection using a panel of probes for 11p13-pter and did not find any allele loss in HCC tumour tissue. A study on 18 primary HCCs and one HCC cell line showed no obvious structural abnormality of RB gene (T'Ang et al, 1988). Further detailed studies are awaited to determine whether WT1, WT2, RB, or other genes on 11p and 13q are involved in the development of HCC and the extent to which these allelic losses are associated with HBV infection.

Buetow et al (1989) have also found HCC allele loss on the long arm of chromosome 4 in seven out of nine patients with HBV. Since rearrangement on chromosome 4 was found in HBV positive HCC (Pasquinelli et al, 1988), they suggested that a tumour suppressor gene for HBV positive HCC might be at 4q32. In a series of HBV positive cases of HCC from China, allele loss was found

at 17p13 near an HBV integration site 17p11.2-12 in 6 out of 10 patients (Slagle et al, 1991). These findings suggest an association between HBV infection and allele loss in HCC but none of these studies has directly compared the allelic loss in tumours with or without HBV infection. There is no evidence that HBV integration is associated with allelic losses on 17p13 (Slagle et al, 1991) or on 11p (Wang & Rogler, 1988). Recent evidence suggests that HBV status may not correlate with allele loss in HCC since simultaneous studies of HCCs from two groups of patients (HBV positive or HBV negative) have not usually revealed different patterns of allele loss (Zhang et al, 1990b; Tsuda et al, 1990; Fujimori et al, 1991; Simon et al, 1991; Walker et al, 1991).

One of the chromosome regions specifically deleted in HCC is 16q22-23. Zhang et al (1990b) reported an allele loss at 16q22 in 8 out of 14 informative cases of HCC irrespective of HBV and non-A, non-B hepatitis virus infection. The same group also found that loss of heterozygosity on chromosome 16 was associated with the progression of HCC (Tsuda et al, 1990). In six early cases of HCC, none had allele loss at 16q22-23, while loss was found in 22 out of 25 (88%) poorly differentiated and advanced HCC. The loss occurred also more frequently in HCCs of large size or with metastasis (Tsuda et al, 1990). Fujimori et al (1991) have also confirmed allele loss on 16q.

TABLE 1.4 LOSS OF HETEROZYGOSITY IN PRIMARY HEPATOCELLULAR CARCINOMA

Chromosome Region	Allele Loss[a]	HBV Infection	Liver Cirrhosis	Reference
1p32-pter	5/6	+ or -[b]	ND[c]	Simon et al, 1991
4p11-q21	8/16	+ or -	+	Zhang et al, 1990b
4p16	2/6	+ or -	+ or -	Walker et al, 1991
4q	7/9	+	ND	Buetow et al, 1989
5q	4/9	-	ND	Fujimori et al, 1991
8p	39/97	ND	ND	Emi et al, 1992
8q	4/9	+	ND	Slagle et al, 1991
10q	6/24	+ or -	ND	Fujimori et al, 1991
11p13-14	1/1	+	ND	Rogler et al, 1985
11p13-15	6/14	+	ND	Wang & Rogler, 1988
11p	6/13	+ or -	ND	Fujimori et al, 1991
11p13-pter	0/8	-	ND	Kiechle-Schwarz et al, 1990
13q12	6/9	+ or -	+ or -	Walker et al, 1991
13q14	3/11	-	+ or -	Walker et al, 1991
13q32	4/8	+ or -	+ or -	Walker et al, 1991
13q12-qter	5/10	+	ND	Wang & Rogler, 1988
16p	5/6	+	ND	Slagle et al, 1991
16q22	8/14	+ or -	+	Zhang et al, 1990b
16q22-23	36/69	+ or -	ND	Tsuda et al, 1990
16q	12/33	+ or -	ND	Fujimori et al, 1991
17p13	6/10	+	ND	Slagle et al, 1991
17p13	14/26	+ or -	ND	Fujimori et al, 1991
17p13	3/5	+	ND	Bressac et al, 1991
17p13	3/14	-	+ or -	Walker et al, 1991
17p13	12/17	+	ND	Scorsone et al, 1992
17p13	3/8	ND	+ or -	Kress et al, 1992
17p13	3/20	+	ND	Hosono et al, 1993
17p13	24/49	+ or -	ND	Nishida et al, 1993

a. No. of allele loss/No. of informative cases

b. +: presence of HBV infection or liver cirrhosis;
-: absence of HBV infection or liver cirrhosis

c. ND: no data

1.3.1.4.2 p53 tumour suppressor gene in HCC

The involvement of the p53 tumour suppressor gene in HCC has been recently documented (Tables 1.4 and 1.5). Aberrations in the p53 tumour suppressor gene seem to be the most common genetic changes in human cancers, including hepatocellular carcinomas (Levine et al, 1991; Hollstein et al, 1991). Allele loss at 17p13 (Table 1.4) frequently occurs in all subtypes of HCC, irrespective of HBV infection (Fujimori et al, 1991), liver cirrhosis (Walker et al, 1991), aflatoxin B_1 exposure (Bressac et al, 1991 and other references in Table 1.5) and ethnic origins (African: Bressac et al, 1991; Caucasian: Walker et al, 1991; Chinese: Slagle et al, 1991; and Japanese: Fujimori et al, 1991). Most of these studies were carried out with probes which identify regions near the p53 gene locus so the conclusions of these studies need to be kept in perspective. Fujimori et al (1991) suggested that there was a second tumour suppressor gene for HCC on 17p, in addition to the p53 gene, but there is no direct evidence for this at present.

Studies on p53 gene mutations have provided convincing evidence that aberrations of the p53 gene are involved in the hepatocarcinogenesis. Mutations of the p53 gene frequently occur in HCC and may be a key step during the multi-step process of HCC development. The abnormal structure and expression of the p53 gene were first observed in several human HCC cell lines by Bressac et al (1990) and subsequently confirmed by Hosono et al (1991). More exciting were two reports that linked the hepatocarcinogen, aflatoxin B_1, with the p53 gene

mutation at a particular base position (Table 1.5). Hsu et al (1991) studied 16 HCCs from patients from Qidong, China, where the contamination of aflatoxin B_1 in the food is high, and found a p53 mutation in eight of the tumours. Moreover, mutation in all eight tumours occurred at the third base position of codon 249 (wild-type: AGG coding for arginine) of the p53 gene. Seven of these mutations were G to T transversions (AGT, coding for serine). Similar mutations were also found in five of 10 cases of HCC from South Africa, an area which also has a high exposure to aflatoxin B_1 (Bressac et al, 1991).

There are two questions arising from these studies (Vogelstein & Kinzler, 1992b). Firstly, since China and South Africa also have high incidence of HBV infection in addition to the high exposure of aflatoxin B_1, are the specific mutations of the p53 gene in HCC linked with aflatoxin B_1 or HBV alone, or both? Secondly, since mutations at codon 249 are rarely found in tumours of the colon, lung, breast and other organs (Levine et al, 1991), is mutation at codon 249 specific to HCC rather than specific to a carcinogen (aflatoxin B_1)? If so, HCC from regions without aflatoxin B_1 exposure should also contain the same mutations. Recent studies have been conflicting: some indicate that the codon 249 mutations are indeed aflatoxin B_1 - specific or aflatoxin B_1 with HBV infection - specific, rather than HBV - specific or HCC - specific (Ozturk et al, 1991), but others have not (Patel et al, 1992; Buetow et al, 1992; Fujimoto et al, 1992; Hulla et al, 1993; see Table 1.5). Mutations at codon 249 were not found in HCC from areas with low exposure of aflatoxin B_1, such as Taiwan which has a high incidence of HBV

infection (Hosono et al, 1991), Australia, in patients with or without HBV infection (Hayward et al, 1991) and Japan which has a high incidence of both HBV and HCV infection (Murakami et al, 1991). The contrast in codon 249 mutations between areas of high and low aflatoxin B_1 exposure became more striking from the study of Ozturk and collaborators (1991). They studied 167 cases of HCC from 14 countries with different levels of exposure to aflatoxin B_1. Twelve tumours had the G to T mutations at codon 249 and 11 of them came from areas with high exposure. In tumours from low aflatoxin exposure areas, including those from areas with a high incidence of HBV infection, only one had codon 249 mutation. Another recent study strengthens the link between aflatoxin B_1 exposure and p53 codon 249 mutation. Scorsone et al (1992) showed that 21 out of 36 (58%) HCC from Qidong, China, an area with high aflatoxin exposure, had the G to T mutation at the third base position of p53 codon 249. Most tumours from the areas with high aflatoxin exposure were associated with HBV infection. It remains to be determined whether the p53 codon 249 mutation is induced by aflatoxin B_1 alone or whether this is due to a combination of aflatoxin and HBV.

Mutations of the p53 gene have been linked to tumour progression, such as in tumours of the colon and brain (Fearon & Vogelstein, 1990; Sidransky et al, 1992b) and may also apply to HCC (Murakami et al, 1991; Nishida et al, 1993). Deletions and mutations of p53 were observed in eight out of 22 advanced cases of HCC, but in none of the 21 early cases (Murakami et al, 1991; Table 1.5). All

the tumours in the study of Scorsone et al (1992) were from patients with advanced HCC, and may account for the high frequency of p53 point mutations and allele loss. A putative gene on chromosome 16q22-23 has been associated with HCC progression (Tsuda et al, 1990). Thus both p53 and a putative chromosome 16 gene may be involved in the progression of hepatocellular carcinoma.

Oda et al (1992) noted that the p53 mutation pattern differed from case to case, hence it could be used as a diagnostic marker for multiple HCC. They found that 17 out of 26 HCC had p53 mutations. The internodule mutation patterns were heterogeneous in 11 cases and homogeneous in 6, suggesting a multifocal origin in the former and a metastatic origin in the later (Oda et al, 1992).

TABLE 1.5 MUTATION OF p53 GENE IN HUMAN AND EXPERIMENTAL HEPATOCELLULAR CARCINOMA

Geographical Origin of Tumour	Mutation Frequency	Aflatoxin B_1 Exposure	HBV	Mutation in Codon 249	Other Codons	References
Human HCC						
Africa	1/3	High	+ or -[a]	1/1 (G→T)	ND[b]	Patel et al, 1992
Australia	0/16	Low	+ or -	0	ND	Hayward et al, 1991
Bangladesh	1/3	High	+ or -	1/1 (G→T)	ND	Patel et al, 1992
China (Shanghai etc)	2/19	High	+	2/2 (G→T)	ND	Ozturk et al, 1991
China etc	3/21	High	+ or -	1/3	2/3	Buetow et al, 1992
	6/75	Low	+ or -	1/6	5/6	
	0/11	ND	+ or -			
Egypt	1/7	Low	+ or -	1/1 (G→T)	ND	Patel et al, 1992
Germany	2/13	Low	ND	0/2	2/2	Kress et al, 1992
Japan						Murakami et al, 1991
early	0/21	Low	+ or -	0	0	
late	8/22	Low	+ or -	0	8/8	
Japan	17/26	Low	ND	3/17 (1:G→T 2:A→T)	14/17	Oda et al, 1992
Japan						Nishida et al, 1993
early (stages I+II)	3/15	Low	-	0/3	3/3	
late (stages III+IV)	14/38	Low	+ or -	0/14	14/14	
Mozambique	8/15	High	+	8/8 (G→T)	ND	Ozturk et al, 1991
Qidong, China	8/16	High	+	8/8 (7: G→T 1: G→C)	0	Hsu et al, 1991
Qidong, China	21/36	High	+	21/21 (G→T)	ND	Scorsone et al, 1992

(Conted. on next page)

TABLE 1.5 (continued)

Geographical Origin of Tumour	Mutation Frequency	Aflatoxin B_1 Exposure	HBV	Mutation in Codon 249	Other Codons	References
South Africa	5/10	High	+	3/5 (G→T)	2/5 1: 157 (G→T) 1: 286 (deletion)	Bressac et al, 1991
Transkei, South Africa	1/24	Low	+	1/1 (G→T)	ND	Ozturk et al, 1991
Taiwan, China	0/10	Low	+	0	0	Hosono et al, 1991
Taiwan, China	15/41	Low	+	4/15 (G→T)	11/15	Sheu et al, 1992
	5/20	Low	-	0/5	5/5	
Taiwan, China	3/20	Low	+	0/3	3/3	Hosono et al, 1993
UK	1/17	Low	+ or -	1/1 (G→T)	ND	Patel et al, 1992
UK	2/19	Low	+ or -	0/2	2/2	Challen et al, 1992
Vietnam	1/3	High	+	1/1 (G→T)	ND	Ozturk et al, 1991
Experimental HCC						
Monkey	1/4	Aflatoxin B_1 Induced	-	0	1/1: 175 (G→T)	Fujimoto et al, 1992
Rat	0/6	As above	-	0	0	Hulla et al, 1993

a. +: presence of HBV infection
-: absence of HBV infection
b. ND: no data

1.3.1.5 HGF and HCC

Hepatocyte growth factor (HGF) is an important regulator of liver regeneration in response to injury and may play a part in developmental liver growth (reviewed in Selden & Hodgson, 1991). It was first described in the serum of rats 24 hours after partial hepatectomy (Nakamura et al, 1984). Selden et al (1986) reported the presence of a similar substance in normal human serum, and in the serum from patients 24 hours after partial hepatectomy. Subsequently, this high molecular weight growth factor was purified and its nucleotide and amino acid sequence established (Nakamura et al, 1989; Miyazawa et al, 1989). Apart from its role in liver regeneration, HGF has a variety of other activities (Strain & Neuberger, 1992). The receptor for HGF was identified as the c-met proto-oncogene product (Bottaro et al, 1991; Naldini et al, 1991), which is a membrane spanning receptor with tyrosine kinase activity.

HGF is a potent mitogen for mature hepatocytes (Nakamura et al, 1984). Serum HGF is elevated in patients with diseases associated with the development of hepatocellular carcinoma, such as chronic hepatitis and liver cirrhosis (Tsubouchi et al, 1991), HGF was, therefore, initially considered a likely candidate for autocrine stimulation of HCC. Contrary to expectation, HGF was found to have low expression in primary HCC, no expression in 8 HCC cell lines, and even to inhibit growth of an HCC cell line (Selden et al, 1991; Shiota et al, 1992). Selden et al (1991) found that the expression of HGF mRNA was lower in HCC tissue

compared to adjacent liver tissue. Shiota et al (1992) introduced an albumin-HGF expressing vector into an HCC cell line. Expression of HGF in HCC cells inhibited their growth in vitro. Tumorigenicity of HGF-expressing HCC cells in nude mice was also decreased. The authors speculated that the elevated HGF expression in patients with diseases predisposing to HCC might actually protect against HCC development. They suggested that the inhibitory effect of HGF on HCC might be related to some transformation event, perhaps alteration of the HGF receptor (Shiota et al, 1992).

HGF has been found to be identical to a previously identified factor, known as scatter factor (Weidner et al, 1991). Scatter factor dissociates and increases the motility of epithelial cells and may be involved in tumour invasiveness and progression. It is of interest that both the HGF gene (Laguda et al, 1991) and certain genes responsible for tumour invasiveness and metastasis (Collard et al, 1987) have been independently assigned to chromosome 7. The role of HGF in tumour development , invasion and metastasis in various tissues, including liver, clearly needs to be investigated further.

1.3.2 Colorectal-liver metastases

1.3.2.1 Primary colorectal tumours

The molecular-genetic alterations in carcinogenesis in the colorectum is particularly well studied (Stanbridge, 1990b). This is partly because of the accessibility of this organ through colonoscopy. The multisteps of tumorigenesis from normal mucosa through hyperplasia, benign adenoma to carcinoma in situ and finally metastases are readily identifiable. Genetic changes from chromosome 5 gene alteration, ras gene mutation and chromosomes 18 and 17 gene losses have been well characterized. A genetic model for colorectal tumorigenesis has been proposed on the basis of the above changes (Figure 1.1; Fearon & Vogelstein, 1990).

Among oncogenes, ras mutation has been reproducibly identified in both carcinomas and large size adenomas of colorectum (Bos et al, 1987; Forrester et al, 1987; Vogelstein et al, 1988). Bos et al (1987) detected ras mutation in over one third of colorectal carcinomas using a combination of DNA hybridization analyses and tissue sectioning techniques. Most mutations were at codon 12 of K-ras. Similarly Forrester et al (1987) found mutations at codon 12 of K-ras in about 40 percent of colorectal carcinomas using RNase A mismatch cleavage analysis. In colorectal adenomas, ras gene mutations occurred in 58 percent of those larger than 1 cm, in only 9 percent of those under 1 cm size, suggesting that

ras mutation may not be the initial step in colorectal tumorigenesis, although it may be an early event (Vogelstein et al, 1988; Fearon & Vogelstein, 1990). Since ras mutation occur in a high percentage of relatively early stage of colorectal tumours, the detection of such mutations in stool DNA may become a means to screen the early colorectal tumours (Sidransky et al, 1992a). Apart from ras, mutations of other oncogenes (eg, myc, cyclins and neu/HER2) have also been found in colorectal carcinomas (Hamilton, 1992; Leach et al, 1993).

Loss of heterozygosity on chromosomes 5, 17 and 18 has been well established in colorectal carcinomas and abnormalities of tumour suppressor genes (MCC, APC, p53 and DCC) have been identified in this type of tumour. Chromosome 5 allele loss usually occurs in early stage of tumour, including adenomas and carcinomas; chromosome 18 allele loss, in both carcinomas and advanced adenomas, and chromosome 17 allele loss, only in carcinomas (Vogelstein et al, 1988). In an allelotype study, LOH in colorectal carcinomas was found on almost every chromosome (Vogelstein et al, 1989). Besides chromosomes 5, 17 and 18, other chromosomes frequently involved are chromosomes 8 (Vogelstein et al, 1989; Emi et al, 1992; van der Bosch et al, 1992), chromosome 22 (Okamoto et al, 1988; Vogelstein et al, 1989) and chromosome 14 (Young et al, 1993). A putative tumour suppressor gene on 14q may be associated with tumour progression in colorectum (Young et al, 1993). The frequency of allele loss has been correlated with prognosis of colorectal carcinomas (Vogelstein et al, 1989; Hamilton, 1992). The more allelic losses are present in a particular tumour, the poorer prognosis

the patient will have. Frequent LOH on chromosomes 17 and 18 may be associated with a high potential of metastases (Kern et al, 1989).

Colorectal carcinoma is among the human cancers in which p53 is well studied. Baker et al (1989) found that common region of LOH on chromosome 17 in colorectal carcinomas was on 17p13, where p53 gene locates, indicating that one copy of p53 gene is lost in these tumours. Another allele of p53 is frequently mutated (Baker et al, 1989; Nigro et al, 1989). Introduction of wild type p53 gene into colorectal carcinoma cells can suppress cell growth in vitro, and in vivo mutations abrogate this suppressing ability (Baker et al, 1990).

Recently a new colon cancer gene has been mapped on chromosome 2p15-16 (Peltomäki et al, 1993). The nature of the gene has not been identified, and possibly it belongs to a new class of cancer genes other than oncogenes and tumour suppressor genes (Marx, 1993; Aaltonen et al, 1993). This gene, tentatively called "FCC", for familial colon cancer, may maintain DNA replication accuracy. Its mutation may cause genetic instability, such as microsatellite alterations throughout the genome (Aaltonen et al, 1993; Thibodeau et al, 1993). Table 1.6 summarizes the genes altered in colorectal carcinomas.

TABLE 1.6 GENES ALTERED IN COLORECTAL CARCINOMAS (Marx, 1993)

Gene	Chromo-some	Tumours with mutations	Class	Action
K-ras	12	30-50%	Oncogene	Intracellular signaling molecule
Cyclins	Various	4%	Oncogene	Help regulate cell cycle
neu/HER2	17	2%	Oncogene	Growth factor receptor
myc	8	2%	Oncogene	Regulates gene activity
MCC	5	>70%	Tumour suppressor	Regulates a G protein
APC	5	>70%	Tumour suppressor	Unknown
DCC	18	>70%	Tumour suppressor	Cell adhesion molecule
p53	17	>70%	Tumour suppressor	Regulates gene activity
"FCC"	2	~15%	?	Maintains DNA replication accuracy

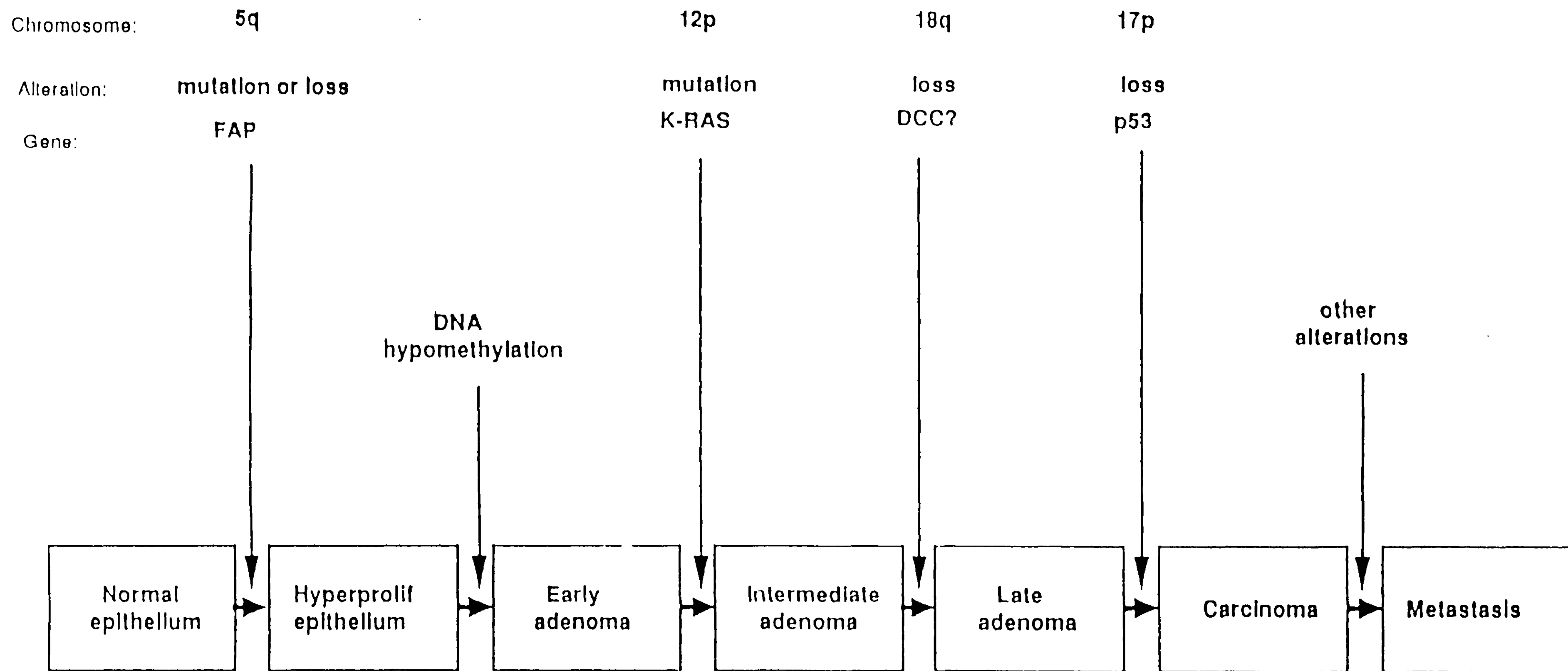


Figure 1.1 A genetic model for colorectal tumorigenesis

(Fearon & Vogelstein, 1990)

1.3.2.2 Colorectal-liver metastases

Colorectal-liver metastases are one of the major causes of death from colorectal carcinomas and one of the most common secondary liver tumours. Although the primary colorectal carcinomas have been well studied, the molecular-genetic background for colorectal liver metastases has not been thoroughly investigated. Distant metastasis may be associated with LOH on chromosomes 17 and 18 in primary colorectal carcinomas (Kern et al, 1989; Hamilton et al, 1992), but it has yet to be identified whether p53 and DCC genes are also involved in metastasis.

A gene related with tumour metastasis, named nm23, has been identified by its reduced expression in highly metastatic K-1735 murine melanoma cell lines and in human breast cancers with nodal metastases (Steeg et al, 1988; Bevilacqua et al, 1989; Hennessy et al, 1991). Two such genes have been identified subsequently and have been mapped on 17q21 (Leone et al, 1991; Backer et al, 1993). nm23 genes may be antimetastatic genes (Hennessy et al, 1991; Backer et al, 1993). Although the association of nm23 expression with lower incidence of metastasis of breast cancers seems clear (Bevilacqua et al, 1989; Hennessy et al, 1991), the role of nm23 genes in colorectal metastasis has been yet fully identified. Allelic deletions of nm23 genes have been observed in colorectal carcinoma (Leone et al, 1991), and the deletions have been associated with distant metastasis of this cancer (Cohn et al, 1991), however the mRNA and protein products are expressed equally in colorectal carcinomas with both high and low metastasis potential (Haut

et al, 1991; Lacombe et al, 1991). No study on the role of nm23 in colorectal metastasis to liver has been reported so far.

In colorectal-liver metastases, overexpression of ras oncogene has been found (Habib & Wood, 1986). There is no reported exclusive study on allele losses in colorectal liver metastases.

1.3.3 Carcinoma of the pancreas

Carcinoma of the pancreas (CaP) is an increasingly common disease for which there is no effective cure except in the rare case when resection is possible (Williamson, 1988). The aetiology is unknown, but recently much progress has been made in understanding of the molecular pathogenesis of this tumour, especially in terms of oncogene activation and abnormalities of growth factors (Lemoine & Hall, 1990). Relatively few studies in cytogenetics and tumour suppressor genes have been performed in CaP.

1.3.3.1 Cytogenetic abnormalities in CaP

About 10 cytogenetically abnormal primary CaP have been reported (Casalone et al, 1987; Teyssier, 1987; Johansson et al, 1989, 1991, 1992; Scappaticci et al, 1992). Most of the studies are performed in exocrine CaP. Chromosomal numerical change is common, with modal number ranging from 43 to 80 (Johansson et al,

1992). The structural changes, including deletions, reciprocal translocations and premature chromosome condensation, involve almost every chromosome, with no CaP-specific marker found (Casalone et al, 1987; Johansson et al, 1992). Non-random aberrations have been seen on chromosomes 1p, 3p, 6q, 8p and 17p (Johansson et al, 1992). In the study in which six primary exocrine CaP with chromosomal rearrangements were reported, four had deletions and unbalanced translocations on chromosome 6q and the authors speculated that deletions of 6q might reflect loss of tumour suppressor genes on the long arm of this chromosome, since 6q deletions are common in other tumours, such as those of salivary gland and ovary (Johansson et al, 1992 and references therein). Another frequent change is the structural rearrangement on chromosome 1p. Four out of the 6 CaP in the above study had deletions and translocations involving 1p, all leading to loss of chromosomal material distal to 1p32. Changes on chromosome 1, and also those on 3p, 8p and 17p, are frequently seen in other solid tumours and they may represent secondary changes in tumour progression (Johansson et al, 1992). Deletions of 17p may involve p53 gene loss.

A case of CaP has been reported in a patient with the "5q-syndrome", that is refractory macrocytic anaemia with deletion of the long arm of chromosome 5 (Berrebi et al, 1984). It is not known from this single observation whether CaP developed coincidently with the 5q-syndrome or the 5q deleted patient had a genetic predisposition to CaP.

In some CaP cell lines, both structural and numerical alterations were seen on chromosome 7p, where the gene for the epidermal growth factor (EGF) receptor has been mapped. These changes might be correlated with the enhanced expression of EGF receptor found in these cell lines (Korc et al, 1986).

Chromosomal abnormalities of endocrine pancreatic tumours have been seen in only one report, in which two pancreatic islet tumours, an insulinoma and a glucagonoma, were studied (Scappaticci et al, 1992). Both tumours were from patients with multiple endocrine neoplasia type 1 (MEN1). The insulinoma had a modal number peak at 84 chromosomes. Deletions of chromosomes 1, 2, 7, 16, and 17 were found. Chromosome 1p was deleted in all cells analyzed. The glucagonoma had loss of chromosomes 14, 16, 19 20, 2, 22 and X in different cells, with no consistent trend (Scappaticci et al, 1992).

1.3.3.2 Oncogene activation in CaP

The role of ras oncogene activation in carcinoma of the pancreas is extensively studied. Almoguera et al (1988) reported that twenty-one out of 22 (95%) exocrine CaP contained c-K-ras genes with mutations at codon 12, detected by RNase A mismatch cleavage analysis after PCR amplification of DNA from frozen tumour specimens and single 5 μm sections from formalin-fixed, paraffin-embedded tumour tissues. This incidence is the highest among human tumours (Lemoine, 1990a,b). Using the same method they detected a high frequency of

c-K-ras mutations in fine needle aspirates (FNA) from CaP (Shibata et al, 1990). They studied FNA from 47 patients with pancreatic mass. Of the 36 patients with pancreatic adenocarcinoma, mutations of c-K-ras oncogene at codon 12 were detected in 18 of 25 (72%) with malignant cytologies, 2 of 8 (25%) with atypical cytologies and 0 of 3 with benign aspiration cytologies. The remaining 11 patients without pancreatic carcinoma did not have mutant c-K-ras genes, that is, no false positives occurred (Shibata et al, 1990). The results suggested that detection of mutant c-K-ras genes in FNA from CaP might be a very useful aid in the differential diagnosis of CaP which is often problematic (Lemoine, 1990a).

In accordance with above reports, a high frequency of mutations at codon 12 of c-K-ras gene has been found in CaP in other studies (Smit et al, 1988; Grünewald et al, 1989; Nagata et al, 1990). Nagata et al (1990) found that thirty-five out of 38 cases had such mutations. Lemoine et al (1992b) recently reported that such mutations may occur at a relatively early stage of CaP. Frequent mutations of c-K-ras codon 12 were found in preinvasive pancreatic carcinomas (5 out of 6, 83%), as well as in invasive ones (12 out of 16, 75%). Similarly such mutations have also been found at an early stage in pancreatic carcinogenesis in an animal model (Cerny et al, 1992). These results strongly indicate an essential role of mutations of c-K-ras in the development of CaP.

The role of other oncogenes in CaP is less clear. Amplifications of c-myc (Yamada et al, 1986) and overexpression of erbB-2 and erb B-3 (Hall et al, 1990;

Lemoine et al, 1992c) have been found in CaP. The products of erb B-2 and erb B-3 oncogenes belong to the type 1 growth factor receptor family (Lemoine et al, 1992a). Their abnormalities in CaP are discussed in next section.

1.3.3.3 Abnormalities in growth factors in CaP

Aberrations of type 1 growth factor receptors and their ligands in CaP are well studied in Dr Lemoine's Laboratory at the Hammersmith Hospital. The type 1 growth factor receptors consist of the epidermal growth factor (EGF) receptors, c-erb B-2 and c-erb B-3 (Lemoine et al, 1992a). The EGF receptors are overexpressed in almost all CaP (Lemoine et al, 1992d). This is in accordance with the result in CaP cell lines (Korc et al, 1986). EGF receptors have four ligands identified so far, including EGF itself and transforming growth factor alpha (Lemoine et al, 1992a), both of which are overexpressed in CaP cells (Barton et al, 1991a; Korc et al, 1991). Thus these overexpressed receptors and ligands may result in an autocrine loop with an increased proliferation signal in CaP. Overexpression of c-erb B-2 and c-erb B-3 has also been found in CaP (Hall et al, 1990; Lemoine et al, 1992c). c-erb B-2 has several ligands, but ligands for c-erb B-3 are not identified yet (Lemoine et al, 1992a). It is not known whether these ligands are also overexpressed in CaP.

The role of another type of growth factors and their receptors, fibroblast growth factors (FGFs) and FGF receptors, is also under study in Dr Lemoine's

Laboratory. There are 7 FGFs and 4 FGF receptors (Lemoine et al, 1992a). Preliminary results have shown that they may also form potential autocrine loops in CaP cells (Leung et al, 1992).

1.3.3.4 Tumour suppressor genes in CaP

Studies on tumour suppressor genes in CaP are less comprehensive than those in other common tumours, such as hepatocellular carcinoma and colorectal carcinoma. Loss of heterozygosity on chromosome 11q13 in both sporadic and familial pancreatic endocrine tumours, related to multiple endocrine neoplasia type 1, has been reported (Teh et al, 1990; Bale et al, 1991). The MEN 1 gene has been mapped on 11q13 (Larsson et al, 1988; Bystrom et al, 1990). LOH in this region may imply that the MEN 1 gene is a tumour suppressor gene.

Abnormalities of the p53 tumour suppressor gene in CaP have been recently documented. In the study of Barton et al (1991b), overexpression of p53 was found in 13 of 22 (60%) frozen primary human CaP, 28 of 124 (23%) human pancreatic cancer samples in formalin-fixed, paraffin-embedded tissue blocks, and 7 of 13 (54%) pancreatic cancer cell lines. Point mutations in exons 5, 6, 7 and 8 of p53 gene in CaP cell lines and mutations at codons 246, 249 and 273 in formalin-fixed, paraffin-embedded CaP material were identified (Barton et al, 1991b). Similarly, Ruggeri et al (1992) found overexpression of this gene in 4 out of 10 (40%) primary CaP and 6 out of 7 (86%) CaP cell lines. Point mutations

were found in all 7 cell lines. These findings indicate that p53 alterations may be an important feature in CaP as in the other human tumours.

The involvement of RB gene in CaP is not clear. In one report (Ruggeri et al, 1992), absence of RB protein expression was found at a low frequency in CaP cell lines and primary CaP. Recently, loss of expression of DCC and mutations of APC in CaP have been reported (Höhne et al, 1992; Horii et al, 1992b), but LOH at APC and MCC loci is not frequent (Neuman et al, 1991; McKie et al, 1993).

1.3.4 Cholangiocarcinoma

Cholangiocarcinoma develops from the epithelium of the intrahepatic bile duct system. One view is that it arises from the same stem cell as hepatocellular carcinoma (Sell & Dunsford, 1989). In contrast to HCC, cholangiocarcinoma is reported as occurring less frequently in most parts of the world. Little is known of the genetic changes of cholangiocarcinoma.

1.3.4.1 Cytogenetic studies of cholangiocarcinoma

There has been no reported cytogenetic study as yet on primary cholangiocarcinoma but two cholangiocarcinoma cell lines studied showed a number of abnormalities (Storto et al, 1990). The PCI:SG 231 and RPMI-7451 cell lines had a modal chromosome number of 65 and 67 respectively. Almost all

chromosomes had numerical and/or structural aberrations in one or both cell lines. Numerical abnormalities common to both lines included trisomies 2, 5, 11, and 20 (three copies of each chromosome). Chromosomes 1, 5, 7, and 12 were commonly involved in structural abnormalities. Three consistent chromosomal breakpoints common to both lines were 7q22, 8p11, and 12p11-12. The significance of these aberrations is obscure. The change on chromosome 12, however, might be involved in oncogene activation (see below).

1.3.4.2 Oncogenes in cholangiocarcinoma

The expression of the proteins encoded by the ras, myc and erb B-2 oncogenes has been observed in most cholangiocarcinomas (Voravud et al, 1989). Of 63 cholangiocarcinomas, 59 (95%) expressed p62 c-myc, 47 (75%) expressed p21 c-ras, and 46 (73%) expressed p190 c-erb B-2. The frequency of Ki-ras oncogene point mutations is much higher in cholangiocarcinoma than in HCC (Tada et al, 1990). Out of nine cholangiocarcinomas, five had point mutations of Ki-ras at codon 12, one at codon 61 (6/9, 67%), whereas none of the 12 cases of HCC had mutations of Ki-ras at codons 12, 13, or 61 (Tada et al, 1990). In another study, Ki-ras codon 12 point mutations were found in four of six (67%) bile duct carcinomas, but only in one of six cases of HCC (16%) (Stork et al, 1991). In two cholangiocarcinoma cell lines, one of the common chromosome breakpoints was 12p12 (Storto et al, 1990) which might lead to deregulated transcription of Ha-ras since this is known to be on 12p12.1-pter (Barbacid, 1987). These findings

suggest that ras activation particularly Ki-ras may be important in the development of cholangiocarcinoma.

1.3.4.3 Tumour suppressor gene in cholangiocarcinoma

There has been no reported study on tumour suppressor genes in cholangiocarcinoma as yet.

1.4 Gene therapy for cancer

Our understanding of cancer has led to the development of a unified working hypothesis of cancer as follows: (1) most or all human cancers are genetic diseases and (2) they result from the expression and/or failure of expression of specific genes (Friedmann, 1992). Once such a concept is accepted, the possibility of gene therapy for cancer arises. Indeed, this new therapeutic approach is predicted as an effective treatment for cancer within 10 years (Lemoine & Sikora, 1993).

Gene therapy has begun in humans for other genetic diseases (Miller, 1992). An indirect gene therapy involving injection of patients' lymphocytes transduced by human cytokine genes has been successfully used in treating some human melanoma (Rosenberg, 1992). Another approach is virally directed enzyme prodrug therapy (VDEPT). This is based on a vector being expressed specifically

in cells of a particular tissue or specifically in tumour cells but not in the normal cells (Gutierrez et al, 1992). The first example of VDEPT was reported by Huber et al (1991) for the treatment of hepatocellular carcinoma. HCC often expresses the gene for alpha-fetoprotein (AFP) but normal liver cells and most other cell types do not express this gene. They fused the AFP gene promoter to the varicella-zoster virus thymidine kinase (TK) gene. TK is an enzyme that can convert the prodrug 6-methoxypurine arabinonucleoside (araM) to the cytotoxic metabolite adenine arabinonucleoside triphosphate (araATP). By utilizing such retroviral vector araM was anabolized to araATP in HCC cells, providing a selective cytotoxic effect (Huber et al, 1991).

Cancer is basically a disorder of the combination of activation of oncogenes and inactivation of tumour suppressor genes. Perhaps the most exciting gene therapy for the future is the direct manipulation of oncogenes or tumour suppressor genes, for example, targeting the mutant oncogenes by antisense nucleic acids and restoring tumour suppressor gene functions (Lemoine & Sikora, 1993). As discussed in Section 1.2.5, replacement of normal tumour suppressor genes can effectively suppress the tumorigenicity. This discovery may not be merely an academic interest. Rather it would provide the best hope for cancer treatment by restoration of tumour suppressor gene functions (Friedmann, 1992).

Many problems must be solved before the direct gene replacement can be used clinically. The first one is which gene we can use. p53 is a good candidate, but

this gene alone may not be sufficient (Friedmann, 1992). Tumour specific genes should be more appropriate, but such genes are not available for most of the tumours. Another obstacle is how to deliver the right gene into a right place. Progress has been made for constructing gene delivery systems (Miller, 1992). Recently, such systems have been reported for hepatocytes (Ferry et al, 1991; Cristiano et al, 1993). There are further problems. After the exogenous gene is transferred to target cells, the quantity of protein produced should be controlled. Overexpression could adversely affect normal tissue. Such control methods are not available yet (Lemoine & Sikora, 1993).

Despite these problems, current molecular-genetic knowledge of carcinogenesis has promised that gene therapy will become at least one of the effective approaches for cancer management (Gutierrez et al, 1992; Friedmann, 1992; Lemoine & Sikora, 1993).

1.5 Aims of the thesis

Loss of tumour suppressor genes plays a pivotal role in carcinogenesis. Such loss can be detected by searching for the consistent loss of heterozygosity in tumour DNA. To date no specific tumour suppressor genes for tumours in hepatopancreatobiliary system has been cloned. When the work for this thesis started in 1989, few if any studies on LOH in these tumours had been reported.

Therefore the objective for this thesis was an extensive searching for consistent LOH in hepatocellular carcinoma, other common or uncommon liver tumours, carcinoma of the pancreas and cholangiocarcinoma.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Most chemicals were purchased from Sigma Chemicals Ltd., UK and Merck Ltd., UK. The ingredients for bacterial culture medium were obtained from DIFCO Laboratories Ltd., UK. Phenol was supplied by RATHBURN Chemicals Ltd, UK.

All buffers comprised "Analar" or biochemical grade reagents and, where appropriate, were sterilised by autoclaving at 15 lbs psi 121°C for 30 minutes.

2.1.2 Enzymes

Restriction endonucleases, large fragment polymerase DNA I, T_4 DNA ligase, and calf intestinal alkaline phosphatase were obtained from GIBCO.BRL Ltd., UK. Ribonuclease A (RNase A), deoxyribonuclease I (DNase I) and protease were supplied by Sigma Chemicals Ltd., UK. Taq polymerase, together with the GeneAmp™ DNA amplification reagent kit, was purchased from Perkin Elmer Cetus for polymerase chain reaction (PCR).

2.1.3 Radionuclides

$\alpha^{32}P$ dATP and dCTP were obtained from Amersham International, Amersham, UK.

2.1.4 Patients and samples

Eighty-two patients were analysed. The sources of patients were the Royal Free Hospital, London (n=28); the Hammersmith Hospital, London (n=45); the Old Court Hospital, London (n=4) and the Nozha Hospital, Cairo (n=5).

Surgical biopsies from the tumour and, if possible, non-tumour tissue were snap frozen in liquid nitrogen at the time of operation. Peripheral blood from most patients was obtained preoperatively and before any blood transfusion. Non-tumour tissues and/or peripheral blood lymphocytes were used as sources of constitutional DNA. Tissue was stored at -70°C and blood was stored at -20°C until DNA extraction. Tumour samples were examined histologically by pathologists to confirm the type of tumour present. The distribution of patients is listed in Table 2.1.

TABLE 2.1 DISTRIBUTION OF PATIENTS ANALYSED

Diagnosis	Number
Liver Tumours	**53**
HCC with liver cirrhosis	12
HCC without liver cirrhosis	9
Colorectal-liver metastases	19
Fibrolamellar carcinoma	5
Hepatocelular adenoma	6
Sarcomatoid liver carcinoma	2
Carcinoma of the Pancreas	**15**
Endocrine tumours	2
Exocrine tumours	13
Cholangiocarcinoma	**14**
Total	**82**

2.1.5 DNA probes

These were obtained by Dr JDA Delhanty and Mr NA Habib from various sources (the suppliers were gratefully acknowledged in the "Acknowledgement") and arrived either as purified plasmid DNA or as agar stabs. Agar stabs were grown up immediately and a large scale plasmid preparation carried out. Those received as plasmid DNA were transformed into a suitable E. coli strain prior to culture.

2.1.6 Primers

"Big" T_3 and T_7 primers of bluescript for PCR of cDNA library were the kind gifts from Dr H Hurst, ICRF Oncology, Hammersmith Hospital.

2.1.7 Plasmid vectors, cDNA library and genomic DNA library

The plasmid vector bluescript KS^+, the normal human liver cDNA library and the host bacterium E coli XL-1 blue were purchased from Stratagene, Cambridge, UK. Human chromosome 5 genomic library and the host bacterium LE392 were purchased from American Type Culture Collection (ATCC), USA. This library was constructed from flow-sorted chromosomes (Deaven et al, 1986).

2.1.8 Other materials

Hybond-N hybridisation membranes were obtained from Amersham International, Amersham, UK; Duralon-UV™ membranes from Stratagene, Cambridge, UK; GeanClean II kits from Bio 101 through Stratech Scientific Ltd, UK. Standard λ phage DNA, DNA ladders and random primered DNA labelling kits were purchased from GIBCO.BRL Ltd., UK.

2.2 Methods

2.2.1 DNA extraction

2.2.1.1 Extraction of DNA from Blood

Frozen blood was thawed slowly on ice and put into 50 ml Falcon tubes (10 ml/tube). To this were added 40 ml of ice-cold lysis buffer and mixed well. The mixture was left on ice for 10 minutes to ensure complete lysis of blood cells. Centrifugation was then performed at 4°C and 2800 rpm for 15 minutes. The supernatant was poured off gently and the pellet was resuspended in 5 ml PK buffer. To this were added 0.5 ml of 10% SDS and 200 µl of proteinase K (10 mg/ml), and then the total volume was pooled to 10 ml with PK buffer. The mixture was incubated for 2 hours at 55°C or overnight at 37°C with very gentle

agitation. After incubation with proteinase K the mixture was allowed to cool to room temperature and was added 1 ml (1/10th volume) of 3M NaAc (sodium acetate). An equal volume of buffered phenol/chloroform was added and gently mixed by inversion for 15 minutes in the Falcon tube. The tube was spun for 15 minutes at 20°C at 2800 rpm and the upper phase (the aqueous phase) was carefully removed to a fresh Falcon tube, with the interface being left behind. The extraction of the aqueous face was repeated with phenol/chloroform 2 or 3 times or until there was no interphase left. Finally the aqueous phase was extracted with chloroform to remove traces of phenol. The supernatant was removed to a clean tube and the DNA was precipitated with two volumes of very cold 100% ethanol. DNA was coiled onto a closed sterile Pasteur pipette and washed in 70% ethanol. DNA was allowed to air dry and dissolved in 0.5 - 1 ml of TE buffer (Appendix 1.I) at 4°C.

The DNA solution was pipetted gently with a wide bore 1 ml pipette tip to ensure a homogeneous solution. Then the optical density (OD) of 20 µl diluted in 1 ml of TE buffer was read at 260 nm (OD_{260}) in the spectrophotometer. The DNA concentration in µg/ml was given by multiplying the reading by 2500. Also the OD was read at 280 nm (OD_{280}). The ratio of OD_{260} : OD_{280} should be equal to or larger than 1.8.

2.2.1.2 Extraction of DNA from tissue

About 0.5 gram of tissue was weighed and washed in tissue buffer. The tissue was cooled in a mortar and pestle with liquid nitrogen, and ground into powder in liquid nitrogen. The powder with liquid nitrogen was transferred into a cold homogeniser. About 2 ml of tissue buffer were added to it as soon as the liquid nitrogen had evaporated. The sample was gently homogenised on ice (5-6 strokes) and transferred to a clean 15 ml Falcon tube. The homogeniser was rinsed twice with 2 ml of tissue buffer each and the rinsed buffer added to the Falcon tube. To the tube was added 20% SDS to a final concentration of 0.5% and proteinase K to a final concentration of 100 µg/ml. The mixture was incubated at 55°C for 2 hours or at 37°C overnight. The sample was cooled down to room temperature, and 1/10th volume of 3M NaAc was added. From this point on the procedure was continued as above.

The solutions and buffers needed for DNA extraction are given in Appendix 1.II.

2.2.2 Probe preparation

2.2.2.1 Preparation of competent cells

A rapid method was used for the preparation of competent cells (Chung & Miller, 1988). An appropriate strain of E coli (eg, XL-1 Blue for plasmid bluescript) was grown to the early log phase (OD_{600}=0.3-0.6) in LB broth (Appendix 1.III), then pelleted by centrifugation (1,000 x g for 10 minutes at 4°C). The bacterial cells

were then resuspended in 1/10th volume of transformation and storage buffer (TSB) (Appendix 1.III) at 4°C, and incubated on ice for about 10 minutes. They were then split into 100 µl aliquots in sterile eppendorf tubes. The aliquots were snap frozen in a dry ice/ethanol bath and stored at -70°C.

2.2.2.2 Plasmid transformation

A 100 µl aliquot of frozen E coli was thawed slowly on ice. To this were added 100 pg of plasmid DNA. The cells were incubated on ice for 5-30 minutes. Heat shock was not necessary. Next, cells were grown to permit expression of the antibiotic resistance gene. 0.9 ml of TSB with 20 mM glucose was added, and the cells incubated at 37°C with shaking (225 rpm) for 60 minutes and plated on an antibiotic - containing agar plate. The plate was inversely incubated at 37°C overnight. Positive colonies were then picked and a large scale plasmid preparation carried out.

2.2.2.3 Large scale preparation of plasmid

A 10 ml aliquot of LB broth was inoculated with one positive colony and grown up during the day. This culture was used to prepare several 1 ml glycerol stocks (15% v/v autoclaved glycerol) for long term storage at -70°C and the rest used to seed 2 x 250 ml flasks of LB broth with appropriate antibiotics. The cultures were grown overnight at 37°C with aeration.

When the culture was well saturated the following day they were harvested at 7,000 rpm for 10 minutes at 4°C. The supernatant was discarded completely and the pellets resuspended in a total volume of 13 ml of solution I. No lysozyme was needed. Then 26 ml of freshly made solution II were added and the mixture incubated on ice for 5 minutes. 13 ml of solution III were added and well mixed. (Details of solutions I, II and III are given in Appendix 1.IV.) The mixture was incubated again on ice for 10 minutes. Following lysis, centrifugation was carried out at 12,000 rpm for 10 minutes. The plasmid containing supernatant retained and the pellets containing cell debris discarded. The volume of supernatant was measured and 0.6 volume of propan-2-ol was added to precipitate the DNA. Following incubation at room temperature for 10 minutes, the precipitate was pelleted at 12,000 rpm for 10 minutes at room temperature, drained thoroughly and dissolved in 3ml of 50mM Tris-HCl pH 8.0, 1mM EDTA to neutralise acid. The total volume was then made up to 4.2 ml. Next 4.7g of CsCl were added and dissolved. Then 100µl ethidium bromide (10mg/ml) were added. The whole sample was spun at 8,000rpm for 10 minutes. The supernatant was put into a Quickseal tube (Beckman). The final weight including tube was 9.3g. The tube was spun in a Sorvall ultracentrifuge at 50,000 rpm at 25°C overnight (17-18 hours).

After spinning, the plasmid bands were visible without use of ultraviolet light as most of the ethidium bromide pelleted. The plasmid bands were removed with syringe. The total volume was made up to 3ml with TE buffer. The ethidium

bromide was removed by extraction with equal volume (3ml) of isoamyl alcohol three times. The plasmid sample was then diluted with TE buffer to 6ml of total volume. DNA was precipitated with 2 volumes of absolute ethanol. After cooling at -20°C, DNA was pelleted by spinning at 10,000 rpm, 4°C for 10 minutes. The pellet was dissolved in TE and 1/10th volume of 2M NaCl added. DNA was precipitated with absolute ethanol again, washed with 70% ethanol, air dried and dissolved in 0.5-1ml TE. The OD_{260} was read and the plasmid DNA stored at -20°C.

2.2.2.4 Preparation of inserts

20µg of plasmid DNA were digested with 100 units of the appropriate restriction enzymes (Appendix 2) and electrophoresed on a 0.9% agarose gel in TAE buffer to separate vector and insert fragments. Both the gel and buffer contained ethidium bromide. The gel was viewed under UV light at 354nm and the required fragment isolated. The DNA was then purified using GeneClean II kit (Bio 101). Briefly, the DNA band was excised from agarose gel (run in TAE buffer) under UV light and weighed. To the tube 3 volumes of NaI stock solution were added and the tube was incubated at 50°C for about 5 minutes until all agarose gel was dissolved. Five to 10 µl of GLASSMILK suspension were added and mixed. The mixture was incubated on ice for 5-10 minutes, shaken to mix at every 2-3 minutes interval. The GLASSMILK bound with DNA was pelleted by spinning 5 seconds and washed 3 times with NEW WASH (NaCl/ethanol/water). The DNA was

eluted by TE buffer. The yield of insert DNA was estimated by running an aliquot on a 1% minigel containing ethidium bromide (100µg/ml) against known amount of a standard λ phage DNA sample.

2.2.3 Restriction digestion of genomic DNA samples

In general restriction digest mixtures were set up as follows:

5 µg genomic DNA (in TE)

4 µl appropriate reaction buffer (10x working strength)

20 units restriction enzyme

Sterile ddH_2O to 40 µl.

The mixture was spun for a few seconds in a microcentrifuge and incubated at 37°C (65°C for Taq I). For Msp I digestion, 50 units instead of 20 units of enzyme were used for each digest and the mixture incubated at room temperature overnight.

Following digestion, 4 µl of sucrose blue loading buffer were added to stop the reaction and the digests run at 20-25V for 10-24 hours on 0.9% (for Msp I and Bgl II digests: 0.6%) agarose gel in TAE running buffer (Appendix 1.I). Both the gel and the buffer contained ethidium bromide at a concentration of 100 µg/ml. Digested DNA samples were run alongside an appropriate molecular weight marker such as Hind III - cut λDNA or the yeast derived 1 kb ladder. The gel was visualised under short wave UV and photographed using an orange filter.

2.2.4 DNA transfer (Southern blotting)

The gel was capillary blotted onto Hybond N or Hybond N+ hybridisation nylon membranes (Amersham) according to manufacturer's specifications. For solutions used see Appendix 1.V.

2.2.5 Radiolabelling of DNA probes for Southern hybridisation

Probes for Southern hybridisation consisted of purified insert DNA labelled to high specificity by the random hexanucleotide method (Feinberg & Vogelstein, 1983) and α^{32}P dCTP (3000 Ci/mmol).

50-100 ng of insert DNA were boiled for 10 minutes to melt the duplex as it must be single stranded for good results. The labelling mixture comprised the following:

11 µl labelling solution buffer (Appendix 1.VI)

1 µl 10mg/ml BSA

1-6 µl insert DNA

Volume made up to 20 µl with sterile ddH_2O.

The mixture was quenched on ice for 2-10 minutes to anneal the random hexanucleotides. 3 - 6 units (1µl) Klenow (GIBCO. BRL) were added along with 30 -50 µCi α^{32}P dATP or dCTP. The tube was spun for several seconds to mix thoroughly and incubated for 3 - 16 hours at room temperature in a lead pot. To

remove the unincorporated labelled nucleotide, the reaction mixture was expanded to 100 µl with 3 x SSC and spun through a G-50 sephadex column (Pharmacia).

2.2.6 Southern hybridisation

2.2.6.1 Prehybridisation and hybridisation

Filters were prehybridised in the prehybridisation solution (Appendix 1.VII) at 65°C for at least 1 hour before addition of the radiolabelled probe DNA. To the probe labelled as above was added sufficient sheared salmon sperm DNA (Sigma Type III) (10mg/ml) to give a concentration of 100 µg/ml in the final reaction mix. The probe and salmon sperm DNA were boiled for 5 minutes and quenched on ice for 5 minutes and then added to the prehybridisation mixture. Hybridisation was carried out at the same temperature for 12 - 24 hours (usually overnight) and the solution was then poured off and retained for future use.

2.2.6.2 Filter wash and autoradiography

Filters were washed in 2 x SSPE (Appendix 1.I), 0.1% SDS at room temperature, then in 1 x SSPE, 0.1% SDS at 65°C for 15 -30 minutes. If required, a further more stringent wash in 0.1 x SSPE, 0.1% SDS was carried out at 65°C to reduce severe background. The filters were then blotted dry, wrapped in cling film and

autoradiographed at -70°C using preflashed Fuji RX-L X-ray for between 1 and 14 days.

2.2.7 Interpretation of autoradiographs: determination of loss of heterozygosity (allele loss)

The detection of chromosomal allele loss depended upon demonstrating a difference in restriction fragment length polymorphism (RFLP) between tumour and non-tumour (constitutional) DNA. If two alleles appeared as two or more separate bands in the resultant autoradiograph of the constitutional DNA, the patient was considered "informative" or heterozygous for the particular RFLP probe. Complete deletion or great loss of intensity of one or more bands in the tumour DNA indicated loss of heterozygosity, or allele loss. If, however, only one band was shown for the constitutional DNA in the resultant autoradiograph, the patient was homozygous for the particular RFLP probe, or scored as "non-informative" because the detection of allele loss was impossible (Figure 2.1). The loss of band intensity was confirmed by examination of the autoradiographs with densitometry. A cutoff level of 50% or more of allele intensity was considered as evidence of LOH. Attention was paid to lymphocyte DNA when it was used as constitutional DNA, since in some cases the intensity of the larger allele of such DNA would be much denser than that of the smaller one (for example, see Figure 6.2, p167). In these cases non-tumorous tissue DNA was always used. All the results were blindly checked by at least one of other investigators.

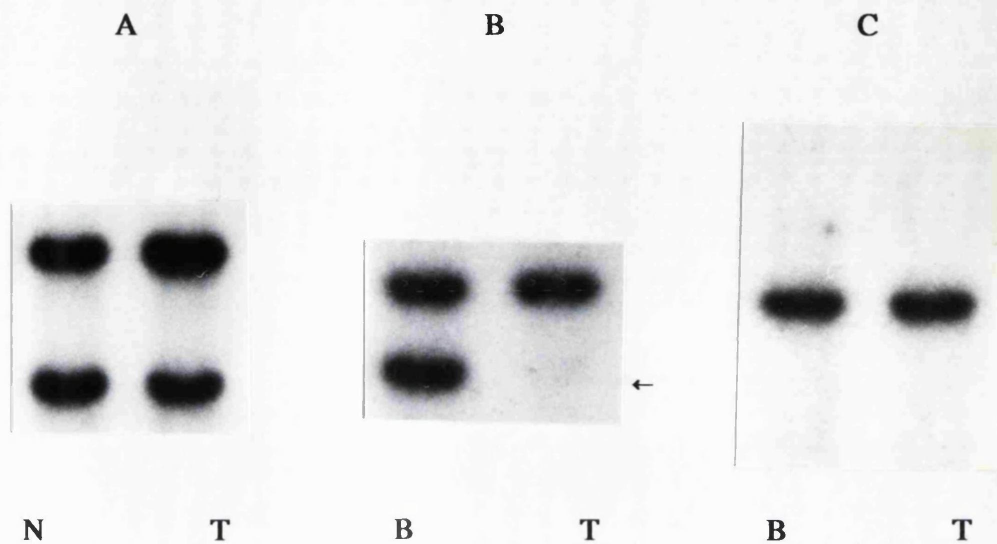


Figure 2.1 Interpretation of autoradiographs. Patients A and B were heterozygous since there were two bands in the constitutional DNA in both patients. There was no band loss in Patient A's tumour DNA, hence no allele loss. The Patient B was shown an allele loss in his tumour DNA (indicated by the arrow). There was only one band in Patient C's constitutional DNA (also the tumour DNA), thus he was homozygous, or non-informative. B = blood lymphocyte DNA; N = non-tumour tissue DNA; T = tumour DNA.

2.2.8 Preparation of plating stock and titration of λ phage DNA libraries

2.2.8.1 Plating stock

A single colony of the appropriate bacterial host (eg, XL-1 blue or LE 392) was inoculated into 15-30 ml LB broth supplemented with 0.2% maltose and 10 mM $MgSO_4$ in a sterile flask, and incubated overnight (or a few hours) with shaking at 37°C. Cells were harvested by centrifugation at 2,000rpm for 10 minutes. The supernatant was carefully removed and the pellet gently resuspended with 7-15 ml of 10 mM $MgSO_4$ without vortexing. The cells were then diluted to OD_{600} = 0.5/ml with 10 mM $MgSO_4$.

2.2.8.2 Titration of the phage libraries

A few serial dilutions of the phage were made with SM buffer (Appendix 1.III). To each 5ml-Falcon tube were added 200 µl of OD_{600} = 0.5/ ml plating stock. The tubes were then inoculated with diluted phage, and incubated at 37°C for 15 minutes in order for the phage to stick on the host cells. 3 ml of 48°C top agar were added to each tube and plated on a 100 mm LB plate supplemented with 0.2% maltose, 10 mM $MgSO_4$ and 50 µg/ml ampicillin. After hardening at room temperature for 10 minutes, the plates were incubated overnight at 37°C. The number of plaques was counted and the concentration (pfu/ml) of the library was

determined based on the dilutions.

2.2.9 Cross screening of two DNA libraries

2.2.9.1 DNA recovery from λ cDNA library (plate lysate technique)

From titre of the library, the amount required for 10^6 colonies was taken from the library. To this was added 1 ml of OD_{600} = 0.5 /ml plating stock and incubated at 37°C for 15 minutes. The host-phage mixture was then added to 7.5 ml of 48°C top agar and plated on a 150 mm LB plate supplemented with 0.2% maltose, 10 mM $MgSO_4$ and 50 µg/ml ampicillin. The plate was invertedly incubated 6-7 hours at 37°C until very small plaques were visible all over plate. The plate was chilled at 4°C for approximately one hour. 10-15 ml of SM buffer were added and the plate shaken gently overnight at 4°C. The supernatant was harvested into a 15ml-Falcon tube. A few drops of chloroform were added and stored at 4°C.

To make DNA, 2 x 500 µl aliquots of the above supernatant was taken and transferred to 2 microfuge tubes. To each tube, 500 µl of 20% PEG/2M NaCl were added and left on ice for at least one hour. The tubes were spun at full speed in microfuge for 10 minutes. The supernatant was removed and the pellet in each tube resuspended in 200 µl of TE. To this were added 200 µl of PK stop (Appendix 1.III) and incubated at 37°C for 30 minutes. The DNA was extracted with phenol/chloroform once and chloroform alone once. To the aqueous phase

were added 40 µl of 3M NaAc and 800 µl of absolute ethanol to precipitate DNA. The tube was vortexed and spun at full speed at microfuge for 10 minutes. The pellet was washed with 70% ethanol and air dried. The DNA was resuspended with 200 µl of TE and OD_{260} checked.

2.2.9.2 cDNA library insert amplification by polymerase chain reaction

The PCR method used was that described by Saiki et al (1985, 1986, 1988). When using this method, it was essential that Gilson tips and microcentrifuge tubes were prepared, autoclaved and handled wearing gloves so that any extraneous DNA contamination was minimised. All reactions were carried out using a Hybaid automated heating block.

For PCR amplification of human liver cDNA library, the primers were the big T_3 and T_7 (30 bp) promoters of bluescript in the phage vector Uni-ZAP™ (Appendix 2). The template DNA was the inserts made from 10^6 λ clones as described above. The reaction mixture was set up as follows:

10x PCR buffer (Appendix 1. VIII)	10 µl (1x)
dNTP (1.25 mM each nucleotide)	16 µl (200 µM each)
Big T_3 (20 µM/µl)	1 µl
Big T_7 (20 µM/µl)	1 µl
DNA template	0.5 µg

ddH_2O made to 99.5 µl

The reaction mixture was spun for a few seconds to mix and incubated at 94°C for 6 minutes for initial denaturation. Then 2.5 units of Taq polymerase (0.5 µl) were added to the reaction tube and the reaction mixture was overlayed with 100 µl mineral oil to prevent evaporation. The reaction mixture was submitted to 35 cycles of PCR (denaturing at 94°C for 30 seconds, annealing at 58°C for 3 minutes and elongation at 70°C for 2 minutes). Upon completion, the paraffin oil was carefully removed and a 5 µl aliquot was run on a 1% agarose minigel in TBE buffer (Appendix 1.I) at 50V for about 30 minutes to visualize the PCR products. The rest of the reaction was stored at -20°C.

2.2.9.3 Purification and oligolabelling of PCR products

Before labelling, the PCR products were purified using GeneClean II kit (Bio 101). 80 µl of the reaction products were taken and 240 µl of NaI stock solution were added. 40 µl of GLASSMILK suspension were added and mixed. The mixture was incubated on ice for 5-10 minutes, shaken to mix at every 2-3 minutes interval. The GLASSMILK bound with DNA was pelleted by spinning 5 seconds and washed 3 times with NEW WASH (NaCl/ethanol/water). The DNA was eluted by TE buffer. The yield of DNA was estimated by running an aliquot on a 1% minigel containing ethidium bromide (100µg/ml) against known amount of a standard λ phage DNA sample.

The oligolabelling of the purified PCR products and the removal of the unincorporated radiolabels were performed as described in Section 2.2.5 this chapter.

2.2.9.4 Plaque lifting from chromosome 5 λ genomic DNA library

The plaque lifting was to transfer DNA from chromosome 5 λ phage plaques grown on LB plates onto Nylon membranes, which were hybridised with radiolabelled PCR products of normal human liver cDNA library.

A. Plating of phages

150 mm LB agar plates supplemented with 0.2% maltose and 10 mM $MgSO_4$ were prepared and stored at 4°C for more than two days before use. According to the titre of the phage library, the phage stock was diluted so that each tube contained an amount of about 50,000 pfu, to which were added 600 µl of plating stock (see 2.2.8.1). The tubes were incubated at 37°C for 15 minutes to ensure the infection of phages to bacterial cells. After added 7.5 ml of 48°C top agar, the tube was rolled between palms of the hands gently but quickly and poured onto the above plate. The plate was swirled immediately to give an even layer. The top agar was allowed to harden at room temperature for about 15 minutes. The plates were incubated in an inverted position overnight at 37°C and chilled inverted for 2 hours at 4°C. The chilling procedure was to help prevent the top agar from

sticking to the Duralon-UV™ membranes.

B. Plaque lifting

Duplicate plaque lifts were made from each plate. The membranes used were Duralon-UV™ (Stratagene, Cambridge) and handled always with gloved hands. Before performing plaque lifting, the membranes of choice were labelled with a pen. Each membrane was labelled with plate number and designation of the order of lift from each plate. For example, the two duplicate filters to be lifted from plate one were labelled 1A and 1B respectively. To ensure future orientation, the first membrane to be lifted was marked with a series of asymmetrically placed dots approximately 5mm from the membrane edge. For example, if the circular membrane was a clock face, one dot would be at 1 to 3 o'clock, two dots would be placed between five and seven o'clock and three dots would be marked between 9 and 12 o'clock (Figure 10.3, Chapter 10).

The first membrane was placed onto the surface of the plate without trapping air bubbles. The filter was gently curved in half and its centre touched to the centre (line of diameter) of the plate and the wetting action allowed to pull the remainder of the membrane onto the plate. Once the membrane was thoroughly wet, holes through the pen marked dots and into the agar were punched with a 16 gauge syringe needle. The plate was then held up to a light and on the underside of the plate markers were made to note the position of the needle

holes.

After 2 minutes, the first membrane was carefully removed with forceps and placed into the denaturing solution (see next section). The next membrane carefully laid onto the top agar and allowed to wet. Again the plate was held up against light (membrane side forward) and the marked dots were used as a guide to repeat the needle holes through the membrane. After 5 minutes the membrane was removed carefully and immersed in denaturing solution.

C. Membrane treatment

Each membrane was denatured for 2 minutes at room temperature by submerging the filter in a solution of 1.5 M NaCl-0.5 M NaOH. Any clinging agar residue was gently rubbed off from the membranes with gloved fingertips to decrease the background after hybridisation. The membranes were denatured separately to ensure that the entire surface was available to the denaturing solution.

The membranes were neutralised for five minutes by submerging them in a solution of 1.5 M NaCl-0.5 M Tris-HCl pH8.0 at room temperature. Again the membranes were not allowed to stick together.

The membranes were rinsed briefly in 0.2 M Tris-HCl, pH7.5-2xSSC at room temperature, blotted on filter paper and baked between sheets of filter paper for

2 hours at 80°C under vacuum.

The master plates were parafilmed and stored upside down at 4°C for subsequent phage isolation.

2.2.9.5 Hybridisation of lifts with labelled PCR products

The following prehybridisation solution mix was used:

10% Dextran Sulfate

1% SDS

1M NaCl

ddH_2O to required volume.

All the reagents were combined and warmed to 65°C. The baked plaque lifts (nylon membranes) were then added to the mix and prehybridised at 65°C for at least one hour before addition of the freshly radiolabelled PCR products (Section 2.2.9.3). To the labelled PCR products (probe) was added sufficient sheared salmon sperm DNA (Sigma Type III) (10mg/ml) to give a concentration of 100 µg/ml in the final reaction mix. The probe and salmon sperm DNA were boiled for 5 minutes and quenched on ice for 5 minutes and then added to the prehybridisation mixture. Hybridisation was carried out at the same temperature for 24 hours. Both membranes of the duplicate lifts from the same plate were hybridised together. Membrane wash and autoradiography were done as described previously (Section 2.2.6.2). For future orientation, washed membranes

were stabilised on a Whatman filter paper and the orientation of the membranes were marked with a radioactive pen before the X-ray films were put down for autoradiography. The signals from radioactive markers on the films were used to define the positions of positive clones after autoradiography. Two autoradiography films of the duplicate membranes from a certain plate were labelled Film A and Film B, for example, Film 1A and Film 1B according to the labels made when plaques were lifted (Section 2.2.9.4.B). If both Film A and Film B showed hybridization signals of a same plaque, this plaque was identified as a positive clone. The position of a positive plaque on the master plate was decided according to the positions of the six dots made before lifting (Section 2.2.9.4.B).

2.2.9.6 Three rounds of screening

Three rounds of cross-screening of the two libraries were carried out. The positive clones of the first screening were picked with the wide ends of sterile pasteur pipette out of the master plates (Section 2.2.9.4) according to the autoradiography results above. Each picked plaque was transferred to 1ml SM buffer (Appendix 1.III) plus a drop of chloroform to kill the bacterium, shaken at room temperature for 1-3 hours to allow phages to release from agar block into SM buffer and then stored at 4^{0}C. For the second round of screening, the above supernatant was diluted. One µl of this was added to 1000 µl SM buffer. Twenty µl of this diluted phage solution were used for plating out on a 90 mm LB agar plate supplemented with 0.2% maltose and 10 mM $MgSO_4$. The plating of

phages, duplicate plaque lifting and membrane treatment were done as described in Section 2.2.9.4. The lifts (membranes) were prehybridised and hybridized with freshly radiolabelled PCR products of human liver cDNA library inserts as done in the above section.

Positive clones from the second round were identified as above and picked with the sharp ends of sterile pasteur pipettes. Each plaque was transferred to 1 ml SM buffer plus a drop of chloroform, and shaken and stored as described above. Twenty µl of a one to 1000 dilution were used for the third screening. This was done as the second round. After the third round the purified positive plaques were picked. All clones were analysed as described in the next section.

2.2.10 Identification of positive clones

2.2.10.1 Small scale preparation of λ clones and preparation of inserts

Phages from a positive clone were grown on a 90 mm LB agar plate. A fresh plaque was taken into 1 ml SM buffer, and shaken at room temperature for 1 hour. To this were added 200 µl of plating stock. The phages and cells were incubated 15 minutes at 37°C to ensure the infection. The mixture was then added to 20 ml LB broth supplemented with 10 mM $MgSO_4$ and 0.2% maltose in a 50 ml-sterile flask. The flask was shaken vigorously overnight at 37°C. In the

following morning, the cell debris was evident. 200 µl of chloroform were added and the flask returned to shaker for a further 15 minutes to kill the residual bacterial cells. The cell debris was removed by spinning 3,000 rpm 15 minutes at room temperature. The supernatant was transferred to a fresh tube and 20 µl of 10 mg/ml RNase A and 20 µl of 10 mg/ml DNase I to digest bacterial sequences. The digestion was performed at 37°C for 30 minutes. To the tube was added one tenth volume (2ml) of 10 x protease K buffer (Appendix 1.III) and 150 µl of 10 mg/ml protease K. This large amount of enzyme was necessary. The tube was incubated one hour at 37°C. DNA was precipitated by adding 0.6 volume (12ml) of isopropanol. The tube was left on ice for 10 minutes and spun 3,500 rpm 20 minutes at 4°C. The supernatant was discarded and the precipitate resuspended in 500 µl of TE buffer.

The DNA was then extracted twice with phenol/chloroform and once with chloroform alone (all chloroform used was chloroform:isoamylalcohol = 24:1). To the aqueous phase were added 1/10th volume of 3M NaAc and 2 volumes of cold absolute ethanol. The tube was left at -70°C for 10 minutes and spun in microfuge at full speed for 10 minutes. The DNA was washed with 70% ethanol, air dried and dissolved in 100 µl of TE buffer.

The DNA was digested with appropriate restriction endonuclease (EcoRI for chromosome 5 λ phage clones) at 37°C for 3 hours. The digests were separated on 0.9% agarose gel, run in TAE buffer with the yeast derived 1 kb ladder.

Under UV light, the insert band was photographed, cut and purified by GeanClean kit (Bio 101) as described in Section 2.2.2.4, this chapter. The size of inserts was identified according to the molecular weight marker run alongside. The purified insert was used to ligate with bluescript KS^+ vectors.

2.2.10.2 Preparation of bluescript KS^+ vectors

Five µg of bluescript DNA were digested with appropriate restriction enzymes at 37°C for 3 hours. To this was added 1 µl of calf intestinal alkaline phosphatase (CIAP) (Gibco.BRL). The digests were incubated at 37°C for 30 minutes and another 1 µl of CIAP was added and incubated at 37°C for further 30 minutes. To the digests an equal volume of phenol/chloroform was added and vortexed thoroughly for at least 1 minute. The aqueous phase was extracted with phenol/chloroform again. One tenth volume of 3M NaAc was added to the aqueous phase and DNA was precipitated with 2.5 volume of absolute ethanol. The sample was cooled at -70°C for 30 minutes and spun in a microfuge for 10 minutes. The pellet was washed with 0.5ml of 70% ethanol and dried in heat block at 65°C for 5-10 minutes. The DNA was dissolved in 100 µl and the concentration was 30-50 ng/µl.

2.2.10.3 Ligation and large scale insert preparation

A typical ligation was set up as follows:

50-100 ng vector DNA (bluescript)

2-300 ng insert DNA (chromosome 5 clones from λ clones)

10 μl of 5 times ligase buffer

2-5 units T_4 DNA ligase (GIBCO.BRL)

Make up to 50 μl with sterile distilled water

The mixture was incubated at 15°C overnight, and half of the ligation was used for transformation.

The preparation of competent cells, transformation, preparation of large scale of plasmid and insert preparation were done as described in the Section "Preparation of probe". Selection of recombinant colonies (positive colonies) of the bluescript clones was based on white/blue colour difference. The recombinant clones were white colonies on the bacterium strain XL1-blue on the Xgal gel plate and could be identified with ease (for detailed discussion, see Chapter 10).

2.2.10.4 Preparation of single copy DNA sequences

From the large scale preparation of plasmid clones (chromosome 5 clones that were expressed in human liver), sufficient amount of insert DNA was obtained. These inserts were digested with those restriction enzymes, such as PstI, HindIII and BamHI, which cut DNA sequences infrequently. The digests were run on a 0.9% agarose in TAE buffer (Appendix 1.I) and visualized by UV light. Most

clones were cut into 2 or more fragments by those enzymes and seen as different bands on the agarose gel. DNA transfer was performed as described in Section 2.2.4. Total human genomic DNA (the source used: normal blood lymphocyte DNA) was radiolabelled as previously described (Section 2.2.5). The radiolabelled total human genomic DNA was used as a probe to hybridize the above separated fragments from the digestion of the insert DNA. The prehybridisation and hybridization were carried out as described in Section 2.2.6. After autoradiography, the band(s) which did not hybridize with the total human genomic DNA was the single copy DNA sequence. The insert DNA of the original plasmid clone was digested with those infrequently cutting enzymes again and separated on agarose gel. The equivalent non-hybridised DNA band(s) was cut from the gel and purified by GeneClean II (Section 2.2.2.4). This single copy DNA sequence was subcloned to plasmids following ligation with bluescript KS^+ as described in the above section (2.2.10.3).

2.2.11 Statistical analysis of data

The significance of the relationship between frequency of allele loss and clinical parameters was checked by the Fisher's exact test (Bland, 1987). The significance of the difference in the frequency of allele loss between different types of tumours was tested by a standard method for comparison of proportions (Bland, 1987).

Chapter 3 Loss of Heterozygosity in Classic Hepatocellular Carcinomas

3.1 Introduction

As discussed in Chapter 1, hepatocellular carcinoma (HCC) is a major cause of death from malignancy in the world, with a particularly high incidence in the Far East and Africa where hepatitis B virus infection, established as an aetiological agent, is common. In Western countries the incidence is lower but increasing. Most HCC are beyond radical resection when detected, and all other forms of the currently available therapies are rarely beneficial.

Most HCC are associated with chronic HBV infection. The HBV genome is integrated into the host DNA and many studies have shown detailed sites of integration looking for a consistent pattern and/or changes which might activate oncogenes. No consistent pattern has been found (Di Bisceglie, 1989; Lancaster, 1992). Loss of specific segments of chromosomal DNA, however, has been shown in HBV positive HCC including regions on chromosomes 4, 11 and 13 (Buetow et al,1989; Wang et al, 1986). Loss of heterozygosity on chromosome 16 and other chromosomes in HCC in both HBV positive and negative patients has been also reported (Chapter 1), but none of these studies has led to identification of HCC-specific tumour suppressor genes yet (Chapter 1).

Most patients with HCC in Western countries have no HBV infection, and little is known about the genetic changes. These patients can present with or without liver cirrhosis and they have different prognoses. Therefore the study in this chapter was performed to search for the consistent allele loss in HCC and to establish whether there were different patterns of allele loss in HCC with or without liver cirrhosis.

3.2 Patients and analysis

Twenty-one patients with HCC (12 with and 9 without liver cirrhosis) were studied. Of the 9 patients with HCC without cirrhosis, 7 were HBV negative and two HBV positive. Ten patients with HCC and cirrhosis were HBV positive and two HBV negative. All these patients had their tumours localised to the liver. None had extrahepatic spread as judged by pre-operative imaging and laparotomy findings. All patients underwent either liver resection or liver transplantation. None of these patients had a tumour of the fibrolamellar type. Surgical biopsies from the tumour and non-tumour liver tissue were snap frozen in liquid nitrogen at the time of liver resection. Lymphocytes from peripheral blood obtained preoperatively and before any blood transfusion were also used as a source of normal DNA. Tissue was stored at -70°C until DNA extraction. None of the patients received chemotherapy or radiotherapy before surgery and tumour samples were examined histologically to confirm the type of tumour present.

Among the 9 patients with HCC without liver cirrhosis, Patient WS, a 74-year-old man, had both primary and recurrent HCC. In 1989, he was noted to have a 10 cm mass in the left lobe of the liver on follow-up ultrasonography for colorectal carcinoma. This was confirmed by computed tomography (CT). Percutaneous biopsy showed it to be a well differentiated hepatocellular carcinoma instead of a secondary from colorectal carcinoma. Laparotomy revealed a large lesion

involving segments II, III and IV of the liver, and a left hepatectomy was performed. Definitive histology showed complete resection of the tumour. A follow-up CT scan, performed 15 months later, revealed a 6 cm nodule in segment VI of the liver, with no extra-hepatic metastasis. These findings were confirmed at laparotomy and local resection of segment VI was performed. Histology showed this to be a recurrent hepatocellular carcinoma. Serum neurotensin and vitamin B_2 binding assay were normal throughout. His hepatitis B virus status, determined by blood assay and Southern analysis of hepatic tissue DNA, was negative. At both operations, biopsies from the tumour and non-tumour liver tissue were collected and snap frozen in liquid nitrogen.

All patients' viral hepatitis status was determined by analysis of serum for hepatitis B markers and by Southern analysis of liver tissue DNA using the HBV genome probe pEco63.

DNA extraction and hybridisation were done as described in Chapter 2. In this study 28 probes for chromosomes 1, 2, 3, 4, 5, 7, 9, 11, 12, 13, 14, 16, 17, 18 and 20 were used (Table 3.1). Loss of heterozygosity was defined as described in Chapter 2.

3.3 Results

3.3.1 Overall allele loss in HCC

Table 3.1 shows the overall pattern of allele loss, or loss of heterozygosity (LOH), in DNA from 21 HCC compared to non-tumour DNA. The analyses showed an informative pattern in 284 of 411 Southern blots (heterozygosity: 69.1%). Overall LOH was present in 42 / 284 (14.8%).

In the 9 patients with HCC without liver cirrhosis a high frequency of LOH only occurred in the regions 5q35-qter and 17p13 (Tables 3.1). The probe for the terminal region of long arm of chromosome 5 (λMS8, 5q35-qter) was informative in 6 cases (HBV positive: 2; HBV negative; 4) and all showed LOH (Figure 3.1). Of the 7 patients informative with the probe p144-D6 for the short arm of chromosome 17 (17p13) 5 showed LOH. Another probe for 17p13, pYNZ.22, detected LOH in 3 out of 8 informative cases.

In the 12 patients with HCC and liver cirrhosis LOH was found on chromosomes 1q, 5p and 17p in about half of the informative cases (Tables 3.1). Out of 10 informative patients 7 showed LOH at 17p13 by the probe p144.D6. Three out of 7 informative cases had LOH detected by pYNZ (17p13). Nine cases in this group were heterozygous with the probe λMS8 (5q35-qter), but none of them exhibited LOH. Of the nine cases, seven were HBV positive and the remaining

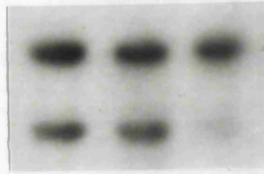
two were not. One patient showed LOH on 5q21-22, but was non-informative for λMS8 (5q35-qter).

TABLE 3.1 LOSS OF CHROMOSOMAL HETEROZYGOSITY IN HUMAN HEPATOCELLULAR CARCINOMA

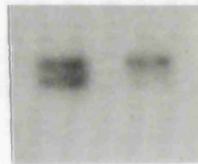
Probe	Locus	Enzyme	HCC without Cirrhosis (n=9)	HCC with Cirrhosis (n=12)	Reference for Probes
λMS1	1p33-35	HinfI	1/7*	0/10	Wong et al, 1987
PB3	1q21-23	MspI	0/3	1/5	Scott et al, 1985
λMS32	1q42-43	AluI	0/6	5/11	Wong et al, 1987
P5G1	2q33-35	TaqI	0/5	0/7	O'Connell et al, 1987
H3H2	3p21	HindIII	0/4	0/5	Caritt et al, 1986
HS3	3q12	HindIII	0/3	0/5	Naylor et al, 1984
F47.3	4q11-13	HaeIII	0/4	0/4	Murray et al, 1983
cMS621	5p	HinfI	0/4	3/4	Armour et al, 1990
ECB27	5q21	BglII	0/4	1/4	Vareso et al, 1989
L5-71.3	5q21 (MCC)	MspI	0/5	0/8	Kinzler et al, 1991a
FB54-D	5q21 (APC)	MspI	0/6	0/9	Kinzler et al, 1991b
YN5.48	5q21-22	MspI	0/3	1/3	Nakamura et al, 1988a
v-fms	5q33-35	EcoRI	0/0	0/0	Xu et al, 1985
λMS8	5q35-qter	HinfI	6/6	0/9	Wong et al, 1987
λMS31	7pter-q22	HinfI	0/4	1/7	Wong et al, 1987
pλg3	7q31.3-qter	HinfI	0/3	1/7	Wong et al, 1987
EFD126.3	9q34	PvuII	0/4	2/8	Nakamura et al, 1987a
H-ras	11p15	BamHI	0/2	0/3	Krontiris et al, 1985
pMS51	11q13	HaeIII	0/4	0/6	Armour et al, 1989
λMS43	12q24.3-qter	HinfI	1/5	0/7	Wong et al, 1987
cMS626	13q	AluI	0/5	0/6	Armour et al, 1990
cMS627	14q	AluI	0/5	0/5	Armour et al, 1990
3'HVR	16p13.3	PvuII	0/5	0/6	Higgs et al, 1986
pulB1148	16q22.1	TaqI	0/3	0/3	vander Straten et al, 1983
p144-D6	17p13	RsaI	5/7	7/10	Kondoleon et al, 1987
pYNZ.22	17p13	RsaI	3/8	3/7	Nakamura et al 1988b
cMS440	18q	HaeIII	0/3	0/2	Armour et al, 1990
cMS617	20q	AluI	0/2	1/3	Armour et al, 1990

* No with allele loss / No of informative cases.

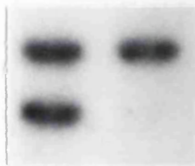
WS



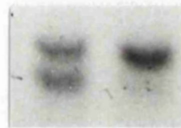
RH



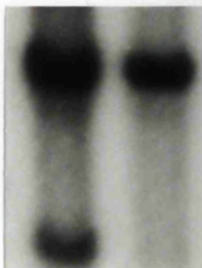
AA



SH



GA



YB

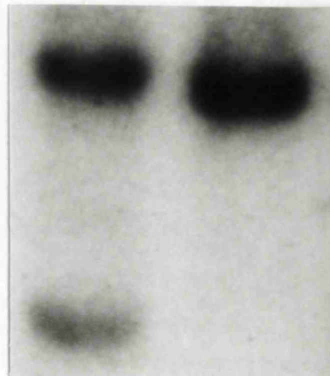


Figure 3.1 Allele loss in 6 informative hepatocellular carcinomas without liver cirrhosis detected by λMS8. B = blood lymphocyte DNA; N = non-tumour liver tissue DNA; T = HCC DNA. Patients' names are indicated above the tracks. All showed allelic losses in tumour DNA (indicated by arrows).

3.3.2 Allele loss on chromosome 5

Previous work on colorectal adenomas and carcinomas has shown that the chromosome 5 region (5q21-22) encompassing the familial adenomatous polyposis coli (APC) gene is deleted in inherited and sporadic colorectal cancer (Miyaki et al, 1990). Recently, two tumour suppressor genes, MCC and APC, for colorectal tumours on the long arm of chromosome 5 (5q21-22) have been cloned, and probes made available (Kinzler et al, 1991a; Kinzler et al, 1991b; Groden et al, 1991). These two genes are frequently lost or mutated in colorectal cancers (Chapter 1). For this reason I compared the pattern of allele loss in non-cirrhotic HCC with that of colorectal liver secondaries using various probes for chromosome 5q including a genomic DNA probe, L5-71.3, from MCC and a cDNA probe, FB54-D, from APC (Figure 3.2). Table 3.2 shows that patients with non-cirrhotic HCC had no allele loss when screened with probes mapped to regions of the chromosome other than 5q35-qter. In 9 cases of primary HCC without liver cirrhosis, five were informative with L5-71.3 , six with FB54-D, but none of them had allele loss. In contrast, in 7 cases of colorectal-liver metastases, allele loss was found in only 2 out of 5 informative cases with λMS8, while most of the informative cases with probes from 5q21-22, including the MCC and APC probes, had allele loss (Table 3.2). Another probe for 5q, v-fms (5q33-35), was not an informative one (Table 3.1) and not used for the comparison analysis. Figure 3.3 shows representative autoradiographs of Southern hybridisations with probes for chromosome 5.

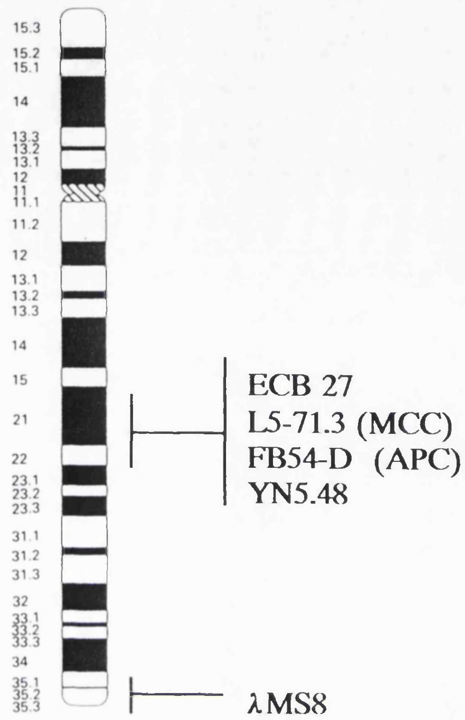


Figure 3.2 Localisation of probes for chromosome 5

TABLE 3.2 ALLELE LOSS ON CHROMOSOME 5q IN HCC WITHOUT CIRRHOSIS AND COLORECTAL-LIVER METASTASES

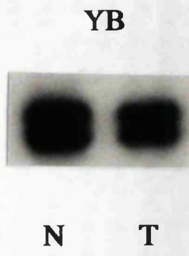
		DNA Probes and Loci[a]				
Patients	HBV Status[b]	ECB27 (5q21) D5S98	L5-71.3 (5q21) (MCC)	FB54-D (5q21) (APC)	YN5.48 (5q21-22) D5S81	λMS8 (5q35-qter) D5S43
HCC without Cirrhosis						
AA	neg	1,2[c]	1,2	1,2	-	1,(2)
WS	neg	1,2	-	1,2	-	1,(2)
RH	neg	-	-	1,2	-	1,(2)
SH	pos	1,2	1,2	-	1,2	(1),2
PP	neg	-	-	1,2	1,2	-
MB	neg	1,2	1,2	-	1,2	-
GA	neg	1,2	-	1,2	-	1,(2)
AL	neg	nd	1,2	-	nd	-
YB	pos	nd	1,2	1,2	nd	1,(2)
Total No		**7**	**9**	**9**	**7**	**9**
Heterozygosity		**5**	**5**	**6**	**3**	**6**
Allele Loss		**0**	**0**	**0**	**0**	**6**
Colorectal-liver metastases						
ET		-	1,2	1,(2)	1,2	1,2
JJ		-	-	(1),2	(1),2	-
AM		-	(1),2	-	-	1,2
MM		(1),2	-	1,(2)	1,(2)	-
FB		-	-	1,2	-	1,2
MK		-	(1),2	-	1,(2)	(1),2
PK		-	-	-	(1),2	1,(2)
Total No		**7**	**7**	**7**	**7**	**7**
Heterozygosity		**1**	**3**	**4**	**5**	**5**
Allele Loss		**1**	**2**	**3**	**4**	**2**

a. References for probes: ECB 27: (Varesco et al, 1989); L5-71.3: (Kinzler et al, 1991a); FB54-D: (Kinzler et al, 1991b); YN5.48: (Nakamura et al, 1988a); λMS8: (Wong et al, 1987).

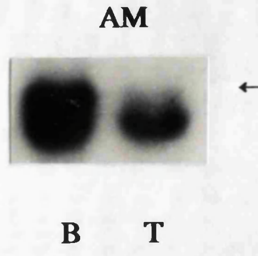
b. HBV status was determined by blood assay and Southern analysis of hepatic tissue DNA, using the HBV genome probe pEco63. neg: negative; pos: positive.

c. Homozygosity in the constitutional DNA (non-informative pattern) is indicated as a dash; where the normal tissue was informative the tumour genotype is shown in the table. Heterozygosity is indicated by 1,2. The continued presence of the larger allelic restriction fragment is indicated by "1" and "2" indicates continued presence of the smaller allelic fragment. Allele loss (deletion or reduction of intensity of a band) is indicated by ( ). "nd" indicates no data.

YB



AM

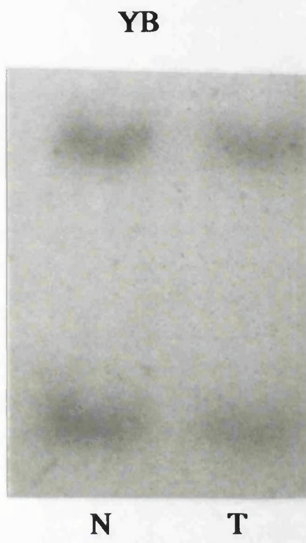


N T

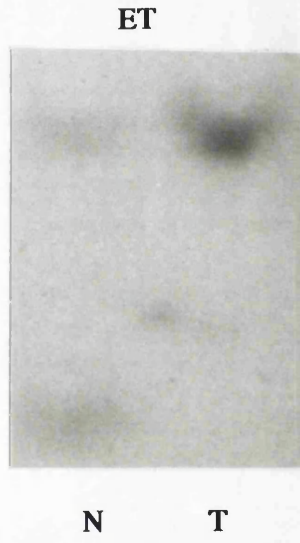
B T

L5-71.3

YB



ET

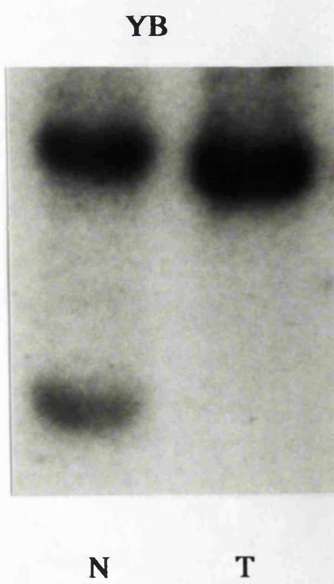


N T

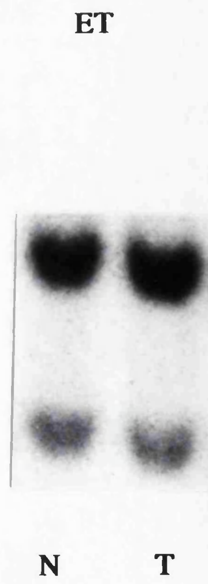
N T

FB54-D

YB



ET



N T

N T

λMS8

Figure 3.3 Representative autoradiographs of Southern hybridisations with L5-71.3 (MCC, 5q21), FB54-D (APC, 5q21) and λMS8 (5q35-qter). Patients' names are indicated above tracks. YB is hepatocellular carcinoma without cirrhosis and ET and AM are colorectal-liver metastases. B = blood lymphocyte DNA; N = non-tumour tissue DNA; T = tumour tissue DNA. Allelic losses in tumour DNA are indicated by arrows.

3.3.3 Allele loss in primary and recurrent HCC in Patient WS

Eleven probes were used for both primary and recurrent tumours. Out of the eleven probes studied, three showed different patterns of LOH between the primary and recurrent hepatocellular carcinomas (Table 3.3, Figure 3.4). The primary tumour had allele loss on the long arm of chromosome 5 (probe: λMS8, 5q35-qter) and the short arm of chromosome 17 shown by the probe p144-D6 (17p13) but not by the probe pYNZ22 (17p13), while the recurrent HCC had allele losses on chromosome 12 shown by λMS43 (12q24.3-qter) and chromosome 17 shown by both p144-D6 and pYNZ22, but no allele loss on chromosome 5 (Table 3.3, Figure 3.4a-c). This patient was shown as homozygous with the probe λMS32, assigned on the long arm of chromosome 1 (1q42-43), for his normal and primary tumour DNA. However, DNA from the recurrent tumour showed three bands, a gain of two, indicating a rearrangement (Figure 3.4d). Thus it was clear that there were different patterns of genetic changes between primary and recurrent tumours.

TABLE 3.3 PATTERN OF DNA CHANGES IN THE PRIMARY AND RECURRENT HEPATOCELLULAR CARCINOMA

Probe Name	Chromosome Locus	First HCC	Second HCC
λMS1	1p33-35	1,2	1,2
λMS32	1q42-43	-	R
λMS8	5q35-qter	1,(2)	1,2
λMS31	7pter-q22	1,2	1,2
pλg3	7q31.3-qter	1,2	1,2
EFD126.3	9q34	1,2	1,2
pMS51	11q13	1,2	1,2
λMS43	12q24.3-qter	1,2	(1),2
3'HVR	16p13.3	1,2	1,2
p144-D6	17p13	(1),2	(1),2
pYNZ22	17p13	1,2	(1),2

Homozygous pattern of normal DNA is indicated as a dash; where the normal DNA was heterozygous the tumour genotype is shown in the table as "1,2". The continued presence of the larger allele is indicated by "1", and "2" indicates the continued presence of smaller allele. Allele loss is indicated by ( ). "R" indicates DNA rearrangement.

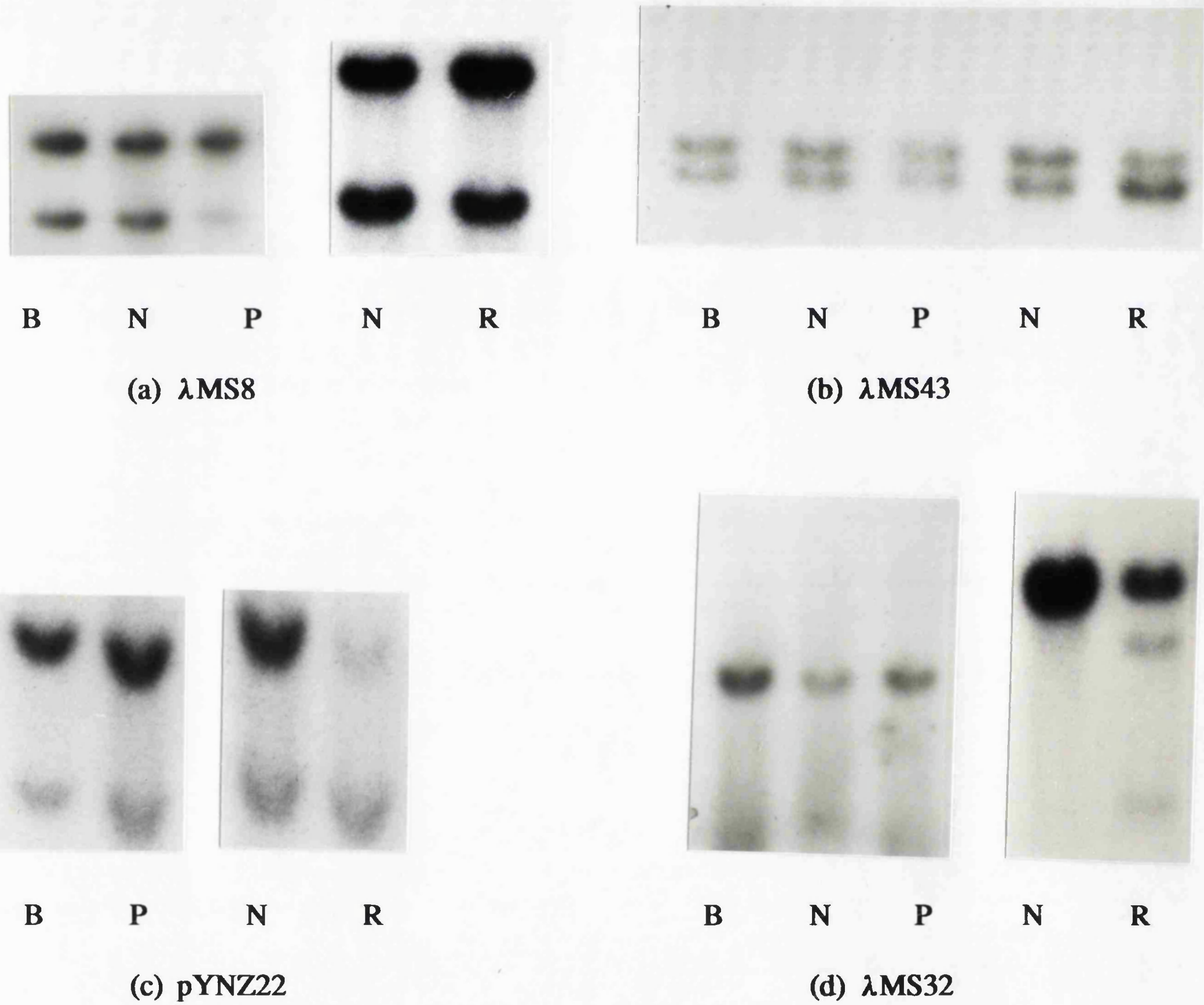


Figure 3.4 Autoradiographs of Southern hybridisation of Patient WS's DNA with: (a) λMS8 (5q35-qter); (b) λMS43 (12q24.3-qter); (c) pYNZ22 (17p13) and (d) λMS32 (1q42-43). B = Blood lymphocyte DNA; N = Non-tumour tissue DNA; P = Primary HCC DNA; R = Recurrent HCC DNA.

3.4 Discussion

3.4.1 Loss of heterozygosity in HCC

This is the first study that shows LOH in the terminal region of the long arm of chromosome 5 (ie, 5q35-qter) in patients with non-cirrhotic HCC and the short arm of chromosome 5 (5p) in patients with cirrhotic HCC (Ding et al, 1991). Patients with non-cirrhotic HCC showed LOH mainly on chromosomes 5q and 17p, while patients with cirrhotic HCC had allele loss on chromosomes 1q, 5p and 17p. Chromosomes 17p and 1q allele losses are shared with many other tumours and are likely to represent "tumour progression" (Sager,1989; Lasko et al, 1991). LOH at 17p13 may represent allele loss of p53 gene (Chapter 1). The presence of a tumour suppressor gene locus on the short arm of chromosome 5 has not been previously reported and may be important in cirrhotic HCC.

It is interesting that the pattern of chromosomal deletion in HCC shown so far correlates with the presence or absence of liver cirrhosis rather than the presence or absence of HBV infection. Since it has been shown that tumours from patients who are seropositive for markers of HBV infection contain integrated HBV DNA sequences it has been argued that the viral genome may be involved in the induction and /or maintenance of the neoplastic phenotype (Chen et al, 1988). The role of virally mediated oncogenesis in HCC has been widely studied, but yet, no conclusive results have emerged (Chapter 1). Therefore it was interesting to

find in this study no absolute differences in LOH pattern between HCC with or without HBV infection in spite of the differences in the aetiology and pathology processes. This lends support to the hypothesis that the development of cirrhosis (with its regenerative capacity) rather than the presence of integrated HBV genome is most important, although the number of patients studied to date is small. It remains to be seen whether tumour suppressor gene loss is different in HCC from cirrhotic patients with different aetiology.

Relatively few chromosome studies have been carried out on HCC (Chapter 1), but investigations on HCC cell lines showed involvement of chromosome 5, regions p14 and q31-33 in rearrangement or deletion (Simon et al, 1982; Simon & Knowles, 1986). Of particular relevance is a chromosome 5 (q34) rearrangement in direct preparations from an HCC arising in a patient without evidence of HBV infection (Simon et al, 1990). In other studies (Buetow et al, 1989; Zhang et al, 1990) frequent allele losses were found on chromosomes 4 and 16 in both HBV positive and negative HCC. Tsuda et al (1990) suggested that LOH on chromosome 16 represents tumour progression. The results in this study did not show LOH on chromosomes 4q, 16p or 16q. This could reflect either the difference in probes used or a difference in the stage of the tumours studied. None of the patients studied had extrahepatic tumour spread and all underwent "potentially curative" resection of the tumours. In agreement with other workers (Kiechle-Schwarz et al, 1990), no evidence for allele loss on 11p was found in this work. A literature survey did not reveal previous screening of the terminal region

of 5q in HCC. In a recent study (Fujimori et al, 1991) allelic loss was reported in HBV negative HCC in the region 5q21 (D5S84), but they did not mention the screening of 5q35-qter.

Since the two cloned tumour suppressor genes, MCC and APC, are located on 5q (Kinzler et al, 1991a; Kinzler et al, 1991b; Groden et al, 1991), it was important to rule out any role of these gene loci in HCC without cirrhosis. The results showed that none of the informative cases of HCC without liver cirrhosis had allele loss detected by the probes from or around MCC and APC probes, while all 6 informative primary HCC with the probe λMS8 (5q35-qter) exhibited LOH. On the other hand, probes from 5q21-22 detected a high frequency of allele loss in colorectal-liver metastases.

In conclusion, these results suggest that one of the tumour suppressor genes in non-cirrhotic HCC could be located on chromosome 5 and appears to be distinct from the loci of the MCC and APC genes.

3.4.2 Different patterns of LOH in primary and recurrent HCC

HCC is one of the most lethal malignancies in the world. At present only surgical resection offers a chance for cure (Tang et al, 1989). However, intrahepatic tumour recurrence rates, following liver resection, can be as high as 50-82% (Kanematsu et al, 1988; Nagasue et al, 1990).

Information on the clonal origin of tumours is important for prevention of recurrence, as management strategies are formed based on the exact cause of this. It has been shown that, genetically, recurrence in HCC can either be monoclonal, with recurrent tumours originating from the primary lesion, or polyclonal, where recurrent tumours represent de novo neoplasms (Chen et al, 1989b). Detecting the integrated hepatitis B virus (HBV) DNA in HCC genome is an effective means of determining the clonal origin of HBV related HCC (Esumi et al, 1986; Chen et al, 1989b). However, this approach is not possible for HBV negative HCC, which is more common than HBV related HCC in the West.

In this chapter, DNA restriction fragment length polymorphism analysis in Patient WS showed differences in DNA changes between primary and recurrent HCCs, suggesting that the recurrent tumour was, in fact, a de novo lesion. The finding of allele loss on chromosome 5 in the primary but not recurrent tumour must indicate that the latter tumour was not a progression from the first. More likely, both neoplasms were of different clonality. Recently Tsuda et al (1992) reported the successful determination of the tumour origins of multiple HCCs using RFLP analysis. Their results and that in this chapter suggest that RFLP analysis can be an effective way for comparing the tumour origins.

Tumour recurrence can be due to incomplete resection of the primary lesion, presence of satellite nodules at the time of primary resection, tumour seeding along needle biopsy track or genuine de novo tumour recurrence. DNA analysis

in this patient allowed us to exclude the possibility of tumour seeding along the needle biopsy track of the original tumour. It also indicated that the first resection was indeed complete. It is of interest that this patient developed three independent primary neoplasms (a colorectal carcinoma and two HCCs), which may indicate genetic predisposition.

It is imperative to study the clonal origin of the recurrent tumours, since strategies for preventing recurrence are dependent on causation. By detecting the integrated HBV DNA in HCC genome, Chen et al (1989b) reported five pairs of primary and recurrent HCC; two showing the same clonal origin, while the other three were of different clonalities. For the first group, more careful diagnostic and treatment procedures, especially extent of surgical resection, are essential in preventing recurrence, while for the second group, one has to identify means of blocking the function of or deleting the effects of persisting carcinogenetic factors, such as HBV infection and progressive genetic changes. Although the techniques for these are not available at present, recent continuing advances in the field of molecular biology and tumour suppressor gene research might lead to some benefit with gene therapy in the future (Chapter 1).

Chapter 4 Multiple Allelic Losses in Sarcomatoid Liver Carcinoma

4.1 Introduction

Sarcomatoid liver carcinoma is an uncommon form of liver tumour and is thought to arise from the sarcomatoid transformation of hepatocellular carcinoma (Kakizoe et al, 1987). The incidence of sarcomatoid changes in primary liver cancers is 2.2-3.9% (Kakizoe et al, 1987; Haratake et al, 1991). Sarcomatoid changes also occur in carcinomas of other organs, such as those in lung and prostate, and often constitute a more aggressive variant (Ro et al, 1992; Shannon et al, 1992).

As yet no chromosome allele loss studies have been reported in sarcomatoid liver carcinoma, nor in any other sarcomatoid variants of carcinomas. In this chapter the study of allele loss in two sarcomatoid liver carcinomas is described.

4.2 Patients and analysis

Because of the rarity of the tumour, only two patients with sarcomatoid liver carcinoma were identified in a three year period from the Royal Free and Hammersmith Hospitals where more than 100 hepatectomies were performed during that time. Clinical details for the two patients are given in Table 4.1. In addition, both patients had high levels of alpha fetoprotein (greater than 6000 ku/l) and there were no signs of lung or other distant metastases before operation. Pathological and immunohistochemical studies of the biopsies showed that both tumours were sarcomatoid liver carcinomas.

Biopsies collection, DNA extraction and analyses were done as described in Chapter 2. Twenty-five RFLP probes for chromosomes 1, 2, 3, 4, 5, 7, 9, 11, 12, 13, 14, 16, 17, 18 and 20 were used. The probes and the appropriate restriction enzymes are listed in Table 4.2.

TABLE 4.1 CLINICAL DETAILS OF TWO PATIENTS WITH SARCOMATOID LIVER CARCINOMA

	Patient MC	Patient MR
Age (year)	62	63
Sex	Female	Female
HBV Infection	Negative	Negative
Liver Cirrhosis	Absent	Absent
Tumour Site	Right lobe of the liver (Segments V & VI)	Right lobe of the liver (Segments V & VI)
Local Invasion	Abdominal wall, Right kidney, Right side of colon	Invasion of the right kidney capsule
Operation	Bisegmentectomy of V & VI of the liver, Right hemicolectomy, Right nephrectomy, Right adrenalectomy	Bisegmentectomy of V & VI of the liver, Excision of Gerota's fascia over right kidney
Tumour Size	14 cm in diameter	10.5 cm in diameter
Tumour Differentiation	Undifferentiated	Undifferentiated
Recurrence	Local recurrence & multiple lung metastases	Multiple local recurrences & multiple lung metastases
Death from the Disease	5 months after operation	6 months after operation

4.3 Results

Table 4.2 shows the allele status in the two sarcomatoid liver carcinomas. Out of 25 probes used, 16 showed informative patterns in each of the patients' normal liver DNA (heterozygosity: 64%). In Patient MC allele loss was found in 10 out of the 16 informative Southern hybridisations (fraction of allele loss: 62.5%). Furthermore, one probe, p144-D6, showed a gain of one novel band in tumour DNA, indicating a rearrangement. This probe has been assigned to the short arm of chromosome 17 (17p13), near the locus of the p53 tumour suppressor gene. For Patient MR, eight out of 16 informative probes showed allelic losses (50%). Altogether, the percentage of allele loss in the two tumours was 56.2%. The common losses for both patients were those on chromosomes 1, 12, 14, 16 and 20. The probe p144-D6, which showed a rearrangement in Patient MC, revealed an allele loss in Patient MR. Figure 4.1 shows the examples of allelic losses. Figure 4.2 shows the rearrangement found in Patient MC.

TABLE 4.2 ALLELE STATUS IN THE TWO SARCOMATOID LIVER CARCINOMAS

Probe	Chromosome Location	Enzyme Digest	Patient MC	Patient MR
λMS1	1p33-35	HinfI	(1),2	1,(2)
PB3	1p21-pter	MspI	1,(2)	1,2
λMS32	1q42-43	AluI	1,2	1,2
P5Gi	2q33-35	TaqI	-	1,2
H3H2	3p21	HindIII	-	1,2
HS-3	3q12	HindIII	-	-
VC63	4q	TaqI	1,(2)	-
pMS621	5p	HinfI	1,2	(1),2
ECB27	5q21	BglII	-	-
YN5.48	5q21-22	MspI	1,(2)	1,2
λMS8	5q35-qter	HinfI	-	-
λMS31	7pter-q22	HinfI	1,2	1,2
pλg3	7q31.3-qter	HinfI	1,2	1,2
EFD126.3	9q34	PvuII	-	-
H-ras	11p15	BamHI	-	1,(2)
pMS51	11q13	HaeIII	1,(2)	-
λMS43	12q24.3-qter	HinfI	(1),2	1,(2)
pMS626	13q	HinfI	1,2	-
pMS627	14q	AluI	(1),2	1,(2)
3'HVR	16p13.3	PvuII	(1),2	(1),2
pulB1148	16q22.1	TaqI	1,(2)	-
p144.D6	17p13	RsaI	R	1,(2)
pYNZ.22	17p13	RsaI	-	-
pMS440	18q	HaeIII	-	1,2
pMS617	20q	AluI	(1),2	(1),2
Number of allelic losses and rearrangement/ number of informative probes			11/16	8/16

Non-informative pattern of normal DNA is indicated as a dash; where the normal DNA was informative the tumour genotype is shown in the table as "1,2". The continued presence of the larger allele is indicated by "1" and "2" indicates the continued presence of smaller allele. Allele loss is indicated by ( ). R indicates DNA rearrangement.

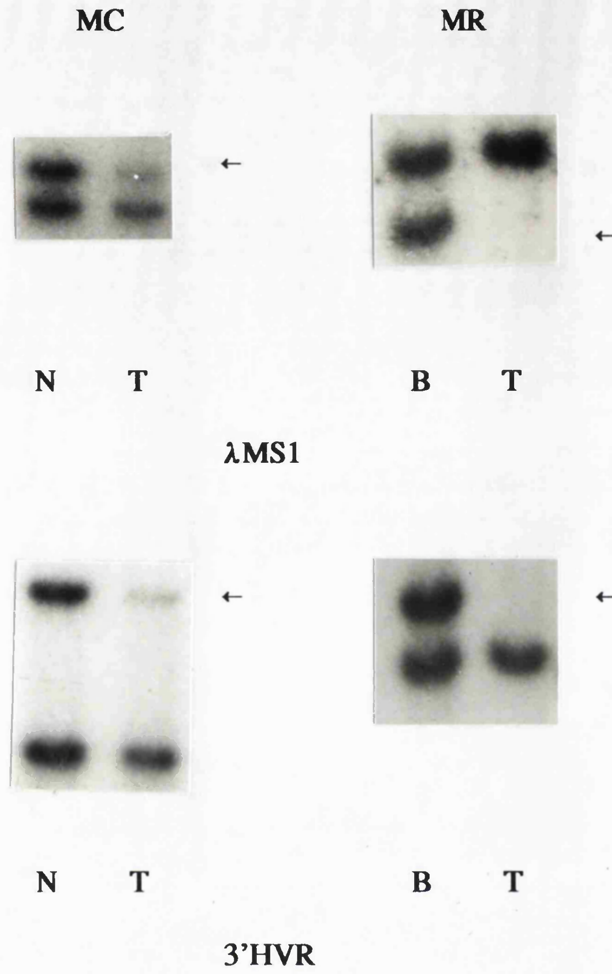


Figure 4.1 Representative allelic losses in sarcomatoid liver carcinomas. B=Blood lymphocyte DNA; N=Non-tumour liver tissue DNA; T=Tumour tissue DNA. Allelic losses are indicated by arrows. Patients' names are above the tracks.

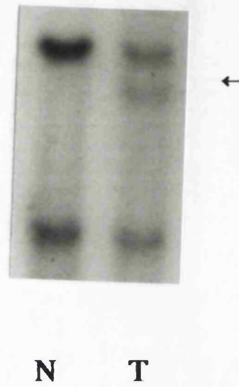


Figure 4.2 DNA rearrangement revealed by the probe p144-D6 (17p13) in Patient MC. N=Non-tumour liver tissue DNA; T=Tumour tissue DNA. The arrow indicates the extra band.

4.4 Discussion

This is the first reported study of allele loss in sarcomatoid carcinomas. It showed multiple allelic losses in two sarcomatoid liver carcinoma. Consistent allele loss in a certain type of tumour in a particular region of genome may represent loss of a tumour suppressor gene. Although data from only two patients may not necessarily represent such loss, the results obtained in this study may still be consistent with the concept that cancer is fundamentally a genetic disease (Lasko et al, 1991), since there were multiple genetic alterations in both tumours. Using the same method as used in this study, I found a much lower frequency of allele loss in "classical" hepatocellular carcinoma (30/186, 16.1%) and an even lower frequency in fibrolamellar carcinoma of the liver (2/55, 3.6%). Work from other groups showed that the percentage of allele loss was 13-20% in HCC (Table 4.3).

In HCC without liver cirrhosis a consistent allele loss in the chromosome region 5q35-qter detected by the probe λMS8 and a frequent loss in the region 17p13 (near the locus of the p53 tumour suppressor gene) with the probes p144-D6 and pYNZ.22 have been found (Chapter 3). The probes λMS8 and pYNZ.22 showed non-informative (homozygous) patterns in both patients in this study (Table 4.2), but interestingly the probe p144-D6 revealed a rearrangement in one patient and a loss in another. Since abnormalities of the p53 tumour suppressor gene have been found in most, if not all, human malignancies (Chapter 1), the findings here may reflect alterations of that gene in the two sarcomatoid carcinoma. Loss of heterozygosity on chromosome 16 in HCC has been associated with advanced

stage disease (Tsuda et al, 1990); the involvement of this chromosome in both cases in the present study may confirm this association. In one patient (Patient MC) at least, the whole chromosome may be lost, as both short and long arms are involved (Table 4.2).

Patients with sarcomatoid carcinoma usually have a poor prognosis (Ro et al, 1992; Shannon et al, 1992). In 14 sarcomatoid liver carcinomas studied by Kakizoe et al (1987), 13 had extrahepatic metastasis (93%), the frequency of metastasis being higher than that in classical HCC. Both patients in this study had large, undifferentiated primary tumours, extensive local invasion, multiple lung metastases, local recurrence and both died of the disease within half year of the operation (Table 4.1). The molecular study found multiple genetic changes (allelic losses and rearrangement). The poor prognoses might be associated with the high frequency of these genetic changes. The association may not be due to chance, since a similar correlation has been observed in other tumours, such as colorectal cancers (Vogelstein et al, 1989), classical and fibrolamellar variant of HCC (Ding et al, 1993a) and carcinoma of the pancreas (Ding et al, 1992a). Confirmation of this association awaits the study of more cases of this rare variant of cancer of the liver and of other organs.

In conclusion this study found multiple allelic losses and rearrangement in two sarcomatoid liver carcinomas and these multiple genetic changes may be associated with the patients' poor prognoses.

TABLE 4.3 COMPARISON OF FREQUENCY OF ALLELE LOSS IN PRIMARY LIVER TUMOURS

Patient Origin	Tumour*	No. of LOH/ No. of Heterozygotes	% Allele Loss	Reference
American	HCC	18/104	17.3	Buetow et al, 1989
Japanese	HCC	47/361	13.0	Zhang et al, 1990
Japanese	HCC	111/792	14.0	Fujimori et al, 1991
Australian	HCC	23/112	20.5	Walker et al, 1991
European	HCC	30/186	16.1	Ding et al, 1991
European	FLC	2/55	3.6	Ding et al, 1993a
European	SLC	18/32	56.2	This chapter

* HCC = hepatocellular carcinoma; FLC = fibrolamellar carcinoma; SLC = sarcomatoid liver carcinoma.

Chapter 5 Loss of Heterozygosity in Fibrolamellar Carcinoma

5.1 Introduction

Fibrolamellar carcinoma (FLC) is a rare variant of hepatocellular carcinoma (HCC). It occurs in younger patients (20-30 years) with an equal sex incidence. Cirrhosis and hepatitis B virus (HBV) infections are rarely seen in patients with FLC and it is thought that the tumour may arise from areas of focal nodular hyperplasia (Vecchio et al, 1984). The prognosis of patients with FLC is better than that of HCC with an average survival of 44 months compared to 6 months in HCC (Craig et al, 1980). It is these differences in clinico-pathological features which would suggest that FLC and HCC have a different pathogenesis.

Loss of heterozygosity (allele loss) in classic HCC has been well studied (this thesis & Zhang et al, 1990b; Fujimori et al, 1991). In FLC, no such studies have been reported. Therefore such a study in FLC with 18 DNA restriction fragment length polymorphism (RFLP) probes was performed and the pattern of allele loss compared with that of HCC.

5.2 Patients and analysis

Due to the rarity of the condition, in the past three years I was able to collect five patients with fibrolamellar carcinoma who underwent surgical resection of their tumours at Hammersmith or the Royal Free Hospitals, despite the fact that both of these hospitals are national referral centres for liver cancers. Patients' clinical data are presented in Table 5.1. None of the patients received chemotherapy or radiotherapy before surgery. Surgical biopsies from tumoral and non-tumoral liver tissues were snap frozen in liquid nitrogen at the time of operation. Lymphocytes from peripheral blood obtained pre-operatively were also used as a source of normal DNA. Tissue was stored at -70°C until DNA extraction. A portion of each tumour sample was examined histologically to confirm the type of tumour present.

DNA extraction and analysis were done as described in Chapter 2. The 18 RFLP probes for chromosomes 1, 5, 7, 9, 11, 12, 13, 16 17 and 18 and the appropriate restriction enzymes are listed in Table 5.2.

5.3 Results

Table 5.2 shows the pattern of allele loss in fibrolamellar carcinoma. Overall, 55/78 Southern blots were informative (heterozygosity: 70.5%) and the allele loss was only 2 out of the 55 informative cases (3.6%). The frequency of allele loss in FLC is significantly lower than that in HCC (30/186, 16.1%) [p=0.03, SE(p_1 -p_2) = 2.7%]. Figure 5.1 shows the two allelic losses, both of which occurred in a single patient (GT). Only that patient had a recurrent FLC (Table 5.1). The chromosomal regions deleted in his tumour were 1q42-43 detected by the probe λMS32 and 5p by cMS621. These two probes also showed a high frequency of allele loss in HCC with liver cirrhosis, as described in Chapter 3.

Patient PP had a synchronous HCC. The HCC of this patient had an allele loss detected by the probe λMS 43 (12q24.3-qter), but his FLC had no similar allele loss (Table 5.3, Figure 5.2). The probe was informative also in all the other 4 patients but showed no allele loss (Table 5.2).

TABLE 5.1 CLINICAL DATA OF 5 PATIENTS WITH FIBROLAMELLAR CARCINOMA

Name	Sex	Age	HBV Status[a]	Liver Cirrhosis	FLC Recurrence	No of Allele Loss in FLC
AB	F	23	-	-	-	0
BL	M	55	-	-	-	0
GT	M	23	-	-	+	2
PP[b]	M	60	-	-	-	0
CL	F	19	-	-	-	0

a. HBV status was determined by blood assay and Southern analysis of hepatic tissue DNA, using the HBV genome probe pEco63.

b. This patient had a synchronous HCC. Two tumours were resected together.

TABLE 5.2 CHROMOSOME ALLELE LOSS IN FIBROLAMELLAR CARCINOMA

Probe	Chromosomal Region	Enzyme Used	Allele Loss*
λMS1	1p33-35	HinfI	0/3
λMS32	1q42-43	AluI	1/3
cMS621	5p	HinfI	1/4
ECB27	5q21	BglII	0/0
YN5.48	5q22	MspI	0/3
λMS8	5q35-qter	HinfI	0/2
λMS31	7pter-q22	HinfI	0/4
pλg3	7q31.3-qter	HinfI	0/3
EFD126.3	9q34	PvuII	0/2
H-ras	11p15	BamHI	0/3
pMS51	11q13	HaeIII	0/4
λMS43	12q24.3-qter	HinfI	0/5
cMS626	13q	AluI	0/4
3'HVR	16p13.3	PvuII	0/4
pulB1148	16q22.1	TaqI	0/1
p144-D6	17p13	RsaI	0/2
pYNZ.22	17p13	RsaI	0/5
cMS440	18q	HaeIII	0/3

* No with allele loss / no of informative cases.

TABLE 5.3 ALLELE STATUS IN PATIENT PP'S HEPATOCELLULAR CARCINOMA AND THE SYNCHRONOUS FIBROLAMELLAR VARIANT

Probe Name	Chromosome Region	HCC	FLC
λMS1	1p33-35	1,2	1,2
λMS32	1q42-43	1,2	1,2
pMS621	5p	1,2	1,2
ECB27	5q21	-	-
YN5.48	5q21-22	1,2	1,2
λMS8	5q35-qter	-	-
λMS31	7pter-q22	1,2	1,2
pλg3	7q31.3-qter	-	-
EFD126.3	9q34	-	-
pMS51	11q13	1,2	1,2
λMS43	12q24.3-qter	**1,(2)**	**1,2**
pMS626	13q	1,2	1,2
3'HVR	16p13.3	1,2	1,2
pulB1148	16q22.1	1,2	1,2
p144.D6	17p13	-	-
pMS440	18q	1,2	1,2

Non-informative pattern of normal DNA is indicated as a dash; where the normal DNA was informative the tumour genotype is shown in the table as "1,2". The continued presence of the larger allele is indicated by "1" and "2" indicates the continued presence of smaller allele. Allele loss is indicated by ( ).

Figure 5.1. Autoradiographs of Southern hybridisations of Patient GT's DNA with λMS32 (1q42-43) and cMS621 (5p). N = non-tumour tissue DNA; T = tumour tissue DNA. Both showed allelic losses in tumour DNA (indicated by arrows).

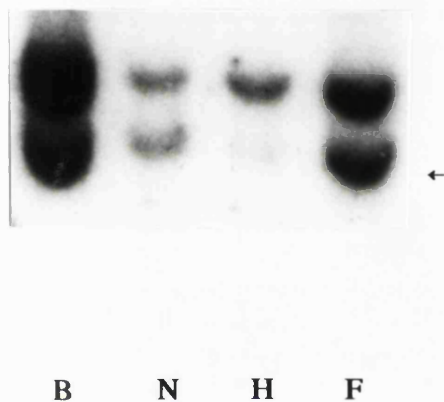


Figure 5.2. Autoradiograph of Southern hybridisation of Patient PP's DNA with λMS43 (12q24.3-qter). B = Blood lymphocyte DNA; N = Non-tumour tissue DNA; H = Hepatocellular carcinoma DNA; F = Fibrolamellar variant DNA. The small allele was deleted in HCC DNA compared with lymphocyte and non-tumour DNA but, the allele was present in the FLC DNA (indicated by the arrow).

5.4 Discussion

This study showed that the frequency of allele loss in fibrolamellar carcinoma was very low (2/55, 3.6%). With the same method, a much higher frequency of allele loss in HCC (30/186, 16.1%) was found. For colorectal carcinomas, patients with a higher frequency of allelic losses had a considerably worse prognosis than did the other patients (Vogelstein et al, 1989). A similar correlation was observed in carcinomas of the pancreas and colorectal liver metastases in this thesis (Chapters 8 and 9). Thus this study showing a much lower frequency of allele loss in FLC than in HCC is in agreement with the above observations since FLC has a much better prognosis than HCC (Craig et al, 1980). Of the 5 patients with FLC in this study, the FLC with two allelic losses recurred while the others did not (Table 5.1).

As described in Chapter 3 HCC with liver cirrhosis showed the highest frequency of allele loss in chromosomal regions 1q42-43, 5p and 17p13, and in HCC without cirrhosis, in 5q35-qter and 17p13. The probes used for the region 17p13, ie, p144-D6 and pYNZ.22, were near the locus of the p53 tumour suppressor gene. The high frequency of allele loss shown by these probes in HCC might represent the p53 gene loss in the tumour. None of the informative FLC had allele loss in 5q35-qter and 17p13 (Table 5.2), the chromosomal regions where the HCC series showed a high frequency of allele loss. It is of interest to note that the two allelic losses in the FLC occurred in 1q42-43 and 5p. A larger study is needed to

determine whether the loss is characteristic of this type of tumour or due to chance (Lasko et al, 1991).

In Patient PP who had a synchronous HCC and FLC, the HCC showed allelic loss in the region 12q24.3-qter, but not the FLC. To date, no tumour suppressor gene has been mapped on chromosome 12 yet. A recent study (Sano et al, 1991) has shown allele loss on that chromosome in about 30% gastric carcinomas with the same probe (λMS43) as I used in the present study. Further study is needed to determine whether there is a possible tumour suppressor gene located on chromosome 12.

The data of this chapter supports the view that FLC is a different clinico-pathological entity from HCC, and suggests that the former has a different molecular-genetic mechanism for the change to its development. Fewer allelic losses in FLC than those in classic HCC may be associated with its better prognosis.

Chapter 6 Allele Status in Hepatocellular Adenoma

6.1 Introduction

Hepatocellular adenoma is a rare liver tumour, but the incidence has increased significantly following the introduction of oral contraceptives in the last 20 years (Bruguera & Rodés, 1991). Ninety percent of patients with hepatocellular adenoma are young women, of whom ninety percent have used an oral contraceptive (Kerlin et al, 1983). The prognosis of this benign tumour is good (Kerlin et al, 1983).

Carcinogenesis is a multi-step process (Fearon & Vogelstein, 1990). In an animal model of liver tumour induction by the HBx gene of hepatitis B virus, the sequential process from multifocal areas of altered hepatocytes, to benign adenomas and eventually to malignant carcinomas was seen (Kim et al, 1991). Although oral contraceptive-associated hepatocellular adenomas may not be premalignant and may undergo reversible change after withdrawal of oral contraceptives, foci or areas of liver cell dysplasia within adenomas are premalignant and may transform into hepatocellular carcinoma (Tao, 1992). In the colorectum most, if not all, malignant tumours (carcinomas) arise from preexisting benign tumours (adenomas), and a genetic model has been proposed for this process (Fearon & Vogelstein, 1990). There are fewer genetic changes in adenomas compared with those in carcinomas, but changes like chromosome

allele loss, or loss of heterozygosity, do occur in colorectal adenomas, though at a much lower frequency (Rees et al, 1989; Fearon & Vogelstein, 1990). Allele loss in one thyroid adenoma and one parathyroid adenoma has also been reported (Kubo et al, 1991; Arnold & Kim, 1989). Allele loss has been well documented in hepatocellular carcinoma (Chapter 3 and the references therein). The aim of this chapter was to find out whether allele loss, or LOH, also occurs in the hepatic adenoma. No such studies have hitherto been reported in the literature except for this study.

6.2 Patients and analysis

Six patients with hepatocellular adenoma were studied. All were female and ages ranged from 16 to 51 years old. Four of them had been on oral contraceptives. All underwent resection of their tumours. One of the patients (MB, aged: 51) had synchronous hepatocellular carcinoma and adenoma and both tumours were resected. She never had oral contraceptives. Surgical biopsies from tumoral and non-tumoral liver tissues were snap frozen in liquid nitrogen at the time of operation. Lymphocytes from peripheral blood obtained pre-operatively were also used as a source of normal DNA. Tissue was stored at -70°C until DNA extraction. A portion of each tumour sample was examined histologically to confirm the type of tumour present. There were no foci or areas of liver cell dysplasia within any of the adenomas. All non-tumorous liver tissues were non-cirrhotic.

DNA extraction and analysis were done as previously described (Chapter 2). Twenty-five RFLP probes for 21 chromosome arms, ie, 1p, 1q, 2q, 3p, 3q, 4q, 5p, 5q, 7p, 7q, 9q, 11p, 11q, 12q, 13q, 14q, 16p, 16q, 17p, 18q and 20q, were used. These 25 probes and the appropriate restriction enzymes are listed in Table 6.1.

6.3 Results

The results obtained from the 25 probes are shown in Table 6.1. No allele loss has been detected in any of the 6 adenomas. For each chromosome locus detected by the probes, at least two patients were heterozygous, but none showed any LOH. Figure 6.1 shows some examples of autoradiographs. For patient MB, her HCC had an allele loss detected by the probe pYNZ.22. This probe has been assigned to the region of the short arm of chromosome 17 (17p13) (Nakamura et al, 1988b), near the p53 tumour suppressor gene (Bressac et al, 1991). The patient's hepatocellular adenoma, resected together with her HCC, did not show such loss (Figure 6.2).

TABLE 6.1 DNA PROBES USED AND NUMBER OF HETEROZYGOTES AT EACH REGION IN HEPATOCELLULAR ADENOMA

Probe	Chromosome Location	Enzyme Digest	No. of Allele Losses/ No. of Heterozygotes
λMS1	1p33-35	HinfI	0/5
PB3	1p21-pter	MspI	0/2
λMS32	1q42-43	AluI	0/6
P5Gi	2q33-35	TaqI	0/2
H3H2	3p21	HindIII	0/3
HS-3	3q12	HindIII	0/2
VC63	4q	TaqI	0/3
pMS621	5p	HinfI	0/4
ECB27	5q21	BglII	0/2
YN5.48	5q21-22	MspI	0/3
λMS8	5q35-qter	HinfI	0/3
λMS31	7pter-q22	HinfI	0/5
pλg3	7q31.3-qter	HinfI	0/6
EFD126.3	9q34	PvuII	0/4
H-ras	11p15	BamHI	0/2
pMS51	11q13	HaeIII	0/5
λMS43	12q24.3-qter	HinfI	0/5
pMS626	13q	HinfI	0/4
pMS627	14q	AluI	0/3
3'HVR	16p13.3	PvuII	0/3
pulB1148	16q22.1	TaqI	0/2
p144.D6	17p13	RsaI	0/3
pYNZ.22	17p13	RsaI	0/4
pMS440	18q	HaeIII	0/3
pMS617	20q	AluI	0/4

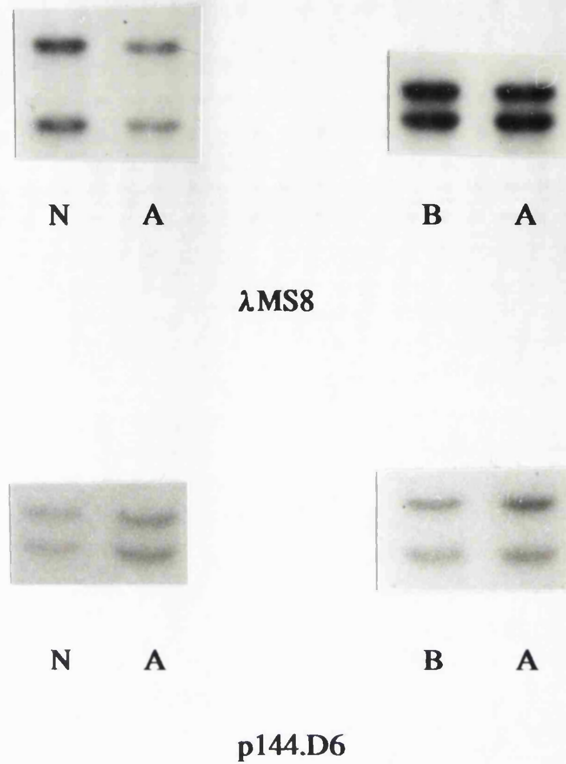


Figure 6.1 Representative autoradiographs of Southern hybridisations in two hepatocellular adenomas. B = Blood lymphocyte DNA; N = Non-tumour tissue DNA; A = Adenoma tissue DNA. None showed allele loss.

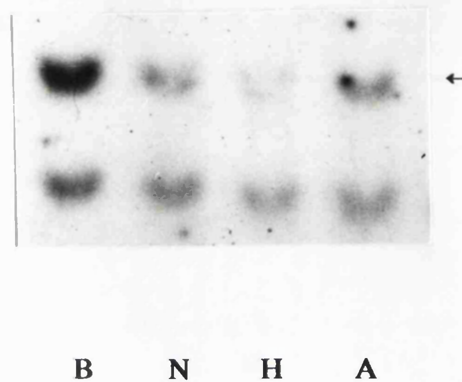


Figure 6.2 Autoradiograph of Southern hybridisation of DNA from Patient MB with pYNZ.22 (17p13). B = Blood lymphocyte DNA; N = Non-tumour tissue DNA; H = Hepatocellular carcinoma DNA; A = Adenoma DNA. The intensity of larger allele was greatly reduced in HCC DNA compared with lymphocyte and non-tumour DNA but, the allele was present in the adenoma DNA (indicated by the arrow).

6.4 Discussion

The results in this chapter showed no evidence for chromosome allele loss in hepatocellular adenoma. In hepatocellular carcinoma without liver cirrhosis, a high frequency of LOH at 5q35-qter and 17p13 has been found (Chapter 3), but none of the heterozygous adenoma in this study had such loss (Table 6.1, Figure 6.1). It is known that in the sequential process of carcinogenesis, early stage tumours have few genetic changes (Fearon & Vogelstein, 1990). Chromosome allele losses have been detected in colorectal adenoma, a premalignant tumour, but the frequency is very low (Rees et al, 1989; Fearon & Vogelstein, 1990). This is to be expected as the initiating event in carcinogenesis is likely to be a point mutation or small molecular deletion (Miyoshi et al, 1992b). Therefore it is not surprising that none of the liver adenomas in this study exhibited LOH since none of them contained foci or areas of liver cell dysplasia, a sign of premalignant status (Tao, 1992).

Allele loss at 17p13 may represent loss of the p53 tumour suppressor gene, which is normally seen in carcinomas, but rarely in adenomas, such as those in the colorectum (Fearon & Vogelstein, 1990), and ovary (Eccles et al, 1990). This chapter and Chapter 3 show that this is also the case in liver primary tumours. Patient MB in this study provided a unique opportunity to observe this phenomenon in a single patient, and it is of interest to note that allele loss at 17p13 detected by pYNZ.22 was found in her HCC DNA, but not in her hepatic adenoma DNA. She was homozygous for another probe at 17p13, p144-D6.

In conclusion, the results of this chapter suggest that if LOH occurs in hepatocellular adenoma it is a rare event.

Chapter 7 Loss of Heterozygosity in Cholangiocarcinoma

7.1 Introduction

Cholangiocarcinoma, the intrahepatic bile duct carcinoma, is thought to arise from the same stem cell as hepatocellular carcinoma (HCC) (Sell & Dunsford, 1989). Cholangiocarcinoma is reported as occurring less frequently than HCC in most parts of the world. The prognosis of cholangiocarcinoma is poor, with the majority of patients dying 6-12 months after diagnosis. The overall survival rate in treated cases at 5 years is below 9% (Czerniak & Blumgart,1989).

Multiple genetic alterations including the activation of oncogenes and the inactivation of tumour suppressor genes are important in carcinogenesis. As reviewed in Chapter 1, expression of oncogenes, including ras, myc and erbB-2, and point mutations at K-ras codons 12 and 61 have been reported in a high proportion of cholangiocarcinomas (Voravud et al, 1989; Tada et al, 1990). Cytogenetic studies on two cholangiocarcinoma cell lines revealed several chromosomal abnormalities (Storto et al, 1990). In this chapter the first study of loss of heterozygosity in cholangiocarcinoma is described.

7.2 Patients and analysis

Fourteen patients with cholangiocarcinoma were studied. All underwent resection of their tumours. None of the patients received chemotherapy or radiotherapy before surgery. Surgical biopsies from tumoral and non-tumoral liver tissues were snap frozen in liquid nitrogen at the time of operation. Lymphocytes from peripheral blood obtained pre-operatively were also used as a source of normal DNA. Tissue was stored at -70°C until DNA extraction. A portion of each tumour sample was examined histologically to confirm the type of tumour present.

DNA extraction and analysis were done as previously described (Chapter 2). The 22 RFLP probes for chromosomes 1, 5, 7, 9, 11, 12, 13, 14, 16 17 and 18 and the appropriate restriction enzymes are listed in Table 7.1.

7.3 Results

Table 7.1 shows the pattern of allele loss in cholangiocarcinoma. Overall, 164/229 Southern blots were informative (heterozygosity: 71.6%) and the overall LOH was 17 out of 164 informative cases (10.4%). Figure 7.1 shows representative examples of allele loss.

As shown in Table 7.1, the 14 cholangiocarcinomas had a higher rate of LOH on chromosomes 1, 5 and 17 than on other chromosomes. Allelic losses were shown in 2 out of 14 informative cases (14.3%) for the region of the short arm of chromosome 1 (1p33-35) detected by the probe λMS1, 3 out of 13 (23.1%) for the region of the long arm of chromosome 1 (1q42-43) by λMS32, 3 out of 10 (30%) for 5q35-qter by λMS8, 4 out of 9 (44.4%) at 17p13 by p144-D6 and 2 out of 5 (40%) also at 17p13 by pYNZ22. No consistent allele loss was revealed by any other probes used.

A comparison of allele loss between cholangiocarcinoma and colorectal liver secondaries using various probes for chromosome 5q, including a genomic probe L5-71-3 for MCC and a cDNA probe, FB54-D, for APC, was performed, as described in Chapter 3. The localisation of probes used was shown in Figure 3.2 (Chapter 3). Table 7.2 shows that patients with cholangiocarcinoma had no allele loss when screened with probes mapped to regions of the chromosome other than 5q35-qter. On the other hand the majority of patients with colorectal liver

metastases showed allele loss with probes from 5q21-22, the region of the chromosome associated with colorectal cancer.

TABLE 7.1 LOSS OF CONSTITUTIONAL HETEROZYGOSITY IN HUMAN CHOLANGIOCARCINOMA

Probe	Chromosomal Region	Enzyme Used	LOH[a]
λMS1[b]	1p33-35	HinfI	2/14
λMS32	1q42-43	AluI	3/13
cMS621	5p	HinfI	0/4
ECB27	5q21	BglII	0/5
L5-71	5q21	MspI	0/7
54-D	5q21	MspI	0/6
YN5.48	5q21-22	MspI	0/4
λMS8	5q35-qter	HinfI	3/10
λMS31	7pter-q22	HinfI	1/13
pλg3	7q31.3-qter	HinfI	0/12
EFD126.3	9q34	PvuII	1/11
H-ras	11p15	BamHI	0/3
pMS51	11q13	HaeIII	0/7
λMS43	12q24.3-qter	HinfI	1/11
p3.8R	13q14.2	HindIII	0/7
cMS626	13q	AluI	0/5
cMS627	14q	AluI	0/5
3'HVR	16p13.3	PvuII	0/8
pulB1148	16q22.1	TaqI	0/3
p144-D6	17p13	RsaI	4/9
pYNZ.22	17p13	RsaI	2/5
cMS440	18q	HaeIII	0/2

(Notes for Table 7.1)

a. No. of LOH / No. of informative cases

b. References for probes: λMS1, λMS32, λMS8, λMS31, pλg3 and λMS43: Wong et al, 1987; cMS621, cMS626, cMS627 and cMS440: Armour et al, 1990; ECB27: Varesco et al, 1989; L5-71: Kinzler et al, 1991a; 54-D: Kinzler et al, 1991b; YN5.48: Nakamura et al, 1988a; EFD126.3: Nakamura et al, 1987a; H-ras: Krontiris et al, 1985; pMS51: Armour et al, 1989; P3.8R: Friend et al, 1986; 3'HVR: Higgs et al, 1986; pulB1148: vander Straten et al, 1983; p144-D6: Kondoloen et al, 1987; pYNZ22: Nakamura et al, 1988b.

TABLE 7.2 ALLELE LOSS ON CHROMOSOME 5q IN CHOLANGIOCARCINOMAS AND COLORECTAL METASTASES IN LIVER

	Probes and Regions or Genes				
Patients	ECB27 (5q21)	L5-71 (MCC)	54-D (APC)	YN5.48 (5q21-22)	λMS8 (5q35-qter)
Cholangiocarcinoma					
1	-	1,2	1,2	1,2	(1),2
2	1,2	-	1,2	1,2	1,2
3	1,2	1,2	-	-	1,2
4	-	1,2	-	1,2	1,2
5	1,2	-	1,2	1,2	-
6	nd	1,2	1,2	-	1,(2)
7	1,2	-	-	nd	1,2
8	1,2	1,2	-	nd	1,(2)
9	nd	1,2	1,2	-	-
10	nd	1,2	1,2	nd	1,2
11	nd	nd	-	nd	1,2
12	nd	nd	-	nd	-
13	nd	nd	nd	nd	-
14	nd	nd	nd	nd	1,2
Total No	**7**	**10**	**12**	**7**	**14**
Heterozygosity	**5**	**7**	**6**	**4**	**10**
Allele Loss	**0**	**0**	**0**	**0**	**3**
Colonic Metastasis					
15	-	1,2	1,(2)	1,2	1,2
16	-	-	1,(2)	(1),2	-
17	-	1,(2)	-	-	1,2
18	(1),2	-	1,2	1,(2)	-
19	-	-	1,2	-	1,2
20	-	1,(2)	-	1,(2)	(1),2
21	-	-	-	(1),2	1,(2)
Total No	**7**	**7**	**7**	**7**	**7**
Heterozygosity	**1**	**3**	**4**	**5**	**5**
Allele Loss	**1**	**2**	**2**	**4**	**2**

(Note for Table 7.2)

Homozygosity in the constitutional DNA (non-informative pattern) is indicated as a dash; where the normal tissue was informative the tumour genotype is shown in the table. Heterozygosity is indicated by 1,2. The continued presence of the larger allelic restriction fragment is indicated by "1" and "2" indicates continued presence of the smaller allelic fragment. Allele loss (deletion or reduction of intensity of a band) is indicated by ( ). "nd" indicates no data.

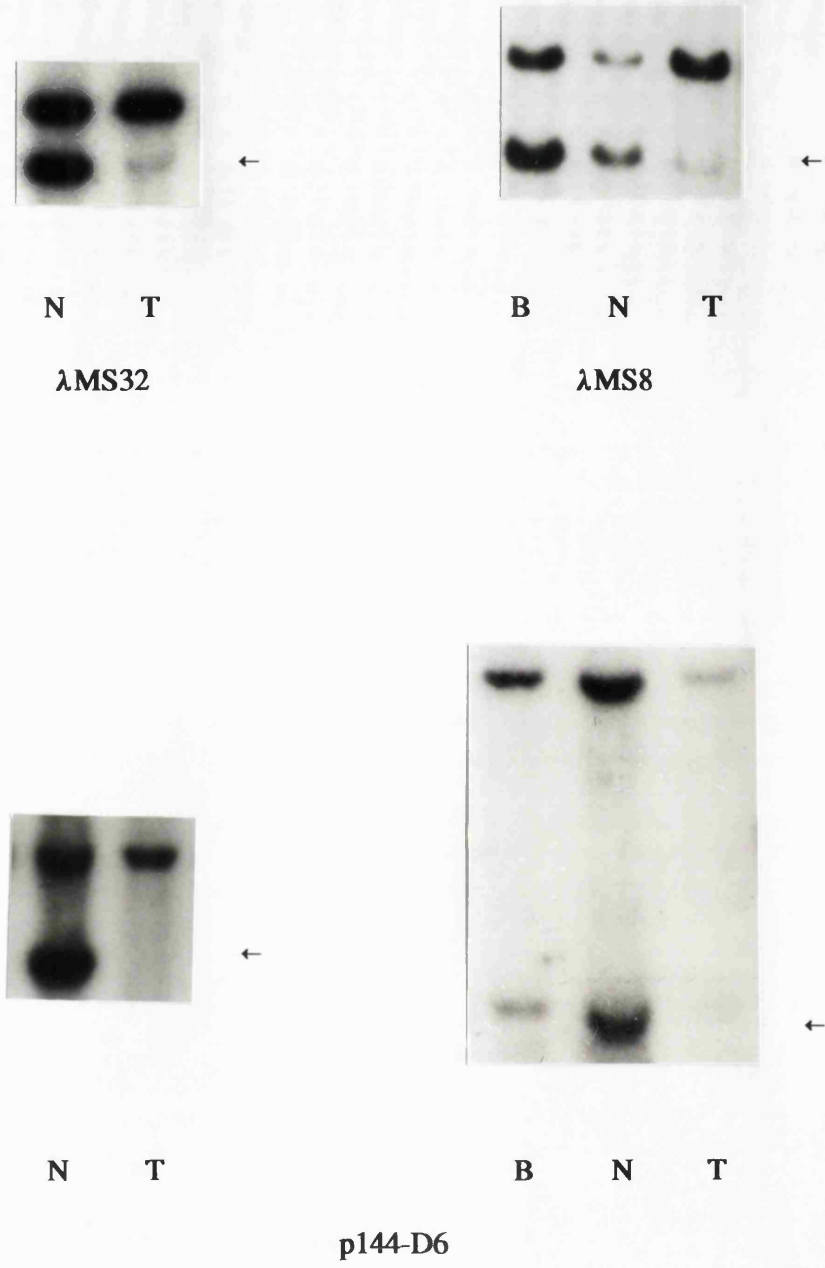


Figure 7.1 Representative autoradiographs of Southern hybridisation in cholangiocarcinoma with λMS32 (1q42-43), λMS8 (5q35-qter) and p144-D6 (17p13). B = blood lymphocyte DNA; N = non-tumour tissue DNA; T = tumour tissue DNA. All showed allele losses in tumour DNA (indicated by arrows).

7.4 Discussion

This is the first reported study of loss of heterozygosity in cholangiocarcinomas. Three out of 22 probes revealed a relatively high rate of LOH in two chromosomal regions, namely, 5q35-qter (30%) and 17p13 (44.4% and 40%). Another region with a relatively high frequency of allele loss but less frequent than the above two regions was 1q42-43 (23.1%). There were also allelic losses at 1p33-35 (2/14, 14.3%), 7pter-q22 (1/13, 7.7%), 9q34 (1/11, 9.1%) and 12q24.3-qter (1/11, 9.1%), but these lower values might represent random losses since rapid division of malignant cells can produce loss of heterozygosity at a certain region by chance (Lasko et al, 1991).

In Chapter 3 allelic losses at 1q42-43 and 17p13 in hepatocellular carcinoma with liver cirrhosis and at 5q35-qter and 17p13 in HCC without liver cirrhosis were described. Hence it is of interest to find LOH in these three regions in cholangiocarcinoma in this study. It has been proposed that HCC and cholangiocarcinoma arise from the same pluripotent liver stem cell (Sell & Dunsford, 1989). These two types of primary liver malignancies, therefore, may share similar genetic changes. Allelic losses on chromosomes 1 and 17p are shared with other tumours and are likely to be involved in "tumour progression" (Sager, 1989; Lasko et al, 1991). Loss of heterozygosity at 5q35-qter in both HCC and cholangiocarcinoma thus might represent a common genetic change in the development of the two tumours. Further study is needed to confirm this finding.

The familial adenomatous polyposis coli (APC) gene is located at 5q21 and the gene has been cloned (Kinzler et al, 1991b; Groden et al, 1991). I previously compared the pattern of allele loss in non-cirrhotic HCC with that of colorectal liver secondaries using various probes for chromosome 5q (Chapter 3, Ding et al, 1991). The majority of LOH in colorectal liver metastases was found at the region 5q21-22 while the LOH in non cirrhotic HCC was at 5q35-qter. In the present study on cholangiocarcinomas allele loss also occurred at 5q35-qter. On the other hand probes from 5q21-22, including DNA probes for the MCC and APC genes, did not show any allele loss in cholangiocarcinoma (Tables 7.1 and 7.2). The possible common region involved in both HCC and cholangiocarcinoma appears to be distinct from that encompassing APC.

There has been no reported direct cytogenetic study as yet on cholangiocarcinoma tissue. Chromosome study on two cholangiocarcinoma cell lines showed a number of abnormalities (Storto et al, 1990). It is of particular interest that chromosomes 1 and 5 were among the most commonly involved chromosomes in structural abnormalities in both cell lines. These findings and the results of RFLP analysis in this study suggest that mutation or deletion of a possible tumour suppressor gene located on chromosome 5, distal to 5q21-22, may be involved in the development of cholangiocarcinoma.

As reviewed in Chapter 1, loss or mutation of the p53 tumour suppressor gene at chromosome 17p has been seen at a very high frequency in a variety of human

malignancies. Loss of heterozygosity occurred in 4 out of 9 cholangiocarcinoma shown by p144-D6, and in 2 of 5 shown by pYNZ22, in this study. Both probes are assigned to the region of 17p13, near the locus of the p53 tumour suppressor gene. This finding makes it likely that loss of the p53 gene is also involved in the development of cholangiocarcinoma. It will be of interest to know if there is any overexpression of mutant p53 or point mutation of the p53 gene in cholangiocarcinoma.

In conclusion, this chapter showed allelic losses on chromosomes 1q42-43, 5q35-qter and 17p13 in cholangiocarcinoma. These losses are shared with HCC.

Chapter 8 Loss of heterozygosity in colorectal liver metastases and its association with clinical features

8.1 Introduction

Colorectal carcinoma is one of the most common cancers in the Western world. In England and Wales alone, about 16,000 men and women die of this disease each year (Office of Population Censuses and Surveys, 1986). Colorectal-liver metastases are one of the major causes of death from colorectal carcinomas and one of the most common secondary liver tumours.

As discussed in Chapter 1, colorectal cancer is the best characterized common adult tumour in terms of molecular-genetic changes. Point mutations of Ki-ras and allele losses, or loss of heterozygosity (LOH), at the tumour suppressor gene sites on chromosomes 5q, 17p, and 18q in colorectal cancer have been well established (Vogelstein et al, 1988; Fearon & Vogelstein, 1990; Sidransky et al, 1992a). Loss and/or mutation of the p53 tumour suppressor gene on chromosome 17p in colorectal tumours have been well documented (Fearon & Vogelstein, 1990). The deleted in colorectal cancer (DCC) (18q), and adenomatous polyposis coli (APC) (5q) genes have been cloned and found to be lost or mutated in this cancer (Fearon et al, 1990; Kinzler et al, 1991b; Nishisho et al, 1991; Groden et al, 1991; Miyoshi et al, 1992a, 1992b). Somatic mutations in the MCC (mutated in colorectal cancer) gene, located 150 kb proximal to APC,

have also been demonstrated in colorectal carcinomas (Kinzler et al, 1991a). LOH not only reflects the genetic changes underlying tumorigenesis, but its frequency in colorectal tumours has been shown to be related to clinical features (Vogelstein et al, 1989; Kern et al, 1989). Patients with a higher frequency of allelic losses have a worse prognosis.

Although primary colorectal carcinomas have been extensively studied, the molecular-genetic changes responsible for colorectal liver metastases are currently unknown. In this chapter the extent of LOH in 19 colorectal liver metastases was studied with 24 restriction fragment length polymorphism (RFLP) probes. The association between LOH and patients' clinical features was also analyzed.

8.2 Patients and analysis

Nineteen patients with colorectal liver metastases were analyzed. All of them underwent liver resection. Although colorectal carcinoma is a common disease, only 25% of these patients have liver secondaries. About 10% of these patients with liver metastases undergo resection. Over the last three years I collected specimens from the above 19 patients from the Royal Free and Hammersmith Hospitals. Only the secondary tumours other than primary colorectal cancers could be collected and studied. Of the 19 patients, 15 were followed-up for more than one year after liver resection. The remaining 4 patients had liver resection less than one year ago and therefore were excluded from the survival analysis.

Surgical biopsies from tumoral and non-tumoral liver tissues were snap frozen in liquid nitrogen at the time of operation. Lymphocytes from peripheral blood obtained pre-operatively were also used as a source of normal DNA. Tissue was stored at -70°C until DNA extraction. A portion of each tumour sample was examined histologically to confirm the type and differentiation of tumour.

DNA extraction and analysis were performed as described in Chapter 2. Twenty-four RFLP probes for 18 chromosome arms, ie, 1p, 1q, 2q, 5p, 5q, 7p, 7q, 9q, 11p, 11q, 12q, 13q, 14q, 16p, 16q, 17p, 18q and 20q, were used. The probes and the appropriate restriction enzymes are listed in Table 8.1. The probes included three specific for the MCC, APC and DCC genes, ie, L5-71, 54-D and p15-65

respectively.

8.3 Results

Table 8.1 shows the overall allele loss in the 19 colorectal liver metastases, and Table 8.2 shows the allele status of each tumour examined. Two hundred and fifty-two of 360 Southern blots were informative (heterozygosity: 70%). Altogether 58 allele losses were found out of the 252 heterozygotes (overall percentage of allele loss: 23.0%, Table 8.2). Figure 8.1 shows examples of LOH.

The expected high frequency of LOH was detected on chromosomes 5q, 17p and 18q. As shown in Tables 8.1 and 8.2, the four probes around or from the MCC and APC genes on chromosome 5q (5q21-22) [ie, ECB27 (5q21), L5-71 (MCC), 54-D (APC) and YN5.48 (5q21-22)] detected LOH in 33.3 to 50% of informative tumours. The size of the deleted region was quite variable. For example, in Patient ET loss was confined to the APC locus, but in Patient MK the deletion extended to include both MCC and the locus detected by λMS8, the terminal 5q region. However no LOH for MCC was detected in the absence of LOH for APC.

Two probes screening the region near the p53 tumour suppressor gene locus on chromosome 17p (17p13), p144.D6 and YNZ.22, showed 66.7% and 61.5% of LOH in this group of tumours respectively. The minisatellite probe pMS440 for

chromosome 18q (Armour et al, 1990), the chromosome arm encompassing the DCC gene, detected allele loss in 8 out of 10 heterozygous patients (80%), while p15-65 (DCC) detected 57.1% LOH (Tables 8.1 and 8.2).

The only other chromosome to show consistent allelic imbalance was chromosome 7 (Tables 8.1 and 8.2). This chromosome has been known to show changes in copy number during colorectal tumorigenesis (Gebhart et al, 1992; Bardi et al, 1991) and to harbour genes related with metastatic ability (Habets et al, 1992; Lakshmi et al, 1991).

Table 8.3 shows the association of the frequency of LOH with clinical features in this series of colorectal liver metastases. The mean number (±SEM) of allele losses in tumours was significantly associated with tumour size ($t = 2.561$, $p < 0.05$), lymph node metastases ($t = 3.122$, $p < 0.01$) and survival time ($t = 3.850$, $p < 0.005$). Overall increased frequency of allele loss was associated with a poorer prognosis. The association of LOH with tumour differentiation was not significant ($t = 1.001$, $p > 0.05$), but the trend was that tumours with poor differentiation had a higher occurrence of loss than those with moderate differentiation (Table 8.3).

TABLE 8.1 DNA PROBES USED AND NUMBER OF ALLELE LOSS IN COLORECTAL LIVER METASTASES

Probe*	Chromosomal Location (Gene)	Enzyme Digest	No. of Allelic Losses/ No. of Heterozygotes
λMS1	1p33-35	HinfI	2/17
λMS32	1q42-43	AluI	0/15
P5Gi	2q33-35	TaqI	0/6
pMS621	5p	HinfI	0/8
ECB27	5q21	BglII	2/4
L5-71	5q21 (MCC)	MspI	2/6
54-D	5q21 (APC)	MspI	4/8
YN5.48	5q21-22	MspI	5/10
λMS8	5q35-qter	HinfI	4/14
λMS31	7pter-q22	HinfI	1/16
pλg3	7q31.3-qter	HinfI	2/10
EFD126.3	9q34	PvuII	1/13
H-ras	11p15	BamHI	0/5
pMS51	11q13	HaeIII	1/14
λMS43	12q24.3-qter	HinfI	1/17
pMS626	13q	HinfI	0/8
pMS627	14q	AluI	0/4
3'HVR	16p13.3	PvuII	2/15
pulB1148	16q22.1	TaqI	1/10
p144.D6	17p13	RsaI	10/15
pYNZ.22	17p13	RsaI	8/13
pMS440	18q	HaeIII	8/10
p15-65	18q21.3 (DCC)	MspI	4/7
pMS617	20q	AluI	0/7

* References for probes: λMS1, λMS32, λMS8, λMS31, pλg3 and λMS43: Wong et al, 1987; pMS621, pMS626, pMS627, pMS440 and pMS617: Armour et al, 1990; ECB27: Varesco et al, 1989; L5-71: Kinzler et al, 1991a; 54-D: Kinzler et al, 1991b; YN5.48: Nakamura et al, 1988a; EFD126.3: Nakamura et al, 1987a; H-ras: Krontiris et al, 1985; pMS51: Armour et al, 1989; 3'HVR: Higgs et al, 1986; pulB1148: vander Straten et al, 1983; p144-D6: Kondoleon et al, 1987; pYNZ22: Nakamura et al, 1988b; p15-65: Fearon et al, 1990.

TABLE 8.2 ALLELE STATUS IN 19 COLORECTAL LIVER METASTASES

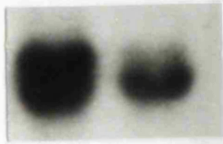
Probe	λMS1	λMS32	P5Gi	pMS 621	ECB27	MCC	APC	YN5.48	λMS8	λMS31	pλg3	EFD 126.3	H-ras	pMS51	λMS43	pMS 626	pMS 627	3' HVR	pulB 1148	p144.D6	YNZ.22	pMS 440	DCC	pMS 617	No. of Probes	Hetero-zygotes	Allele Loss	% Loss
Chromosome	1p	1q	2q	5p	5q	5q	5q	5q	5q	7	7q	9q	11p	11q	12q	13q	14q	16p	16q	17p	17p	18q	18q	20q				
Patient Name																												
ET	1,2	1,2	1,2	1,2	-	1,2	1,(2)	1,2	1,2	1,2	-	-	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,(2)	1,(2)	1,(2)	1,(2)	1,2	24	21	5	23.8
JJ	1,2	-	-	1,2	-	-	(1),2	(1),2	-	1,2	-	-	-	1,2	1,2	1,2	-	1,2	1,2	1,(2)	(1),2	1,(2)	1,(2)	-	24	14	6	42.9
AM	1,2	1,2	-	1,2	-	(1),2	-	-	1,2	1,2	-	1,2	-	1,2	1,2	1,2	-	1,2	1,2	1,2	1,2	(1),2	-	1,2	24	16	2	12.5
MM	1,2	1,2	-	1,2	(1),2	-	1,(2)	1,(2)	-	1,2	-	1,2	-	1,2	1,2	1,2	-	1,2	-	(1),2	-	(1),2	(1),2	1,2	24	16	6	37.5
FB	1,2	1,2	-	-	-	-	1,2	-	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,(2)	-	1,(2)	-	1,2	24	16	2	12.5
MK	1,2	1,2	1,2	1,2	-	(1),2	-	1,(2)	(1),2	-	-	1,2	1,2	1,2	1,2	1,2	-	1,(2)	-	1,(2)	1,(2)	-	1,2	-	24	16	6	37.5
PK	1,2	1,2	1,2	-	-	-	-	(1),2	1,(2)	1,2	1,(2)	1,2	1,2	-	1,2	1,2	1,2	1,2	1,2	-	-	(1),2	1,2	1,2	24	16	4	25.0
DM	1,2	1,2	1,2	1,2	-	1,2	1,2	1,2	-	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	1,2	(1),2	-	1,(2)	1,2	24	21	2	9.5
RV	1,2	1,2	1,2	-	1,2	1,2	1,2	1,2	1,(2)	-	-	-		1,2	1,2			1,2	-	(1),2	-	1,(2)	-	-	21	13	3	23.1
MMo	1,2	1,2	-	1,2	1,(2)	-	-	-	1,2	1,2	-	-		-	-			1,2	1,2	-	1,2	1,2	-	1,2	21	11	1	9.1
AH	1,(2)	1,2	1,2	1,2	-	1,2	(1),2	(1),2	1,2	1,2	1,2	1,2		1,2	1,(2)			1,(2)	-	1,(2)	(1),2	1,(2)	-		20	17	8	47.1
CO	1,2	1,2			-	-	1,2	1,2	1,2	1,2	-	1,2		1,2	1,2			1,2	-	1,2	-	1,2	1,2		18	13	0	0
MB	1,2	1,2			-			-	1,2	1,2	1,2	1,2		1,2	1,2			-	1,2	1,2	1,2				14	11	0	0
ES	1,2	1,2			1,2			1,2	1,2	1,2	1,(2)	1,(2)		1,2	1,2			1,2	-	(1),2	1,2				14	13	3	23.1
PO	1,2	-							-	1,2	1,2	-		1,(2)	1,2			-	-	1,(2)	1,(2)				12	7	3	42.9
JT	1,(2)	-							1,(2)	1,(2)	1,2	1,2		-	-			1,2	-	1,(2)	1,(2)				12	8	5	62.5
BG	1,2	1,2							1,2	-	-	1,2		1,2	1,2			-	1,2	-	1,2				12	8	0	0
BM	-	1,2							-	1,2	1,2	-		-	1,2			-	(1),2	-	(1),2				12	6	2	33.3
PS	-	-							1,2	1,2	1,2	1,2		-	1,2			1,2	-	1,2	-				12	7	0	0
Total No.	**19**	**19**	**11**	**11**	**14**	**12**	**12**	**14**	**19**	**19**	**19**	**19**	**8**	**19**	**19**	**8**	**8**	**19**	**19**	**19**	**19**	**12**	**12**	**10**	**360**	**252**	**58**	**23.0**
No. of Heterozygotes	**17**	**15**	**6**	**8**	**4**	**6**	**8**	**10**	**14**	**16**	**10**	**13**	**5**	**14**	**17**	**8**	**4**	**15**	**10**	**15**	**13**	**10**	**7**	**7**	**252**			
No. of Allele Loss	**2**	**0**	**0**	**0**	**2**	**2**	**4**	**5**	**4**	**1**	**2**	**1**	**0**	**1**	**1**	**0**	**0**	**2**	**1**	**10**	**8**	**8**	**4**	**0**	**58**			
% Allele Loss	**11.8**	**0**	**0**	**0**	**50**	**33.3**	**50**	**50**	**28.6**	**6.3**	**20**	**7.7**	**0**	**7.1**	**5.9**	**0**	**0**	**13.3**	**10**	**66.7**	**61.5**	**80**	**57.1**	**0**	**23.0**			

Homozygosity in the constitutional DNA (non-informative pattern) is indicated as a dash; where the normal tissue was informative the tumour genotype is shown in the table. Heterozygosity is indicated by 1,2. The continued presence of the larger allelic restriction fragment is indicated by "1" and "2" indicates continued presence of the smaller allelic fragment. Allele loss is indicated by ( ). Blank entries indicate no data. Percentage loss is number of allelic losses divided by number of heterozygotes.

TABLE 8.3 ASSOCIATION OF LOH WITH CLINICAL FEATURES IN COLORECTAL LIVER METASTASES

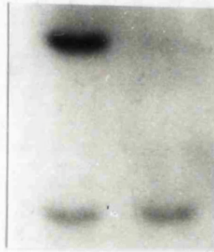
Tumour*	Number of Cases	Number of Allelic Losses (Mean ± SEM)	Significance
Size			
≤ 5cm	7	1.43 ± 0.68	$p < 0.05$
> 5cm	12	4.00 ± 0.65	
Metastasis to Lymph Nodes			
Absence	9	1.56 ± 0.53	$p < 0.01$
Presence	10	4.40 ± 0.72	
Differentiation			
Moderate	16	2.81 ± 0.63	NS**
Poor	3	4.33 ± 0.88	
Survival Time after Operation***			
> 1 year	7	1.71 ± 0.64	$p < 0.005$
≤ 1 year	8	5.13 ± 0.61	

* Features of liver metastases.
** NS: not significant.
*** The follow-up period for four patients was shorter than 1 year, therefore they were excluded.



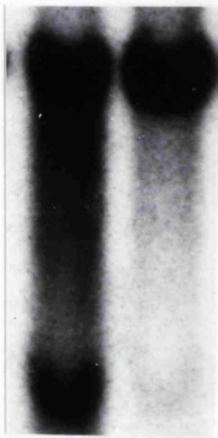
N T

L5-71



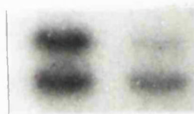
N T

54-D



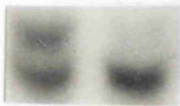
B T

p144-D6



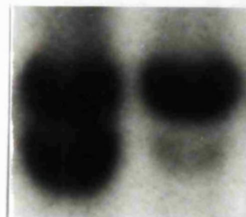
N T

pYNZ.22



N T

pMS440



B T

p15-65

Figure 8.1 Representative autoradiographs of Southern hybridisations of DNA from patients with colorectal liver metastases with L5-71 (5q21, MCC), 54-D (5q21, APC), p144-D6 (17p13), YNZ.22 (17p13), pMS440 (18q) and p15-65 (18q21.3, DCC). B = Blood lymphocyte DNA; N = Non-tumour tissue DNA; T = Tumour tissue DNA. All show allele losses in tumour DNA (indicated by arrows).

8.4 Discussion

This is the first study of loss of heterozygosity and the association of the frequency of allele loss with clinical features carried out exclusively on liver metastases from colorectal carcinoma. The results showed that these secondary tumours had a similar high frequency of LOH on chromosomes 5q, 17p and 18q, as has been shown for primary colorectal tumours (Vogelstein et al, 1988; Fearon & Vogelstein, 1990). No consistent LOH has been found in any other chromosomes apart from a lower frequency on chromosome 7. Scattered allelic losses at other sites probably represent random losses (Lasko et al, 1991). It is not certain at this stage whether all the losses in the secondary tumours are inherited from the primary tumours or whether some of them occurred after metastasis. A study of both primary and secondary tumours from same patients would help to elucidate this problem.

In primary colorectal tumours, allele loss on chromosome 5q may occur in relatively early stages of tumorigenesis, such as adenomas and early stage carcinomas (Rees et al, 1989; Fearon & Vogelstein, 1990). With the successful cloning of APC, mutation and/or deletion of this gene were found in a high proportion of both colorectal adenomas and carcinomas, providing evidence that inactivation of APC is a very early, possibly initiating, event (Powell et al, 1992). This study has shown that four probes from the region including the MCC and APC genes, ie, 5q21-22, detected LOH in 33.3 - 50% of colorectal liver metastases

(Tables 8.1 and 8.2). The results are consistent with those found in primary tumours and support the hypothesis that events involving chromosome 5 are early events since there is no increased frequency in metastatic tissue.

Kern et al (1989) reported that primary colorectal carcinomas with allele loss on 17p and 18q might have an increased propensity to metastasize to a distant organ; no such association was seen with deletion on chromosome 5q. In our study, higher frequency of allele loss on 17p and 18q compared with that on 5q in the liver secondaries may support the observation by Kern et al (1989).

Considering that at least two genes concerned with metastasis have been mapped to chromosome 7 (Habets et al, 1992; Lakshmi et al, 1991) the relatively low frequency of LOH on that chromosome is perhaps surprising. However, it is probable that these genes act dominantly and that increased expression or copy number is the main mechanism of activation.

The frequency of allele loss in primary colorectal tumours has been shown to be associated with prognosis; tumours with a high rate of LOH constituting a more aggressive subset (Vogelstein et al., 1989; Kern et al., 1989). I analyzed such an association in colorectal liver metastases in this study, and found that patients with larger tumours, metastasis to lymph nodes and shorter survival time had an increased frequency of LOH in their tumours (Table 8.3). Tumours with poor differentiation may also have more allele losses, but the number in this group (3

cases) was small and the difference was not statistically significant.

In conclusion, the results showed that colorectal liver metastases have a high frequency of coincident LOH on chromosomes 5q, 17p and 18q. Deletion of all three tumour suppressor genes at these sites may accelerate tumour progression and together with other unidentified changes increase the likelihood of liver metastasis with a fatal outcome.

Chapter 9 Loss of Heterozygosity in carcinoma of the Pancreas

9.1 Introduction

Carcinoma of the pancreas (CaP) is an increasingly common disease. The prognosis of CaP is poor with an overall mean survival of 3-4 months; only about 5% of patients survive for 2 years. Few tumours are amenable to resection with the chance of "cure". Neither radiotherapy nor cytotoxic drugs improve the prognosis significantly.

As discussed in Chapter 1, there are few reports about allele loss in CaP, in contrast to the comprehensive studies of other common malignancies, such as those in breast, colorectum and lung (Chapter 1). Allele losses on chromosome 11 in both sporadic and familial pancreatic endocrine tumours, related to multiple endocrine neoplasia type 1 (MEN 1), have been reported (Bale et al, 1991; Teh et al, 1990). There have been preliminary reports of allele loss on 5q for exocrine CaP (Michelassi et al, 1989; Westbrook et al, 1990). It is of interest to know whether allele loss on chromosome 11 or other chromosomes also occurs in exocrine CaP, whether there is any difference in allele loss between exocrine and endocrine CaP, and whether there is any association between allele loss and clinical course in patients with CaP. In this chapter allele loss in both exocrine and endocrine CaP, and the relationship between allele loss and clinical parameters were studied.

9.2 Patients and analysis

Fifteen patients with carcinoma of the pancreas were studied, including two with endocrine CaP and 13 with exocrine CaP. Of the 13 with exocrine CaP 12 had tumours of the head of pancreas while the remaining one had a tumour of the ampulla of vater. All underwent resection of their tumours (either by partial or total pancreatectomy) except one patient with peritoneal secondaries that had palliative bypass (hepaticojejunostomy and gastrojejunostomy). Of the 13 patients with exocrine CaP, four had their tumours localised to the pancreas while the other nine had metastases in local lymph nodes or extension of their tumours in adjacent portal vein. Judged by the operating surgeons, seven patients had small tumours that were resected radically while the remaining six had large tumours or late diseases such that their surgical procedures should be considered palliative. All patients, if applicable, were followed-up for detection of post-operative recurrence. The data were available until one year after tumour resection.

Surgical biopsies from the tumoral and non-tumoral pancreas tissues were snap frozen in liquid nitrogen at the time of operation. Lymphocytes from peripheral blood obtained pre-operatively were also used as a source of normal DNA. Tissue was stored at -70°C until DNA extraction. None of the patients received chemotherapy or radiotherapy prior to surgery and tumour samples were examined histologically to confirm the type of tumour present and the degree of differentiation of tumour cells.

DNA extraction and analyses were done as described in Chapter 2. The 21 RFLP probes for chromosomes 1, 5, 6, 7, 9, 11, 12, 13, 14, 16, 17 and 18 and the appropriate restriction enzymes are listed in Table 9.1. The fractional allele loss (FAL) was defined in a tumour as the number of chromosomal arms on which allelic loss was observed divided by the number of chromosomal arms for which allelic markers were informative in the patient's normal cells (Vogelstein et al, 1989).

9.3 Results

Table 9.1 shows the overall allele loss in both exocrine and endocrine CaP; and the results of allele loss obtained in each tumour are shown in Table 9.2. Overall, 179/262 Southern blots were informative (heterozygosity: 68.3%) and the overall LOH was 22/179 informative cases (12.3%). Figure 9.1 shows representative examples of allele loss.

Both tumours from the two patients with endocrine CaP had multiple allelic losses, with deletions on 5 chromosomal arms each (Tables 9.1 and 9.2). The FAL was 0.375 and 0.455 for the two tumours respectively. The common regions deleted were at 1p33-35 (probe: λMS1), 1q42-43 (λMS32) and 11p15 (H-ras). One of the two patients (Patient JJ) had allele loss at 11q13 (probe: pMS51), where the MEN 1 gene maps (Larsson et al, 1988), while the other (HA) was non-informative for that marker. Patient HA showed LOH at 5q21-22, in the region of the adenomatous polyposis coli (APC) gene, but both of the two probes used for this region (ECB27 and YN5.48) showed a homozygous pattern for patient JJ and were hence uninformative. Patient JJ had a loss in 6q27, while Patient HA was homozygous for the marker for this region.

As shown in Tables 9.1 and 9.2, the 13 exocrine CaP had LOH in three out of 12 informative cases (25%) in the region 1p33-35, five out of seven (71.4%) in 6q27, one out of 11 (9%) at 9q34 and two out of seven (28.6%) at 11q13, hence both

exocrine and endocrine tumours exhibited LOH at 1p32-33, 6q27 and 11q13, the latter of which is close to the MEN 1 gene. The probe P3.8R for the RB1 gene at 13q14.2 showed no allele loss, nor did another probe, cMS626, screening 13q in either exocrine and endocrine tumours. For both groups, there was no allele loss found at 17p13 (where the p53 tumour suppressor gene maps), shown by the two probes used (p144-D6 and pYNZ22). The FAL in exocrine tumours ranged from 0 to 0.25 (Table 9.2).

The possible relationship between allele loss and some clinical parameters in exocrine CaP was analyzed (Table 9.3). Among seven small tumours (<3cm), three allelic losses were found, while six large tumours (>3cm) had eight LOH ($p < 0.05$). Similarly tumours with poorer differentiation, metastasis and recurrence had more allelic losses, and the differences were all statistically significant (Table 9.3).

TABLE 9.1 LOSS OF CHROMOSOMAL HETEROZYGOSITY IN HUMAN CARCINOMA OF THE PANCREAS

Probe	Locus	Enzyme	Exocrine CaP (n=13)	Endocrine CaP (n=2)	Reference for probe
λMS1	1p33-35	HinfI	3/12*	2/2	Wong et al, 1987
λMS32	1q42-43	AluI	0/11	2/2	Wong et al, 1987
cMS621	5p	HinfI	0/5	0/2	Armour et al, 1990
ECB27	5q21	BglII	0/4	0/1	Varesco et al, 1989
YN5.48	5q21-22	MspI	0/4	1/1	Nakamura et al, 1988a
λMS8	5q35-qter	HinfI	0/10	0/1	Wong et al, 1987
D6S193	6q27	(PCR)	5/7	1/1	Takiguchi et al 1993
λMS31	7pter-q22	HinfI	0/8	0/2	Wong et al, 1987
pλg3	7q31.3-qter	HinfI	0/5	1/2	Wong et al, 1987
EFD126.3	9q34	PvuII	1/11	0/2	Nakamura et al, 1987a
H-ras	11p15	BamHI	0/3	2/2	Krontiris et al, 1985
pMS51	11q13	HaeIII	2/7	1/1	Armour et al, 1989
λMS43	12q24.3-qter	HinfI	0/11	0/2	Wong et al, 1987
P3.8R	13q14.2	HindIII	0/8	0/2	Friend et al, 1986
cMS626	13q	HinfI	0/5	0/2	Armour et al, 1990
cMS627	14q	AluI	0/5	0/1	Armour et al, 1990
3'HVR	16p13.3	PvuII	0/10	1/1	Higgs et al, 1986
pulB1148	16q22.1	TaqI	0/0	0/0	vd Straten et al, 1983
p144-D6	17p13	RsaI	0/9	0/2	Kondoloen, et al, 1987
pYNZ22	17p13	RsaI	0/6	0/2	Nakamura et al, 1988b
cMS440	18q	HaeIII	0/5	0/1	Armour et al, 1990

* No of allele loss/ No of informative cases

TABLE 9.2 ALLELE LOSS IN INDIVIDUAL TUMOURS

Patient Name & Age (year)	Chromosomal arms on which allelic markers were lost	Arms with no allele loss	FAL
Endocrine CaP (n=2)			
JJ (32)	1p, 1q, 6q, 11p, 11q, 16q	5p, 5q, 7p, 7q, 9q, 12q, 13q, 14q, 17p,18q	6/16 (0.375)
HA (47)	1p, 1q, 5q, 7q, 11p	5p, 7p, 9q, 12q, 13q, 17p	5/11 (0.455)
Exocrine CaP (n=13)			
CJ (48)	6q, 9q	1p, 1q, 5p, 7p, 7q, 12q, 13q, 14q, 16p, 17p, 18q	2/13 (0.154)
LA (49)	6q	1p, 1q, 5p, 5q, 7p, 7q, 9q, 11p, 13q, 14q, 16p, 17p	1/13 (0.077)
BA (68)	1p, 6q	1q, 5p, 5q, 7p, 9q, 11p, 13q, 16p, 17p, 18q	2/12 (0.167)
BE (52)	6q, 11q	1p, 1q, 5p, 5q, 7p, 7q, 9q, 12q, 13q, 14q, 16p, 17p, 18q	2/14 (0.143)
SD (55)		1q, 5p, 5q, 6q, 7p, 7q, 9q, 11p, 11q, 13q, 16p, 17p	0/12 (0.000)
KE (50)	6q	1p, 1q, 5q, 7q, 11q, 12q, 13q, 16p	1/9 (0.111)
PD (60)		1p, 1q, 5q, 7p, 9q, 13q, 16p, 17p, 18q	0/9 (0.000)
GP (61)		1p, 1q, 5q, 6q, 7p, 9q, 11q, 13q, 16p, 17p, 18q	0/11 (0.000)
CV (51)	1p	1q, 5q, 7p, 9q, 13q, 16p, 17p	1/8 (0.125)
NW (67)		1p, 1q, 5q, 6q, 9q, 11q, 16p, 17p	0/8 (0.000)
MF (33)	11q	1p, 1q, 5q, 9q, 12q, 17p	1/6 (0.167)
PF (56)	1p	1q, 5q, 12q, 17p	1/4 (0.250)
KW (67)		1p, 9q, 11q, 12q, 17p	0/5 (0.000)

TABLE 9.3 ASSOCIATION OF ALLELE LOSS WITH CLINICAL COURSE IN HUMAN EXOCRINE CARCINOMA OF THE PANCREAS

Tumour	No of Cases	No of Allele Loss	Significance
Size*			
Small	7	3	$p < 0.05$
Large	6	8	
Differentiation of Tumour Cells			
Well	2	0	
Moderate	5	4	$p < 0.05$
Poor	2	3	
Unclassified	4	4	
Metastasis**			
Presence	9	9	$p < 0.05$
Absence	4	2	
Recurrence			
Presence	5	7	$p < 0.05$
Absence	4	1	
Not Applicable	4	3	

* Size: <3cm = small, >3cm = large
** Metastasis: regional lymph nodes or liver deposits

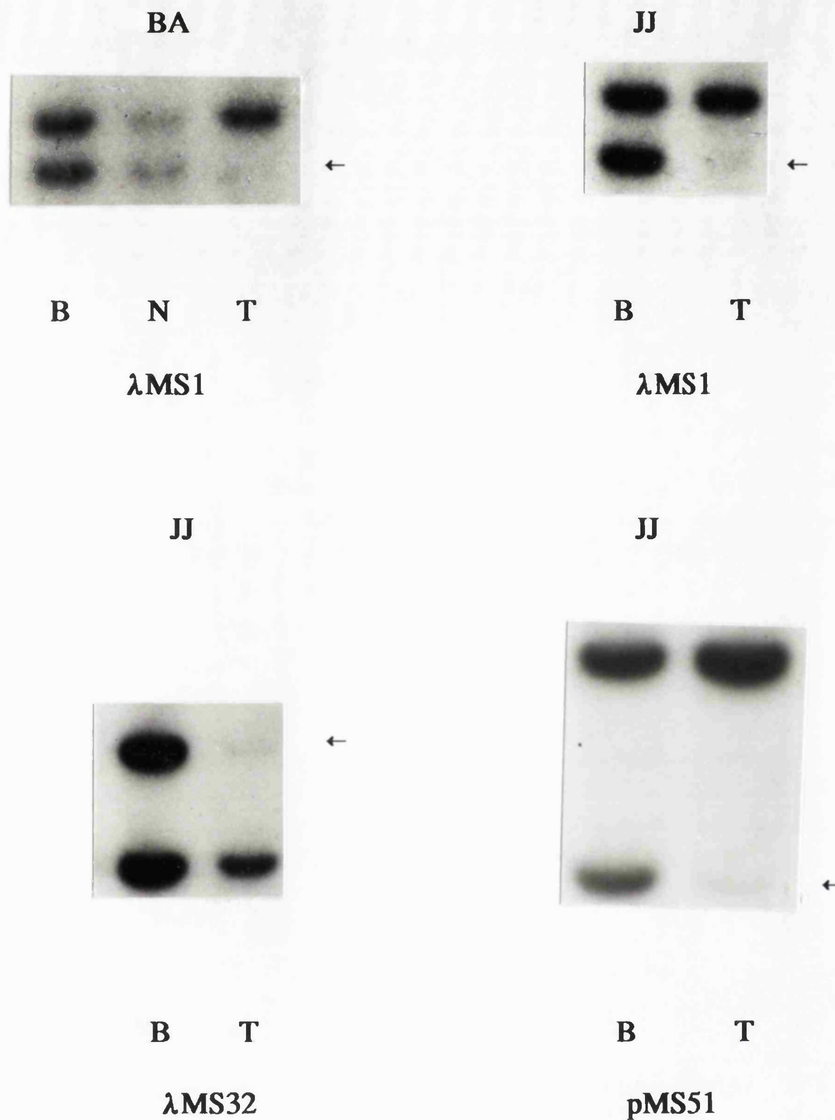


Figure 9.1 Representative autoradiographs of Southern hybridisation in carcinoma of the pancreas with λMS1 (1p33-35), λMS 32 (1q42-43) and pMS 51 (11q13). B = Blood lymphocyte DNA, N = Non-tumour tissue DNA, and T = Tumour tissue DNA. All showed allelic losses in tumour DNA (indicated by arrows). Patient BA had exocrine CaP while Patient JJ had endocrine CaP.

9.4 Discussion

This is the first study comparing the frequency of LOH in exocrine and endocrine carcinomas of the pancreas. The results indicated that both cases of endocrine CaP had multiple allelic losses and that this loss of heterozygosity occurred at a significantly higher frequency than that occurring in a group of patients with exocrine tumours. This may be related to the fact that endocrine CaP can be due to MEN 1, which usually has a background of genetic predisposition. The ages of those two patients at the time of diagnosis were younger than those of the patients with exocrine CaP (Table 2), which may also suggest that these patients with endocrine tumours were more likely to have a genetic predisposition.

The results showed loss of heterozygosity on chromosomes 1p33-35, 6q27 and 11q13 in both exocrine and endocrine carcinomas of the pancreas. Allele loss at 11q13 has been revealed in both sporadic and familial tumours arising in the endocrine pancreas (Teh et al, 1990; Bale et al, 1991). The informative patient with endocrine CaP in this chapter also had allele loss in this region. Interestingly, there was LOH shown by the marker at this region in two of seven informative cases of exocrine CaP, which has not been reported before. Whether the change in this region is involved in the development of exocrine CaP needs further study. The loss of one copy of the H-ras gene at 11p15 in CaP has not been previously reported, which occurred in both cases of endocrine CaP in the present study and may be important in that type of tumour.

There are relatively few cytogenetic studies on CaP, but one study of particular interest showed that four out of 6 CaP cytogenetically studied had deletions and translocations involving 1p, all leading to loss of chromosomal material distal to 1p32 (Johansson et al, 1992). Allele loss at 1p33-35 was shown by the probe λMS1 in this study in both exocrine (three out of 12 informative cases, Table 9.1) and endocrine (2/2, Table 9.1) CaP, which may indicate a possible tumour suppressor gene located there for both types of CaP, but as this region is frequently involved in advanced cancers of other types, its loss may be related to tumour progression (Sager, 1989; Johansson et al, 1992). More cases are needed to confirm the preliminary finding. It is of interest that allele loss also occurred on chromosome 1q in both endocrine CaP, but not in exocrine tumours, which may suggest that loss of genetic material there may also be important for endocrine tumours, but not for exocrine ones.

An exciting finding was the high frequency of allele loss on chromosome 6q. This chromosome arm was frequently found abnormal in cytogenetic study (Johansson et al, 1992). Both the cytogenetic and molecular results suggested that a putative tumour suppressor gene for carcinoma of the pancreas could locate on this chromosome arm.

Loss or mutation of the p53 tumour suppressor gene at 17p13 has been seen at very high frequency in several common human malignancies (Chapter 1). A recent study in exocrine CaP also showed high frequency of overexpression of mutant

forms of p53 by immunohistochemistry and of point mutations of the p53 gene by direct sequencing of genomic DNA (Barton et al, 1991b). Hence it was surprising to find that there was no allele loss shown by either probe (p144-D6 or pYNZ22) at 17p13 in either group of CaP in our study. This was in agreement with the finding of Westbrook et al (1990), who did not find any LOH with pYNZ22 in 7 informative pancreatic adenocarcinomas. It will be of interest to know if there is any overexpression of mutant p53 or point mutation of the p53 gene in the series of CaP studied in this thesis.

Frequent rearrangement or loss of the prototype tumour suppressor gene, retinoblastoma (RB), also occurs in some other types of tumours (Horowitz et al, 1990), but no allele loss occurred at this locus in the two groups of CaP in this study.

Westbrook et al (1990) reported allele loss in 2 out of 7 informative exocrine CaP on chromosome 5 and suggested that the genetic changes associated with allele loss on that chromosome might be a common denominator in the development or progression of the gastrointestine cancers including those of colorectum and pancreas. In this study, the one informative endocrine CaP showed allele loss at 5q21-22, close to the APC gene, but four probes on chromosome 5 did not reveal LOH in the exocrine CaP group.

Vogelstein et al (1989) reported that for colorectal carcinomas, patients with more

LOH had a considerably worse prognosis than did the other patients. In this chapter, the possible correlation between frequency of LOH and some clinical parameters within the group of exocrine CaP was analyzed (Table 9.3). There was a significant correlation found between the frequency of allele loss and the tumour size, tumour differentiation, and presence or absence of tumour metastasis and recurrence. Tumours with more allelic losses appeared to be more aggressive.

In conclusion, the results in this chapter showed that both exocrine and endocrine CaP had allele loss in the regions 1p33-35, 6q27 and 11q13. In the group of exocrine CaP patients with more allelic losses in their tumours had poorer prognosis.

Chapter 10 Identification of Chromosome 5 Clones for HCC

10.1 Introduction

Consistent loss of heterozygosity in DNA of a certain tumour in a particular chromosomal region may represent a rough location of a tumour suppressor gene for this tumour (Lasko et al, 1991; Chapter 1). The results in Chapter 3 and Chapter 7 strongly suggest that a putative tumour suppressor gene for hepatocellular carcinoma, and possibly also for cholangiocarcinoma, may be located in the terminal region of the long arm of chromosome 5 (5q35-qter). In this chapter, the pilot study for cloning this gene is described and the future strategy for completion of the cloning proposed.

The human genome consists of about 3×10^9 base pairs. At a rough estimate, the average density of genes transcribed in a given tissue is only four per one million base pairs, thus the isolation of a disease gene would be facilitated by focusing efforts on expressed sequences. Specifically, identifying sequences from the appropriate chromosome region expressed in the affected tissue could be the initial step in a strategy to isolate a disease gene (Hochgeschwender & Brennan, 1991). Tumour suppressor genes are normal cellular genes and are expressed in normal tissues (Sager, 1989). The strategy used in this project for cloning HCC - tumour suppressor gene started from isolation of sequences from chromosome 5 expressed in the normal human liver.

Wong et al (1989) reported a novel protocol - cross screening of two libraries - for identifying sequences from a particular chromosome expressed in a given tissue. They attempted to isolate human X-chromosome-specific sequences that are expressed in chorioretinal tissue of the human eye and which may underlie X-linked chorioretinal diseases. For doing this, they amplified the inserts of the human chorioretinal cDNA library (expressed sequences of the chorioretinal tissue) by polymerase chain reaction. The PCR product was then used to screen the X-chromosome genomic library to identify sequences which were common to both libraries. Using this method, they systematically isolated 18 human X-chromosome clones that were expressed in human chorioretinal tissue (Wong et al, 1989).

Using the similar strategy described by Wong et al (1989), I screened a human chromosome 5 genomic library with PCR amplified inserts of a normal human liver cDNA library. Twenty-one positive clones were obtained after the cross screening. These were human chromosome 5 sequences that were expressed in normal human liver. They were bacteriophage (phage) clones, and thus subsequently recloned into plasmid vectors to facilitate further use. The size of the inserts was determined. Three single copy DNA sequences were obtained from six of the 21 clones. Figure 10.1 illustrates the strategy of library cross screening and subsequent treatment of positive clones.

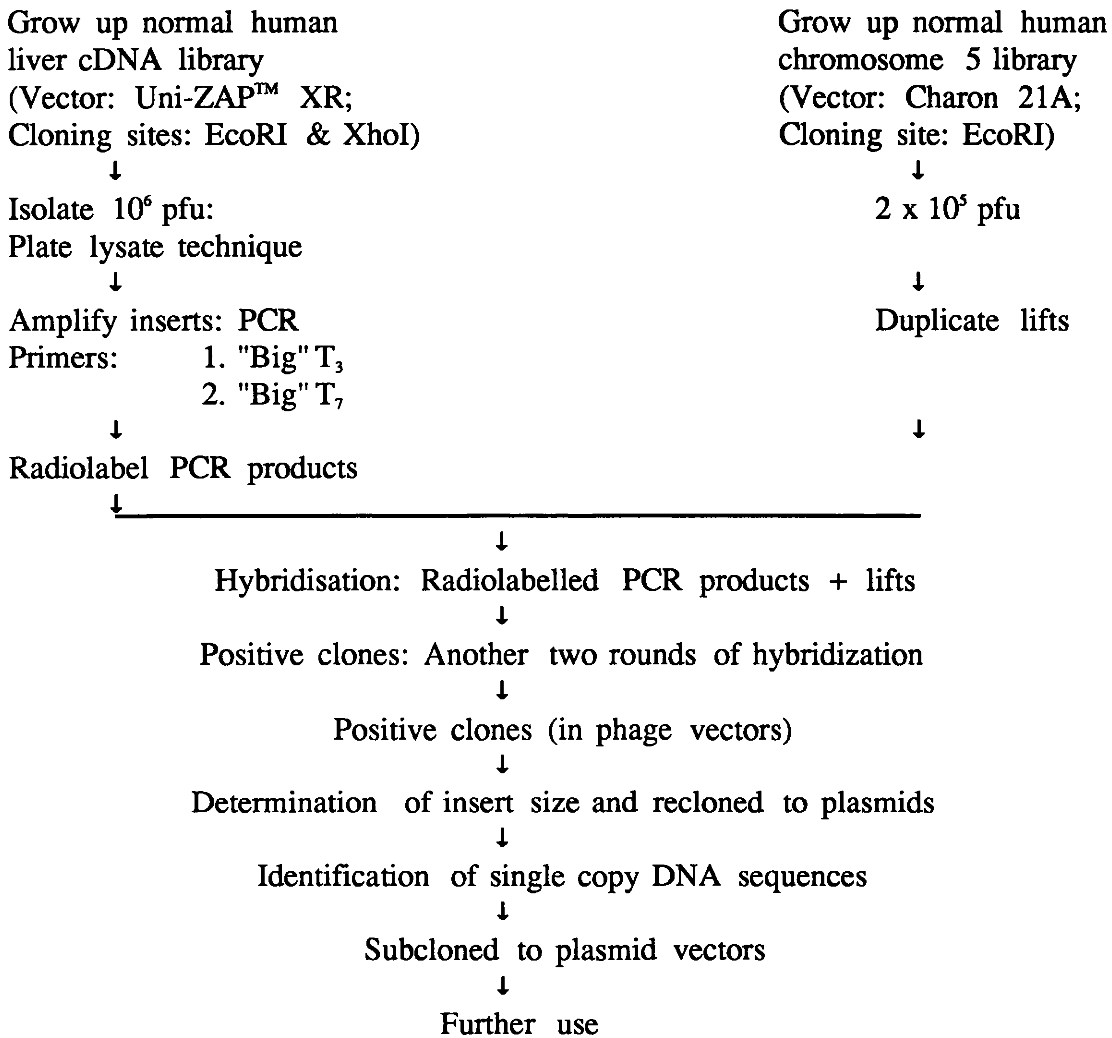


Figure 10.1 Strategy of library cross screening and identification of positive clones

10.2 Cross screening of DNA libraries

10.2.1 Specificity of the two libraries

The human liver cDNA library was established from a 49 year old white, normal male (Stratagene). The vector was Uni-ZAP™XR. This is an insertion lambda phage vector equipped with multiple cloning sites (polylinker) within the plasmid bluescript that can be zapped out from the phage (Short et al, 1988). The cloning sites of this library were EcoRI and XhoI. These sites were flanked by T_3 and T_7 RNA polymerase promoters. Average insert size is 1.5 kb. The titre of the library was 4.5 x 10^7 pfu/µl (for titration of phage libraries, see Section 2.2.8.2, Chapter 2).

Human chromosome 5 genomic library was constructed from flow-sorted chromosomes (Deaven et al, 1986). The vector of the library was phage Charon 21A and the cloning site was EcoRI. The size of inserts was 4 kb average and the inserts were cut with EcoRI. The titre was 1.71 x 10^6 pfu/µl.

10.2.2 Amplification of cDNA library inserts by PCR

DNA was recovered from 10^6 λ clones of the library (Chapter 2). A total of 45 µg DNA in 300 µl TE (0.15 µg/µl) was obtained. 0.5 µg of DNA was used as template for each of the four PCR reactions. Since the cloning sites were flanked

by T_3 and T_7 RNA polymerase promoters, the sequences of these promoters were used as primers. In order to raise the specificity, big primers (30pb) were used. The reaction was performed as described in Chapter 2. The amplified DNA was checked by running on a minigel (Figure 10.2). They were smears of DNA around the size 1.5 kb, in accordance with the library's average insert size.

10.2.3 Three rounds of screening of genomic DNA library with PCR products of cDNA library

Duplicate plaque lifts were made from 2 x 10^5 colonies of the chromosome 5 genomic DNA library as described in Chapter 2. DNA was fixed on nylon membranes by baking the membranes at 80°C for 2 hours. The PCR products of cDNA library inserts were purified and radiolabelled as probes to hybridise the above duplicate membranes (Chapter 2). After the first round of screening, 35 positive clones were identified as shown on both Film As and Film Bs (for identification of positive clones, see Section 2.2.9.5, Chapter 2). Following another two rounds of screening, twenty-one purified positive clones were picked out after the third hybridisation (Figure 10.1). Figure 10.3 shows a representative autoradiograph of positive clones.

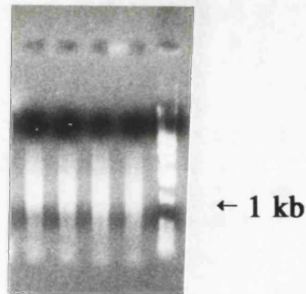


Figure 10.2 The PCR products of inserts of human liver cDNA library.

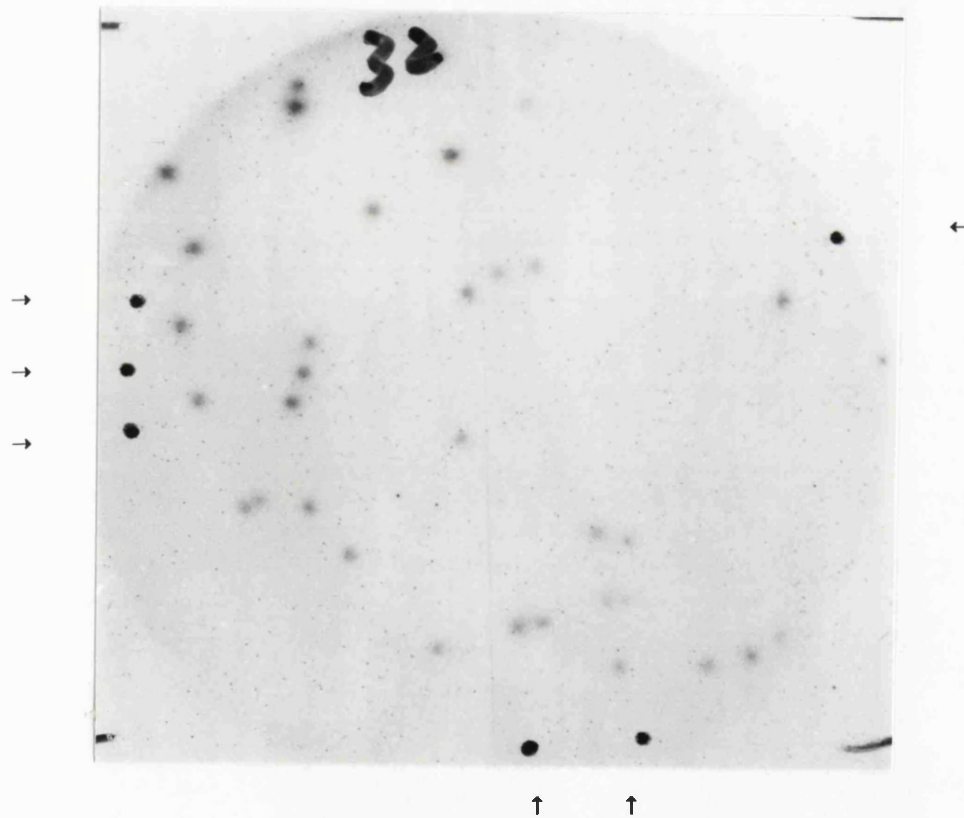


Figure 10.3 The representative autoradiograph showing the positive clones. The dark dots indicated by arrows are those made for orientation.

10.3 Characterization of positive clones

10.3.1 Determination of the insert size

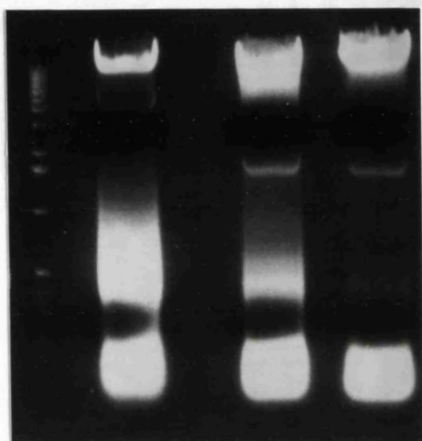
The positive clones obtained were in λ phage vector Charon 21A. Small scale preparation from each of these λ clones was made as described in Section 2.2.10.1, Chapter 2. The cloning site of these clones was EcoRI. The DNA from small scale preparation was digested with this enzyme and run alongside the yeast derived 1 kb ladder in TAE buffer. The insert size was determined according to the position run on the gel (Figure 10.4a). Table 10.1 shows the insert size of the 21 clones, ranged from 1.1 to 5.0 kb.

TABLE 10.1 PARTIAL CHARACTERISTICS OF POSITIVE CLONES

Clone No	Insert size (kb)*	Number of the known restriction sites			Single copy sequences
		PstI	HindIII	BamHI	
1	4.4	1	2	1	No single copy identified yet
2	4.0	1	1	0	1.8 kb HindIII fragment, 0.9 kb PstI fragment
3	5.0	0	0	0	No single copy identified yet
4	4.5	1	0	0	No single copy identified yet
5	1.6	0	0	0	No single copy identified yet
6	3.0	1	0	0	1.1 kb PstI fragment

* Insert size (kb) of other clones: No7: 4.0; No8: 1.1; No9: 4.5; No10: 2.8; No11: 4.0; No12: 2.8; No13: 2.8; No14: 2.0; No15: 2.5; No16: 3.8; No17: 3.8; No18: 2.0; No19: 3.0; No20: 3.2; No21: 1.8.

a



b

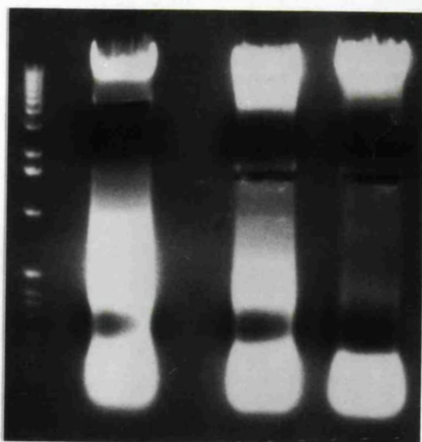


Figure 10.4 The separation of insert from phage vector and the determination of insert size. a. Insert separation; b. Insert bands were excised.

10.3.2 Cloning of λ clones into plasmid

In order to facilitate the subsequent use of these λ clones, they were recloned to plasmid vectors. DNA from small scale preparation of λ clones was digested with EcoRI and the insert was separated from the λ phage vector by electrophoresis (Figure 10.4a). The insert band was excised from the gel (Figure 10.4b) and the DNA purified by Geanclean II (Section 2.2.10.1, Chapter 2). The insert was then ligated with plasmid vector bluescript KS^+. This vector was prepared by cutting with EcoRI as described in Section 2.2.10.2, Chapter 2. The ligated plasmid clones were then used to transform the XL-1 blue E coli and a small scale preparation of the clones was made to check whether the ligation was successful. DNA from the small scale preparation was digested with EcoRI and separated on agarose gel. The size of bluescript KS^+ was 2958 bp (Short et al, 1988) and the inserts from the positive clones could be separated with the vector (Table 10.1). Figure 10.5 showed the examples of separation of inserts from bluescript (~2.9kb). The XL-1 blue transformed with the ligated plasmid clones were stored at -70°C in glycerol.

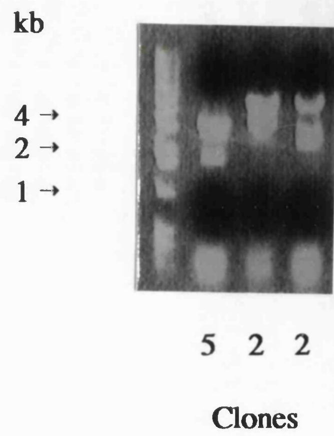


Figure 10.5 Separation of inserts of Clone 2 (insert size: ~4kb) and Clone 5 (insert size: ~1.6kb) from the vector bluescript (size: ~2.9kb).

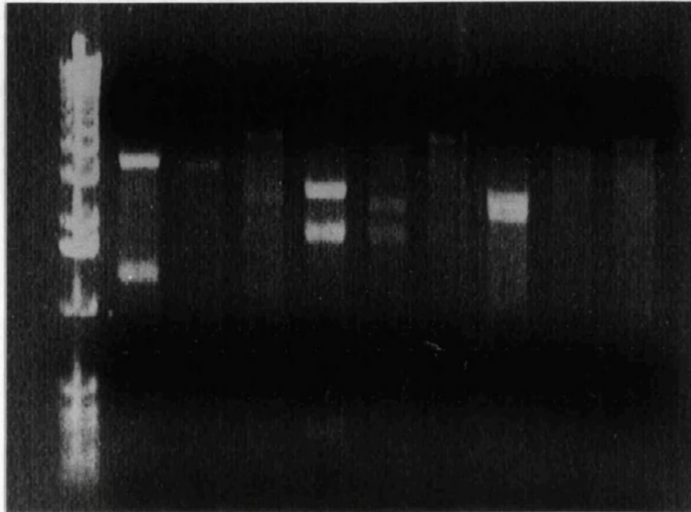
10.3.3 Preparation and subcloning of single copy DNA sequences

Repeated sequences are found dispersed throughout eucaryotic genomes. Repetitive Alu and Kpn sequences, for example, are interspersed and occur approximately 900,000 and 100,000 times per genome, respectively (Weiner et al, 1986). Therefore, large DNA sequences, such as the clones obtained above, will invariably contain these repetitive DNA sequence elements. If the entire clone were to be used as a hybridisation probe the presence of repetitive DNA sequences will confound the results obtained (Nisson et al, 1991). Thus it was necessary to obtain single copy sequences from those positive clones for further use as hybridisation probes. Large scale preparation of plasmid clones was made and sufficient insert DNA was obtained from six of the clones (Clones No 1-6). They were digested with PstI, HindIII and BamHI respectively. These restriction enzymes cut DNA sequences infrequently. The digested DNA was separated on agarose gel, blotted and hybridised with total human genomic DNA (Section 2.2.10.4, Chapter 2). Three single copy DNA sequences were obtained so far and cloned into bluescript again. Figure 10.6 shows the results of digestion of Clones No1-3 and autoradiograph after hybridisation with total human genomic DNA. Clone No2, for example, was cut by HindIII once (hence one HindIII site, Table 10.1), and the two HindIII fragments were of size of 1.8 kb and 2.2 kb respectively (Figure 10.6a). On the autoradiograph, only the 2.2 kb fragment was hybridised with total human genomic DNA, while the 1.8 kb fragment was not (Figure 10.6b). The latter was a single copy sequence.

a

kb

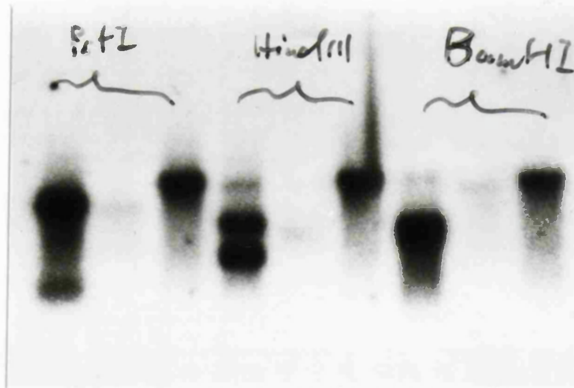
4 →
3 →
2 →
1 →



1 2 3 1 2 3 1 2 3

Pst I HindIII BamHI

b



1 2 3 1 2 3 1 2 3

Figure 10.6 Determination of single copy sequence.

10.4 Discussion

As Dr Bert Vogelstein put it, identification of a tumour suppressor gene is "a Herculean task" (see Marx, 1991). Completing the project for isolation of the putative tumour suppressor gene from chromosome 5 for HCC may need further 3-4 years and is obviously beyond the scope of this thesis. The fact that it took more than one year to finish the work described in this chapter alone may support the estimation of the time needed for completion of the project.

To identify a gene from the human genome which consists of about 3×10^9 base pairs is a kind of job of finding the needle in a haystack (Hochgeschwender & Brennan, 1991). The strategy of cross screening of two libraries proposed by Wong et al (1989) would make the job easier since it can dramatically narrow the scope of searching. By this method, twenty-one chromosome 5 clones which were expressed in normal human liver were obtained in this chapter.

These clones were originally in phage vector Charon 21A and were not convenient for further use. Therefore effort was made to reclone the inserts from phage vectors into plasmid bluescripts. The bluescripts were chosen because they have several advantages for future uses of these clones (Short et al, 1988). They have been designed to simplify commonly used cloning and sequencing procedures. These include construction of nested deletions for DNA sequencing, generation of RNA transcripts in vitro and site specific mutagenesis. They have an extensive

polylinker with 21 unique restriction recognition sites. Flanking the polylinkers are T_3 and T_7 RNA polymerase promoters that can be used to synthesize RNA in vitro. The sequences of these promoters can serve as primers for sequencing or PCR the inserts. The polylinker and T_3 and T_7 promoter sequences are present in the N-terminal portion of a LacZ gene fragment (Sambrook et al, 1989). Bluescripts which have no inserts in the polylinker will grow as blue colonies on the appropriate strains of bacteria (ie, strains containing the lacZ-delta-M15 on an F' episome, eg, XL1-blue). Bluescripts with inserts will grow as white colonies on the same strain, since the inserts disrupt the coding region of the LacZ gene fragment. This colour difference makes it easy to select recombinant clones (Chapter 2).

It is necessary to obtain single copy DNA sequences from the positive clones. As mentioned above, repeated sequences are found dispersed throughout eucaryotic genomes. Most phage and cosmid clones contain at least one representative of a family of highly repeated sequences, eg, the Alu or Kpn family (Sealey et al, 1985). The presence of such repetitive elements limits the uses of clones as hybridisation probes. The procedures employed in this chapter have proved to be successful to isolate the single copy DNA sequences. The immediate future work is to analyze other clones in this way to obtain single copy sequences from them.

It is surprising that only 21 chromosome 5 clones that are expressed in normal

liver have been obtained. A chromosome 5 library may contain upwards of 10^8 bp of DNA, and the number of genes expressed in normal liver tissue from this chromosome should be about 1.5 - 2 orders of magnitude higher than the number of positive clones obtained. The result might arise from the limitations of the approach used. For the cross-screening of the two libraries, the probes used were the complex mixture of cDNA, some of which might be present in low copy number. Thus it was possible that some cDNA failed to hybridise the corresponding genomic chromosome 5 DNA, leading to the underestimation of the number of positive chromosome 5 clones that are expressed in normal liver tissue. Using the similar method, the original investigators who introduced the approach of cross-screening obtained only 18 chromosome X clones that were expressed in chorioretinal tissue of the human eye (Wong et al, 1989). Such a low number of positive clones might also be an underestimation, because of the limitations of this approach.

For final identification of the putative tumour suppressor gene, therefore, alternative experiment routes should be used. One of the preferred strategies is to utilize the chromosome 5q35-ter - specific cosmid clones (Saltman et al, 1992). Four hundred and fifty such clones have been sent to us by Dr M Lovett from America. The probe λMS8 will be hybridized with these cosmid clones. Positive clones after hybridization will be those around λMS8, the marker detecting a high frequency of LOH in HCC and cholangiocarcinoma. Thus they will be those of interest. Single copy sequences will be obtained from these clones, and analyzed

to get CA/GT dinucleotide repeats [$(dC\text{-}dA)_n.(dG\text{-}dT)_n$ sequences, Weber, 1990]. These highly heterogenous repeats will be used for polymorphism analysis of HCC DNA. If any CA/GT sequence reveal loss of heterozygosity in HCC DNA, the clone from which the CA/GT repeat is isolated will be selected. It may be near the putative tumour suppressor gene. Starting from the clone chromosome walk along the genomic chromosome 5 library can be done to obtain the flanking clones. The expression pattern of these clones in different tissues, including normal liver, HCC and cholangiocarcinoma, then can be determined to finally decide which clone(s) is the candidate tumour suppressor gene. The final proof is to introduce the candidate into the HCC cell lines to suppress the tumorigenicity. All these procedures are technically feasible.

Chapter 11 General Discussion and Future Work

There is no doubt that inactivation of tumour suppressor genes plays an important role in tumorigenesis. The task now is to search for and identify such genes in human genome. Demonstration of specific loss of chromosomal constitutional heterozygosity in tumour DNA has proved to be a useful means for such searching in retinoblastoma, Wilms' tumour, colorectal carcinoma, and in breast, lung and other cancers (Chapter 1). The hypothesis for this thesis was that consistent LOH would occur in tumours of liver, pancreas and biliary systems and detection of such loss might lead to localization of tumour suppressor genes in these tumours. The investigation described in this thesis thus focused on the extensive search of consistent LOH in these tumours and preparation for isolation of a putative tumour suppressor gene from chromosome 5 for hepatocellular carcinoma. The main findings have been presented and discussed in Chapters 3-10. Significant LOH has been found in different types of tumours of the above systems, including that on chromosome 5q35-qter in HCC and cholangiocarcinoma and on chromosomes 1, 6 and 11 in carcinoma of the pancreas. These losses in above tumours were not reported before. The allele status in three types of rare liver tumours, ie, fibrolamellar carcinoma, hepatocellular adenoma and sarcomatoid liver carcinoma has been studied. LOH in colorectal liver metastases has been investigated and associated with some clinical data. Finally, priming work has been carried out for isolation of the putative tumour suppressor gene on chromosome 5.

Chapter 11

11.1 Demonstration of LOH as a useful means to search for tumour suppressor genes

11.1.1 Selection of restriction fragment length polymorphism DNA probes

As discussed in Chapter 1 and described in Chapters 2-9, detection of LOH in tumour DNA depends on DNA probes which can recognize DNA restriction fragment length polymorphism. Thus, a battery of probes should be used to survey systematically tumour cell genomes looking for repeated LOH (Weinberg, 1991). However, there are already more than 5,000 known RFLP probes (Williamson et al, 1991). A certain group must be selected from this huge repertoire of probes for a particular study.

In this thesis the probes were chosen based on two principles: those that were highly heterozygous and those that showed LOH in other human tumours or were near regions of interest. The first group included the hypervariable minisatellite or variable number of tandem repeat (VNTR) probes (Jeffreys et al, 1985; Nakamura et al, 1987b). These probes may detect heterozygosities ranging from 90-99%, such as probes λMS1 and λMS32 for chromosome 1, λMS8 for chromosome 5, λMS31 and pλg3 for chromosome 7 and λMS43 for chromosome 12 (Wong et al, 1987). Indeed, the majority of patients proved informative with these probes in this study (Chapters 3-9). The second group of probes included

probes from or around the regions of known genes, such as albumin (F47.3 and VC63, chromosome 4), APC and MCC (ECB27, L5-71.3, FB54-D and YN5.48, chromosome 5), H-ras (H-ras, chromosome 11), RB (P3.8R and pMS626, chromosome 13), p53 (p144.D6 and pYNZ.22, chromosome 17) and DCC (p15-65 and pMS440, chromosome 18).

11.1.2 Chromosomal region-specific and tumour-specific LOH

There are two general features of consistent LOH in tumours, ie, such losses are relatively chromosomal region-specific and tumour-specific. These features are the bases of application of LOH determination for detecting specific tumour suppressor gene losses.

In the initial study of LOH in human tumour, it was recognized that LOH in retinoblastoma was limited on chromosome 13, while probes from chromosomes 3, 11, 12, 15, 17, 18 and 20 did not detect any LOH in retinoblastoma (Cavenee et al, 1983). This observation has been expanded to all other tumours studied (Lasko et al, 1991), although for most tumours, especially common adult cancers, consistent LOH may occur in several regions in the genome. This may reflect the fact that cumulative genetic changes are needed for tumour development (Fearon & Vogelstein, 1990). Colorectal tumours, for example, exhibit consistent allele losses on chromosomes 5, 17 and 18, and possibly on chromosomes 8, 14 and 22 (Chapter 1). Nevertheless, such consistent losses are still limited on a few regions

of the genome, indicating the possible location of genes of interest. Losses with low frequency may occur in any region and may be the changes by chance (Lasko et al, 1991).

Another feature of LOH is that such loss is relatively tumour-specific. In some chromosomal regions, especially 3p24, 11p15, 13q14 and 17p13, LOH is shared by disparate tumours (Lasko et al, 1991). Tumour suppressor genes from these regions, eg, RB1 and p53, particularly the latter, are pleiotropic for many tumours (Chapter 1). On the other hand, some losses are confined to a single tumour type or closely related tumours. Allele loss on 9q34, for example, has been found only in bladder cancers so far (Tsai et al, 1990; Cairns et al, 1993); and loss on 12q24-qter, only in gastric cancer (Fey et al, 1989; Sano et al, 1991). This type of losses may imply the existence of tissue-specific tumour suppressor genes.

The data in this thesis also showed these two features of LOH. In HCC without liver cirrhosis, consistent LOH was confined to 5q35-qter and 17p13, while probes for all other regions detected no consistent loss. Allele loss in the region 17p13 is shared by most, if not all, cancers (Chapter 1 and discussed above). Chromosome 5q35-qter allele loss seems relatively specific for HCC. Allele loss in this region has been found in a few of primary colorectal tumours (Rees et al, 1989) and colorectal liver metastases (Chapter 8, this thesis). Since majority of colorectal liver metastases had allele loss in the region 5q21-22, and almost all such tumours with loss in 5q35-qter also had loss in 5q21-22 (Chapter 8), loss in

5q35-qter may be a coexistent phenomenon of loss in 5q21-22 in colorectal liver metastases, or these patients may have the whole 5q deleted. Cytogenetic study has shown a high frequency of chromosome 5 deletion in colorectal carcinomas (Reichmann et al, 1985a, 1985b).

LOH in the region 5q35-qter did not occur in HCC with liver cirrhosis (Chapter 3), fibrolamellar carcinoma (Chapter 5), hepatocellular adenoma (Chapter 6), and carcinoma of the pancreas (Chapter 9). Interestingly, such loss occurs in 3 out of 10 informative cholangiocarcinoma (30%, Chapter 7). This tumour is believed to arise from the same tumour stem cell for HCC. It is possible that loss in the region 5q35-qter is a common denominator in the development of HCC without liver cirrhosis and cholangiocarcinoma. Neither HCC nor cholangiocarcinoma exhibited allele loss at MCC and APC gene loci (5q21-22, Chapters 3 and 7). The tumour suppressor gene on chromosome 5 for HCC and cholangiocarcinoma appears to be distinct from the MCC and APC genes (Ding et al, 1991, 1993b, 1993c).

In colorectal tumorigenesis, chromosome 5 allele loss may be an initial step, since such loss can be seen in colorectal adenoma and early stage of carcinomas (Rees et al, 1989; Fearon & Vogelstein, 1990). LOH in 5q35-qter may not be such an early event in hepatocarcinogenesis, since hepatocellular adenoma analyzed did not have such loss, although HCC may not necessarily arise from hepatocellular adenoma and the number of hepatocellular adenoma analyzed is too small to

draw any conclusion yet.

As discussed in Chapter 8, the expected frequent LOH on chromosomes 5, 17 and 18 were detected in colorectal liver metastases. So far no other specific loss for these metastatic tumours have been found. Allele loss of the antimetastatic gene, nm23, in primary colorectal carcinoma has been found (Leone et al, 1991). It would be of interest to study this gene in colorectal liver metastases. Unfortunately the probes for this gene are not available to us yet though effort has been made to obtain them.

Very few studies of LOH have been conducted in carcinoma of the pancreas. Specific LOH on chromosomes 1, 6 and 11 was found in both endocrine and exocrine CaP in this thesis (Chapter 9). Chromosomes 1 and 6 were cytogenetically abnormal in CaP (Johasson et al, 1992). Thus possible tumour suppressor genes may locate on these chromosomes for CaP. The probe used for chromosome 11q (pMS51, 11q13) in this thesis is near the MEN 1 gene (Larsson et al, 1988). Allele loss detected by this probe may imply that abnormalities of MEN 1 gene are involved in both endocrine and exocrine CaP.

11.1.3 Limitations of detection of LOH

One of the mechanisms of allele loss is the localised chromosomal alteration such as point mutations, small deletions or gene conversions (Cavenee et al, 1983;

Lasko et al, 1991). These changes are unlikely to be detected with RFLP analysis. As mentioned above there are more than 5,000 RFLP probes available, the LOH data is undoubtedly biased due to the probes selected. As it is practically impossible to cover the entire genome with probes in any given case, the very real risk exists that the examined tumours may harbour many other DNA level changes, and perhaps more important ones, than those detectable by the chosen test battery (Heim, 1992). Certainly, these risks can be decreased if one casts a broader net (the use of as many probes as possible).

The DNA samples used for detection of LOH in tumours are normally obtained from all cell types within the specimen, be they stromal or truly neoplastic, single clonal or multiclonal, and after the extraction there is no way to differentiate between the material deriving from the different sources. The results of the analysis may incorporate features of non-tumorous components (Heim, 1992). Some partial deletions observed (reduction of band intensity other than complete band loss in autoradiographs of Southern hybridisations) may reflect such contaminations of non-tumorous tissue in the tumour samples. Within all neoplastic cells, there may be subclones of tumours. The analysis on the DNA extracted together will fail to reveal genomic differences among different subsets of tumour parenchyma cells.

These limitations of LOH analysis will lead to underestimation of genetic changes in the tumour genome. However, if such analysis does show any repeated changes

in particular tumours, these changes should be significant in the pathogenesis in such tumour types.

11.2 Isolation of the putative tumour suppressor gene from chromosome 5

The most exciting finding in this thesis was that most, if not all, hepatocellular carcinomas without liver cirrhosis had allele loss on chromosome 5q35-qter and such loss also occurred in cholangiocarcinomas but not in HCC with liver cirrhosis, fibrolamellar carcinomas, hepatocellular adenomas and carcinomas of the pancreas. Literature search shows that LOH in this region has not been found in any other tumours except for a few cases of colorectal carcinomas, in which most allele losses occur in the region 5q21-22. This strongly suggests that a putative tumour suppressor gene for HCC without liver cirrhosis, and possibly for cholangiocarcinoma, is located in the terminal region of the long arm of chromosome 5 (5q35-qter). Therefore extensive preliminary work has been carried out for isolation of this gene. By cross screening of a normal human cDNA library and a normal chromosome 5 genomic library, I have obtained 21 chromosome 5 clones that are expressed in normal human liver. The rationale for doing so is that tumour suppressor genes are normal cellular genes that are expressed in normal tissues (Sager, 1989). These clones have been cloned into plasmid bluescripts, which will facilitate further work (Chapter 10). A procedure for isolating single copy DNA sequences from the positive clones has been

described and three single copies have been identified with this method. Because of the limitations of the approach used, however, the number of positive clones might be an underestimation (discussed in chapter 10). Alternative strategies should be employed. The one being used now is discribed in Chapter 10.

11.3 The clinical application of molecular genetic investigation of cancer

The nature of cancer is not fully understood and cancer as a disease is still a major challenge to human health (Vile, 1992). The recent progress in molecular genetic study of cancer, however, has advanced our understanding of carcinogenesis. The concept of cancer as a set of genetic diseases has been established (Chapter 1). This is not only because of the academic interest, but it may lead to more effective preventive and therapeutic strategies for cancer than those currently used (Travis, 1992). The goal of the overall project which includes the work described in this thesis is to identify specific tumour suppressor genes for tumours in liver, pancreas and biliary systems. The success of this project may pave the way for future gene therapy with the identified genes for these tumours eventually. The first of such genes could be the one for HCC without liver cirrhosis and it may become a useful tool in gene therapy.

Currently the applications of molecular genetic data can serve as prognostic factors (Vogelstein et al, 1989; Ali & Callahan, 1990; Starzynska et al, 1992) and

may help the diagnosis of cancer (Shibata et al, 1990; Sidransky et al, 1992a; Oda et al, 1992). As observed by Vogelstein et al (1989) in colorectal carcinomas, the more frequent LOH in tumours have been associated with poorer prognoses in primary liver tumours (Chapters 4 and 5, Ding et al, 1993a, 1993d), colorectal liver metastases (Chapter 8, Ding et al, 1993e) and carcinoma of the pancreas (Chapter 9, Ding et al, 1992a) in this thesis. Another application of RFLP analysis is to detect tumour origins (Vogelstein et al, 1985). The origins of multiple hepatocellular carcinomas were determined by the patterns of allele loss in different HCC (Tsuda et al, 1992). In this thesis, different patterns of allele loss in primary and recurrent HCC were shown and suggested that the recurrent tumour was a de novo neoplasia (Chapter 3, Ding et al, 1992b). Similar differences were seen in the synchronous HCC and fibrolamelar carcinoma (Chapter 5, Ding et al, 1992c) and the synchronous HCC and adenoma (Chapter 6, Ding et al, 1993f, 1993g).

11.4 Conclusion and future work

In conclusion, the work described in this thesis suggests that a putative tumour suppressor gene for hepatocellular carcinoma and possibly for cholangiocarcinoma may be located in the terminal region of the long arm of chromosome 5 (5q35-qter) and this gene appears to be distinct from the MCC and APC genes which are also on the long arm of chromosome 5 (5q21-22) (Chapters 3 and 7). The 21 chromosome 5 clones could be the basis to isolate this gene (Chapter 10). The

allelic losses on chromosomes 1q, 5p and 17p in HCC with liver cirrhosis (Chapter 3), on chromosomes 5q, 17p and 18q in colorectal liver metastases (Chapter 8) and on chromosomes 1p, 6q, and 11q in carcinoma of the pancreas (Chapter 9) may be important in the development and/or progression of these tumours. LOH in fibrolamellar carcinoma (Chapter 5) and hepatocellular adenoma (Chapter 6) may be a rare event, if it occurs.

Due to the limited specimen and time availability for the work in this thesis, the number of each tumour analyzed was limited (although the total number of patients studied was 82). The conclusions drawn from the thesis should be confirmed in larger studies in future, with more patients and more relevant probes. Such study may lead to more detailed localisation of putative tumour suppressor genes in the above tumours and provide the basis for identification of these genes. The association of the expanded LOH data with prognoses of all tumours studied should be analyzed. Above all, the immediate future work should concentrate on the completion of the identification of the putative tumour suppressor gene on chromosome 5q35-qter. The plans for this have been proposed in Chapter 10.

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Appendix 1 Buffers and Solutions

I. Buffers and solutions for general use

TE buffer

10 mM Tris pH 7.5, 8.0
1 mM EDTA (disodium salt)

STE buffer

150 mM NaCl
10 mM Tris pH8
10 mM EDTA (disodium salt)

20xSSC

3M NaCl
0.3M Na_3 citrate
pH to 7.0

1xTAE

40mM Tris.HCl pH 7.8-8.1
2mM EDTA (disodium salt)
20 mM NaOAc
6.25% (v/v) glacial acetic acid

1xTBE

89mM Tris.HCl pH8
89mM Boric acid
20mM EDTA (disodium salt)

20xSSPE

200mM Na_2PO_4 pH7
3.6M NaCl
20mM EDTA (disodium salt)

Agarose gel loading buffer (10x)

40% sucrose
0.025% wv bromophenol blue
0.025% w/v xylene cyanol

II. Buffers, solutions and reagents required for extraction of DNA

Lysis buffer:

0.32 M Sucrose
10 mM Tris pH7.5

5 mM $MgCl_2$
1% (w/v) Triton x 100

Proteinase K buffer (PK buffer or NaCl/EDTA buffer)
0.075 M NaCl
0.024 M EDTA pH7.5

Tissue buffer
50 mM Tris pH 7.5
100 mM NaCl
1 mM EDTA

Chloroform
All chloroform used is chloroform : iso-amyl alcohol 24 : 1

Phenol/Chloroform
50% Buffered Phenol, 50% Chloroform.
Buffering Phenol:
- Make up 1 litre 1 M Tris.HCl pH 8.0
- Mix 900ml above with 1 litre water saturated phenol, set down and remove the buffer
- Dilute remaining 100ml Tris.HCl buffer to 1 litre, adding 2ml 2-mercaptoethanol
- Extract phenol with 500ml 0.1 M Tris.HCl pH 8.0. 0.2% 2-mercaptoethanol twice
- Check pH of top buffer > 7.6
- Make 1 litre chloroform:iso-amyl alcohol 24:1
- Add to phenol until it is 50:50 (calculate from volume of chloroform used as buffer is excluded from phenol at this stage)
- Remove extra buffer, store the solution in dark bottle at 4°C up to 1 month

Ethanol
70% and 100%

NaAc
3 M, pH 7.0

Proteinase K
10mg/ml

SDS
10% (w/v) and 20% (w/v)

III. Buffers and solutions for bacterium, plasmid transformation and DNA libraries

NZY broth

Per litre:

5g NaCl
2g $MgSO_4.7H_2O$
5g Yeast Extract
10g NZ Amine (casein hydrolysate)
autoclave

LB broth

Per litre:

10 g Bacto tryptone
5 g Bacto-yeast extract
10 g NaCl
Adjust to pH 7.5 with NaOH
Autoclave

NZY or LB top agar

Same as NYZ or LB broth except with 0.7% agarose. Autoclave.

NZY or LB plates

Add 15g Difco Agar/litre NZY or LB broth.
Autoclave and pour.
Allow ~80 ml/150mm plate, ~30 ml/90mm plate.

SM buffer

Per litre:

5.8 g Nacl
2.0 g $MgSO_4$-$7H_2O$
50 ml 1 M Tris-HCl pH7.5
5 ml 2% gelatin
Autoclave.

PK stop (λ DNA recovery by plate lysate technique)

1 M Tris-HCl pH7.5	0.5 ml
10% SDS	1 ml
0.5 M EDTA	200 µl
10 mg/ml PK	200 µl
ddH_2O	3.1 ml
Total	5 ml, aliquot, stored at -20°C

10x Protease K buffer (small scale preparation of λ clones)

NaCl	3M
EDTA	0.5M

Tris 1M
Adjust pH to 7.6-7.8 with concentrated HCl.

TSB (transfomation and storage buffer)
10%polyethylene glycol (MW=3,350)
5%DMSO
20mM Mg^{++} (10mM $MgCl_2$ + 10mM$MgSO_4$)
LB broth to required volume

IV. Solutions required for plasmid preparation

Solution I

50mM Glucose	
20mM Tris.Cl	(pH8.0)
10mM EDTA	(pH8.0)
(Store at 4^0C)	

Solution II
0.2N NaOH
1%SDS
(Freshly made at room temperature)

Solution III

5M KAc	60ml
HAc	11.5ml
H_2O	28.5ml

V. Solutions required for Southern blot

HCl
0.25 M

Denaturing solution
1.5 M NaCl
0.5 M NaOH

Alkali transfer buffer
0.25 M NaOH
1.5 M NaCl

20 x SSC
3 M NaCl

0.3 M Na_3 citrate

VI. Solutions for oligolabelling DNA

Labelling solution buffer (LS)

DTM/OL/Hepes
25: 7: 25

DTM

100µM dATP (or dCTP)
100µM dGTP
100µM dTTP
Each in 250 mM Tris.HCl ph 8.0, 25mM $MgCl_2$, 50mM ß-mercaptoethanol filtered and stored at -20°C

OL

90 OD units/ml hexanucleotide in 1mµ Tris HCl pH7.5, 1mM EDTA. Pharmacia $d(N)_6$ (500 OD units) dissolved in 550 µl Tris HCl. Filtered and stored at -20°C.

Hepes

1M pH6.6 autoclaved.

BSA

10mg/ml (acetylated to remove nucleases, GIBCO.BRL).

VII. Standard hybridisation solutions

Prehybridisation Mix 1

1M NaCl
5-10% w/v dextran sulphate (Pharmacia LKB, Uppsala, Sweden)
1% SDS
Made up to required volume with sterile deionized water.

Prehybridisation Mix 2

5xSSPE
5xDenhardt's solution
0.5% w/v SDS

Denhardt's solution

BSA (Fraction V, Sigma) 4mg/ml

Polyvinyl pyrrolidone (Pharmacia)	4mg/ml
Polyethylene Glycol 400 (Pharmacia)	4mg/ml

Salmon sperm DNA

Sigma type III DNA from salmon testes was made up to 10mg/ml with sterile deionized water and then sheared by passing through a 1.1 mm needle several times, boiled for 10 minutes and then frozen at -20°C. Before use the appropriate amount was added to the labelled probe, boiled for 5 minutes, quenched on ice for 5 minutes and added to the prehybridisation mix.

VIII. Solutions for PCR

10x PCR buffer

100mM Tris.HCl pH 8.3
500mM KCl
15mM $MgCl_2$
0.01% (w/v) gelatin (Sigma)

dNTP Mix, 1.25mM each nucleotide, triphosphate

dATP, 10mM	125µl
dCTP, 10mM	125µl
dGTP, 10mM	125µl
dTTP, 10mM	125µl
Distilled sterile water	500µl

Appendix 2 Probes and Primers

I. Restriction fragment length polymorphism DNA probes

Location	Symbol	Probe name	Vector	Site	Size (kb)	RFLP digest	Constant bands (kb)	Alleles (kb)	Frequency	PIC	Reference
1p33-35	D1S7	λMS1	L47.1	BamHI	4.6	HinfI		Hypervariable Alleles: 2.0-22kb	VNTR	0.98	Wong et al, 1987
1q21-23	APOA2	PB3 (pAIIE9)	pKT218	PstI	0.43	MspI		A1 3.0 A2 3.7	0.81 0.19	0.26	Scott et al, 1985
1q42-43	D1S8	λMS32	L47.1	BamHI	5.9	AluI		Hypervariable > 50 alleles	VNTR	0.97	Wong etal, 1987
2q33-35	CRYG1	p5G1	pBR322	HindIII	3.35	TaqI	9, 8.5, 5	A1 3.5 A2 3.3 B1 2.2 B2 1.1 C1 2.0 C2 1.35, 0.6 C3 0.9, 0.6	0.68 0.32 0.33 0.67 0.48 0.50 0.02	0.34 0.34 0.40	O'Connell et al, 1987
3p21	D3F-15FZE	H3H2	Ch4	HindIII	2.0	HindIII	8.0, 3.8	A1 2.3 A2 2.0	0.46 0.54	0.37	Caritt et al, 1986

(Continued)

Location	Symbol	Probe name	Vector	Site	Size (kb)	RFLP digest	Constant bands (kb)	Alleles (kb)	Frequency	PIC	Reference
3q12	D3S1	HS3	Ch16A	EcoRI/ SacI	2.3	HindIII		A1 ? A2 ?	0.77 0.23	0.29	Naylor et al, 1984
4q11-13	ALB	F47.3	pBR322	PstI	0.9	HaeIII	2.2, 1.4, 0.25	A1 3.8 A2 3.6	0.47 0.53	0.37	Murray et al, 1983
5p	D5S110	cMS621	pUC13	EcoRI/ HindIII		MboI AluI		Hypervariable >10 alleles		0.92	Armour et al, 1990
5q21	D5S98	ECB27	pUC18	SalI	2.8	BglII		A1 11.9 A2 10.5	0.38 0.62	0.36	Varesco et al, 1989
5q21-22	D5S81	YN5.48	pUC18	TaqI	2.4	TaqI		A1 6.0 A2 3.6			Nakamura et al, 1988
						MspI		B1 9.0 B2 8.0	0.5 0.5	0.38	
5q33-35	CSF1R	v-fms	pUC9	EcoRI	3.1	EcoRI	3.0, 2.5	A1 29 A2 16, 13	0.14 0.86	0.21	Xu et al, 1985
5q35-qter	D5S43	λMS8	L47.1	BamHI	7.0	HinfI		Hypervariable >15 alleles	VNTR	0.90	Wong et al, 1987

(Continued)

I. (Continued)

Location	Symbol	Probe name	Vector	Site	Size (kb)	RFLP digest	Constant bands (kb)	Alleles (kb)	Frequency	PIC	Reference
7pter-q22	D7S21	λMS31	L47.1	BamHI	5.7	HinfI		Hypervariable Alleles 3.5-13 kb	VNTR	0.99	Wong et al, 1987
7q31-qter	D7S22	pλg3	pUC13	BamHI	7.1	HinfI		Hypervariable Alleles 1.5-22 kb	VNTR	0.97	Wong et al, 1987
9q34	D9S7	EFD 126.3	pUC182	BamHI	4.2	PvuII		VNTR >4 alleles			Nakamura et al, 1987a
11p15	HRAS	H-ras	pBR322	PstI	0.8	BamHI		A1 6.9 A2 7.5	0.69 0.12	0.47	Krontiris et al, 1985
11q13	D11S97	pMS51	pAT153	EcoRI/ BamHI	3.1	HaeIII		Hypervariable 9 alleles 1.3-4.3kb	VNTR	0.77	Armour et al, 1989
12q24.3-qter	D12S11	λMS43	L47.1	BamHI	8.3	HinfI		Hypervariable Alleles 3.5-16 kb	VNTR	0.94	Wong et al, 1987
13q	D13S103	cMS626	BS KS+	BamHI	?	AluI		Hypervariable Alleles 2.6-6.5 kb	VNTR	0.77	Armour et al, 1990
14q	D14S44	cMS627	BS KS+	BamHI	?	AluI		Hypervariable Alleles 1-20 kb	VNTR	0.96	Armour et al, 1990

(Continued)

Location	Symbol	Probe name	Vector	Site	Size (kb)	RFLP digest	Constant bands (kb)	Alleles (kb)	Frequency	PIC	Reference
16p13.3	D16S85	3'HVR	pSP64	HincII	4.0	PvuII		VNTR 30 alleles		0.96	Higgs et al, 1986
16q22.1	HP	pulB1148	pBR322	PstI	1.38	TaqI	7.0	A1 7.2 A2 5.8	0.58 0.42	0.37	vander Straten et al, 1983
17p13.3	D17S5	pYNZ22	pBR322	BamHI	1.7	RsaI	VNTR	>10 alleles 1.3-2.3		0.86	Nakamura et al, 1988
						MspI	VNTR	>10 alleles 1.5-3.0		0.86	
17p13	D17S34	p144D6	pSP65	BamHI	5.5	RsaI	VNTR	A1 5.3 A2 3.8 A3 3.5 A4 3.4 A5 3.3 A6 3.2 A7 3.1 A8 3.05 A9 3.0 A10 2.8 A11 2.55	0.08 0.02 0.16 0.02 0.02 0.05 0.16 0.05 0.05 0.02 0.02	0.86	Kondoleon et al, 1987
									A12 2.5	0.02	

(Continued)

I. (Continued)

Location	Symbol	Probe name	Vector	Site	Size (kb)	RFLP digest	Constant bands (kb)	Alleles (kb)	Frequency	PIC	Reference
18q	D18S31	cMS440	pUC18	BamHI	6	MboI AluI HaeIII		Hypervariable VNTR >10 alleles		0.72	Armour et al, 1990
18q21.3	DCC	p15-65	BS KS+	MspI	2.5	MspI		A1 10.5 A2 9.7 A3 7.8 A4 7.0	0.17 0.04 0.49 0.30		Fearon et al, 1990

II. PCR primers

"Big" T_3 (30 bp)

5'-CTCGAAATTAACCCTCACTAAAGGGAACAA-3'

"Big" T_7 (30bp)

3'-TAAGCGGGATATCACTCAGCATAATGTTAA-5'

Appendix 3 Publications Related to the Thesis

Published papers

1. Ding S-F, Habib NA, Dooley J, Wood C, Bowles C, Delhanty JDA.
Loss of constitutional heterozygosity on chromosome 5q in hepatocellular carcinoma without cirrhosis.
British Journal of Cancer 64: 1083-1087, 1991.

2. Ding S-F, Habib NA, Delhanty JDA, Bowles L, Greco L, Wood C, Williamson RCN, Dooley JS.
Loss of heterozygosity on chromosomes 1 and 11 in carcinoma of the pancreas.
British Journal of Cancer 65: 809-812, 1992.

3. Ding S-F, Wong K, Davidson B, Dooley J, Dhillon AP, Wood CB, Habib NA.
Differences in the tumour DNA analysis of a hepatocellular carcinoma and a synchronous fibrolamellar variant.
International Journal of Oncology 1: 191-193, 1992.

4. Ding S-F, Jalleh RP, Wood CB, Bowles L, Delhanty JDA, Dooley J, Habib NA.
Different DNA changes in primary and recurrent hepatocellular carcinoma.
Gut 33: 1433-1435, 1992.

5. Ding S-F, Delhanty JDA, Bowles L, Dooley JS, Wood CB, Habib NA.
Infrequent chromosome allele loss in fibrolamellar carcinoma.
British Journal of Cancer 67: 244-246, 1993.

6. Ding S-F, Delhanty JDA, Bowles L, Dooley JS, Wood CB, Habib NA.
Loss of constitutional heterozygosity on chromosomes 5 and 17 in cholangiocarcinoma.
British Journal of Cancer 67: 1007-1010, 1993.

7. Ding S-F, Delhanty JDA, Carrillo A, Dalla Serra G, Bowles L, Dooley JS, Wood CB, Habib NA.
Lack of demonstrable chromosome allele loss in hepatocellular adenoma.
International Journal of Oncology 2: 977-979, 1993.

8. Ding S-F, Jalleh RP, Dooley J, Wood CB, Habib NA.
Chromosome 17 allele loss in hepatocellular carcinoma but not in

synchronous liver adenoma. European Journal of Surgical Oncology 19: 195-197, 1993.

9. Ding S-F and Habib NA.
Malignant tumours of the liver and biliary system. In: Cancer: a molecular approach. Eds. by NR Lemoine, T Cooke & J Neoptolemos. Oxford: Blackwell Scientific Publication pp95-105, 1994.

10. Ding S-F, Delhanty JDA, Dooley JS, Bowles L, Wood CB, Habib NA.
The possible tumour suppressor gene on chromosome 5q for hepatocellular carcinoma is distinct from the MCC and APC genes.
Cancer Detection and Prevention 17: 405-409, 1993.

11. Ding S-F, Carrillo A, Dooley JS, Delhanty JDA, Bowles L, Dalla Serra G, Wood CB, Habib NA.
Multiple allelic losses in sarcomatoid liver carcinoma.
International Hepatology Communications 1: 295-301, 1993.

12. Ding S-F, Michail NE, Habib NA.
Genetic changes in hepatoblastoma.
Journal of Hepatology 1993 (In press).

13. Ding S-F, Delhanty JDA, Zografos G, Michail NE, Dooley JS, Habib NA.
Chromosome allele loss in colorectal liver metastases and its association with clinical features.
British Journal of Surgery 1993 (In press)

Presentations: to learned societies

1. Ding S-F, Bowles L, Dooley JS, Habib NA, Wood C, Delhanty JDA.
Chromosome 5 allele loss in hepatitis B negative primary hepatocellular carcinoma.
Second European Workshop on Cytogenetics and Molecular Genetics of Human Solid Tumors. Leuven, Belgium. July 1990.
Cancer Genetics and Cytogenetics 52 (2): 240, 1991.

2. Ding S, Habib N, Wood C, Bowles L, Delhanty J, Dooley J.
Chromosome 5 allele loss in hepatitis B virus negative hepatocellular carcinoma.
British Society of Gastroenterology (BSG). Southampton, UK. September 1990.
Gut 31 (10): A1211, 1990.

3. Ding S-F, Dooley S, Delhanty J, Bowles L, Wood C, Habib N.
Chromosome allele loss in hepatocellular carcinoma.

British Association for the Study of the Liver (BASL). London, UK. February 1991.
Gut 32 (7): A838, 1991

4. Selden C, Ding SF, Habib N, Hodgson H J F.
Expression of hepatocyte growth factor associated with hepatocellular cancer.
British Society of Gastroenterology (BSG). Manchester, UK. April 1991.
Gut 32 (5): A576, 1991.

5. Ding S-F, Dooley J, Delhanty J, Bowles L, Wood C, Habib N A.
Allele loss of chromosomes 1, 5, and 17 in cholangiocarcinoma.
British Society of Gastroenterology (BSG). Manchester, UK. April 1991.
Gut 32 (5): A600, 1991.

6. Ding S-F, Habib NA, Dooley J, Wood C, Bowles L, Delhanty J.
Different pattern of genomic DNA loss in colorectal-liver metastasis and hepatocellular carcinoma.
British Society of Gastroenterology (BSG). London, UK. September 1991.
Gut 32 (10): A1249, 1991.

7. Ding S-F, Habib NA, Delhanty J, Bowles L, Wood C, Williamson RCN, Dooley J.
Allele loss on chromosomes 1 and 11 in carcinoma of the pancreas.
Pancreatic Society of Great Britain and Ireland. Glasgow, UK. November 1991.
Gut 33 (3): A428, 1992.

8. Ding S-F, Dooley JS, Delhanty JDA, Bowles L, Wood C, Habib NA.
The possible tumour suppressor gene on chromosome 5q for hepatocellular carcinoma is distinct from the MCC and APC genes.
British Society of Gastroenterology (BSG). Warwick, UK. September 1992.
Gut 33 (Suppl. No 2): S8, 1992.

9. Ding S-F.
Genetic changes in human hepatocellular carcinoma.
Meeting of the Central London Toxicology Group on Hepatocarcinogenicity. St Bartholomew's Hospital, London. 15th October 1992.
Invited talk.

10. Ding S-F, Delhanty JDA, Carrillo A, Dalla Serra G, Zografos G,

Bowles L, Dooley JS, Wood CB, Habib NA.
Chromosome allele loss in colorectal liver metastases and its association with clinical features.
British Society of Gastroenterology (BSG). Manchester, UK. March 1993.
Gut 34 (Suppl. No 1): S8, 1993.

Br. J. Cancer (1991), **64**, 1083-1087

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Loss of constitutional heterozygosity on chromosome 5q in hepatocellular carcinoma without cirrhosis

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Summary Suppressor gene loci involved in the development of hepatocellular carcinoma (HCC) have not been fully identified. The aim of this study was to look for consistent allele loss, or loss of heterozygosity (LOH), in HCC which might represent such gene loci. We have prepared DNA from tumour and non-tumour material from 16 patients with HCC (nine with and seven without liver cirrhosis). Tumour DNA was compared with non-tumour DNA by Southern analysis performed with a panel of 22 probes recognising restriction fragment length polymorphisms assigned to chromosomes 1, 4, 5, 7, 9, 11, 12, 13, 14, 16, 17, 18 and 20. Non-tumour DNA from five of the seven patients with HCC without cirrhosis was heterozygous with the probe Lambda MS8 (5q35-qter), and in all five there was LOH in tumour DNA. Probes for other regions of chromosome 5 have as yet shown no LOH in this group of patients. Cirrhotic HCC patients exhibited LOH on chromosomes 1q and 5p but not in the region 5q35-qter. Both groups of HCC showed LOH on chromosome 17p13. Screening with other probes has not shown any consistent LOH in either group as yet. A comparison of LOH on chromosome 5 in seven patients with colorectal metastasis in the liver showed a different pattern, which suggests that the proposed tumour suppressor gene locus for HCC without cirrhosis on chromosome 5 appears to be distinct from the familial adenomatous polyposis coli gene.

Hepatocellular carcinoma (HCC) is a major cause of death from malignancy in the world, with a particularly high incidence in the Far East and Africa where hepatitis B virus (HBV) infection, established as an aetiological agent, is common. In Western countries the incidence is lower but increasing. Most HCC are beyond radical resection when detected, and all other forms of the currently available therapies are rarely beneficial. For these reasons the cellular and molecular changes leading to HCC demand study, with a view to identification of patients at particular risk, earlier detection of tumours and, in the long-term, successful therapy.

In most tumours carcinogenesis is the result of an interaction between genetic and environmental factors, and appears to be a multistep process. Several mutations may be necessary and in broad terms two interacting mechanisms seem to be involved: oncogene activation or mutation, and loss of tumour suppressor factors. Mutant oncogenes introduced into cultured cells are capable of inducing malignant transformation. In patients with tumours, mutation of an oncogene may allow production of proteins which perpetuate proliferation. Evidence is accumulating now that excess proliferation is opposed by the products of tumour suppressor genes, and that these are at least as important as oncogenes in carcinogenesis. Their presence was predicted by Knudson's model for sporadic and inherited forms of retinoblastoma (Knudson, 1971). The gene involved in retinoblastoma has now been identified and the gene product characterised (Friend *et al.*, 1986; Lee *et al.*, 1987). Loss of tumour suppressor has now been implicated in Wilms' tumour (Rose *et al.*, 1990), acoustic neuroma (Seizinger *et al.*, 1986) and also carcinomas of the colon (Fearon *et al.*, 1990; Kinzler *et al.*, 1991), lung (Kok *et al.*, 1987) and breast (Mackay *et al.*, 1988).

For the common tumours such as carcinomas of the colon, breast and lung, the evidence for loss of tumour suppressor genes has accumulated from the demonstration of a consistent loss of a region of genomic DNA in tumour tissue ('allele loss') when compared with the individual's normal DNA. Allele loss, or loss of heterozygosity (LOH), on chromosomes 5, 17 and 18 has been found in colonic carcinoma (Vogelstein *et al.*, 1988). Introduction of a normal chromosome 5 or 18 into this tumour, *in vitro*, suppresses tumorigenicity (Tanaka *et al.*, 1991). Two candidate colon tumour suppressor genes, DCC and MCC on chromosomes 18 and 5 respectively, have been identified (Fearon *et al.*, 1990; Kinzler *et al.*, 1991).

Most HCC are associated with chronic HBV infection. The HBV genome is integrated into the host DNA and many studies have detailed sites of integration looking for a consistent pattern and/or changes which might activate oncogenes. No consistent pattern has been found (Di Bisceglie, 1989). Loss of specific segments of chromosomal DNA, however, has been shown including regions on chromosomes 4, 11 and 13 (Buetow *et al.*, 1989; Wang *et al.*, 1986) and these may be the sites of tumour suppressor genes. In HCC without HBV infection, which is more common in Western countries, little is known about the genetic changes. They usually present without liver cirrhosis and have a different prognosis. Therefore we performed this study to establish whether there are different consistent patterns of allele loss in HCC with or without liver cirrhosis.

Materials and methods

Patients and biopsies

We have studied 16 patients with HCC (nine with and seven without liver cirrhosis) and seven patients with liver metastases from colorectal primary tumours. Of the seven patients with HCC without cirrhosis, six were HBV negative and one HBV positive. All nine patients with HCC and cirrhosis were HBV positive. All these patients had their tumours localised to the liver with no extrahepatic spread based on preoperative imaging and findings at laparotomy. All underwent either liver resection or liver transplantation. None of these patients had a tumour of the fibrolamellar type. Surgical biopsies from the tumour and non-tumour liver tissue were snap frozen in liquid nitrogen at the time of liver resection. Lymphocytes from peripheral blood obtained preoperatively and before any blood transfusion were also used as a source of normal DNA. Tissue was stored at $-70°C$ until DNA

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extraction. None of the patients received chemotherapy or radiotherapy before surgery and tumour samples were examined histologically to confirm the type of tumour present.

Patient's viral hepatitis status was determined by analysis of serum for hepatitis B markers and by Southern analysis of liver tissue DNA using the HBV genome probe pEco63.

DNA extraction and hybridisation

DNA was prepared from blood and tissue samples by standard phenol/chloroform methods (Sambrook *et al.*, 1989). Samples were digested with the appropriate restriction endonuclease and were size fractionated by electrophoresis through 0.6–0.9% agarose gels. The DNA was transferred to Hybond-N hybridisation filters (Amersham) according to the manufacturer's specifications. DNA probes were radiolabelled with alpha-^{32}P-dCTP (3,000 $C_i$mol^{-1}) by the random hexanucleotide primer method (Feinberg & Vogelstein, 1983) to a high specific activity. Hybridisations were performed at 65°C in 1% SDS, 1 M NaCl and 5% dextran sulphate (W:V) for 16–24 h. Filters were washed to stringency of 2 × SSC 1% SDS (W:V) at 64°C and were autoradiographed at −70°C using Fuji: RX-L X-ray film.

The detection of chromosomal DNA loss depends upon demonstrating a difference in restriction fragment length polymorphism (RFLP) between tumour and non-tumour ('normal') DNA. Most of the probes used were selected because they are hypervariable, that is the majority of individuals will be heterozygous and hence informative at these loci (Wong *et al.*, 1987). A deletion in the region studied may then be seen as a loss of a band (or loss of intensity of a band). In this study we used 22 probes for chromosomes 1, 4, 5, 7, 9, 11, 12, 13, 14, 16, 17, 18 and 20 and of these 12 were hypervariable (Table I). The remainder were chosen because they were shown to be of importance in studies of other tumours. The analyses showing a partial or complete loss of a band are reported in this study as showing allele loss, while gene rearrangements with partial or complete gain of a band in tumours were not included.

Results

Table I shows the overall pattern of allele loss, or loss of heterozygosity (LOH), in DNA from 16 HCC compared to non-tumour DNA. The analyses showed an informative pattern in 186 of 268 Southern blots (heterozygosity: 69.4%). Overall LOH was present in 30/186 (16.1%). Figure 1 shows representative examples of LOH.

In the seven patients with HCC without liver cirrhosis a high frequency of LOH only occurred in the regions 5q35-qter and 17p13 (Tables I and II). The probe for the terminal region of long arm of chromosome 5 (Lambda MS8, 5q35-qter) was informative in five cases and all showed LOH. Of the four patients informative with the probe p144-D6 for the short arm of chromosome 17 (17p13) three showed LOH.

In the nine patients with HCC and liver cirrhosis LOH was found on chromosomes 1q, 5p and 17p in at least half of the informative cases (Tables I and II). Out of six informative patients five showed LOH at 17p13 by the probe p144.D6. Five cases in this group were heterozygous with the probe Lambda MS8 (5q35-qter), but none of them exhibited LOH. Instead, a single patient (No. 6, Table II) showed LOH on 5q21–22, but was non-informative for Lambda MS8 (5q35-qter).

Previous work on colorectal adenomas and carcinomas has shown that the chromosome 5 region (5q21–22) encompassing the familial adenomatous polyposis coli (APC) gene is deleted in inherited and sporadic colorectal cancer (Miyaki *et al.*, 1990). For this reason we compared the pattern of allele loss in non-cirrhotic HCC with that of colorectal liver secondaries using various probes for chromosome 5q (Figure 2). Table II shows that patients with non-cirrhotic HCC had no allele loss when screened with probes mapped to regions of the chromosome other than 5q35-qter. On the other hand only two of five patients with colorectal liver metastases (informative with probe lambda MS8) showed allele loss in that region, but the majority of these patients showed allele loss with probes from 5q21–22, the region of the chromosome associated with colorectal cancer.

Table I Loss of chromosomal heterozygosity in human hepatocellular carcinoma

Chromosome	*Probe*	*Locus*	*Enzyme*	*HCC without Cirrhosis (n = 7)*	*HCC with Cirrhosis (n = 9)*	*Reference*
1	λMS1	1p33–35	HinfI	1/5*	0/8	Wong *et al.*, 1987
	PB3	1q21–23	MspI	0/2	1/2	Scott *et al.*, 1985
	λMS32	1q42–43	AluI	0/3	3/5	Wong *et al.*, 1987
4	F47.3	4q11–13	HaeIII	0/4	0/4	Murray *et al.*, 1983
5	pMS621	5p	HinfI	0/4	3/4	Armour *et al.*, 1990
	ECB27	5q21	BglII	0/4	1/4	Vareso *et al.*, 1989
	YN5.48	5q21–22	MspI	0/3	1/3	Nakamura *et al.*, 1988*a*
	λMS8	5q35-qter	HinfI	5/5	0/5	Wong *et al.*, 1987
7	λMS31	7pter-q22	HinfI	0/4	1/7	Wong *et al.*, 1987
	pλg3	7q31.3-qter	HinfI	0/3	0/7	Wong *et al.*, 1987
9	EFD126.3	9q34	PvuII	0/2	1/4	Nakamura *et al.*, 1987
11	H-ras	11p15	BamHI	0/2	0/3	Krontiris *et al.*, 1985
	pMS51	11q13	HaeIII	0/4	0/6	Armour *et al.*, 1989
12	λMS43	12q24.3-qter	HinfI	1/5	0/7	Wong *et al.*, 1987
13	pMS626	13q	AluI	0/5	0/6	Armour *et al.*, 1990
14	pMS627	14q	AluI	0/5	0/5	Armour *et al.*, 1990
16	3'HVR	16p13.3	PvuII	0/5	0/6	Higgs *et al.*, 1986
	pulB1148	16q22.1	TaqI	0/3	0/3	vander Straten *et al.*, 1983
17	p144-D6	17p13	RsaI	3/4	5/6	Kondoleon *et al.*, 1987
	pYNZ.22	17p13	RsaI	1/5	2/4	Nakamura *et al.*, 1988*b*
18	pMS440	18q	HaeIII	0/3	0/2	Armour *et al.*, 1990
20	pMS617	20q	AluI	0/2	1/3	Armour *et al.*, 1990

*No. with allele loss; No. of informative cases.

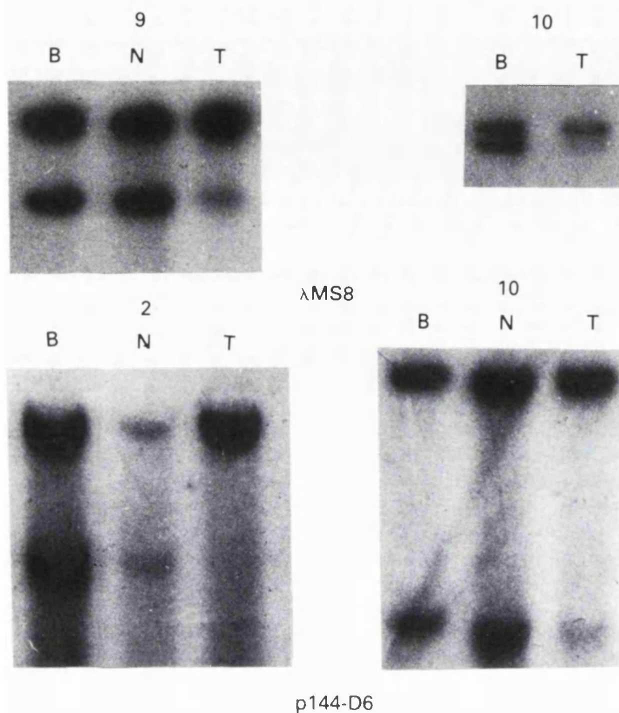

Figure 1 Autoradiographs of Southern hybridisations with MS8 and p144-D6. Patient numbers are indicated above the tracks. **B** = blood lymphocyte DNA; **N** = non-tumour tissue DNA; **T** = tumour tissue DNA. No. 2 is HCC with cirrhosis, and nos 9 and 10 are HCC without cirrhosis. All show allele losses in tumour DNA.

Discussion

This is the first report that shows LOH on the terminal region of the long arm of chromosome 5 (i.e. 5135-qter) in patients with non-cirrhotic HCC and the short arm of chromosome 5 (5p) in patients with cirrhotic HCC. Patients with non-cirrhotic HCC showed LOH mainly on chromosomes 5q and 17p, while patients with cirrhotic HCC had allele loss on chromosomes 1q, 5p and 17p. Chromosomes 17p and 1q allele losses are shared with many other tumours and are likely to represent 'tumour progression' (Sager, 1989). The presence of a tumour suppressor gene locus on the short arm of chromosome 5 has not been previously reported and may be important in cirrhotic HCC.

It is interesting that the pattern of chromosomal deletion in HCC shown so far correlates more on the present or absence of liver cirrhosis rather than the presence or absence of HBV infection. Since it was shown that tumours from patients who are seropositive for markers of HBV infection contain integrated HBV DNA sequences it has been argued that the viral genome may be involved in the induction and/or maintenance of the neoplastic phenotype (Chen *et al.*, 1988). The role of virally mediated oncogenesis in HCC has been widely studied, but yet, no conclusive results have emerged. Therefore it was interesting to find in our study no absolute differences in LOH pattern between HCC with or without HBV infection in spite of the differences in the aetiology and pathology processes. This lends support to the hypothesis that the development of cirrhosis (with its regenerative capacity) rather than the presence of integrated HBV genome is most important, although the number of patients studied to date is small. It remains to be seen whether tumour suppressor gene loss is different in HCC from cir-

Table II Allele loss on chromosome 5 in HCC and colonic metastases in liver

	Probes and loci			
Patients	*pMS621* *5p*	*ECB27* *5q21*	*YN5.48* *5q21–22*	*Lambda MS8* *5q35-qter*
HCC with cirrhosis				
1	1,2	–	–	1,2
2	1,(2)	–	1,2	–
3	–	1,2	–	–
4	–	1,2	–	1,2
5	1,(2)	1,2	1,2	1,2
6	1,(2)	(1),2	(1),2	–
7	nd	–	nd	–
22	nd	–	nd	1,2
23	nd	–	–	1,2
Total no.	6	9	7	9
Heterozygosity	4	4	3	5
Allele loss	3	1	1	0
HCC without cirrhosis				
8	1,2	1,2	–	1,(2)
9	–	1,2	–	1,(2)
10	1,2	–	–	1,(2)
11	1,2	1,2	1,2	(1),2
12	1,2	–	1,2	–
13	nd	1,2	1,2	–
14	nd	nd	–	1,(2)
Total no.	5	6	7	7
Heterozygosity	4	4	3	5
Allele loss	0	0	0	5
Colonic metastasis				
15	1,2	–	1,2	1,2
16	1,2	–	(1),2	–
17	1,2	–	–	1,2
18	1,2	(1),2	1,(2)	–
19	–	–	–	1,2
20	1,2	–	1,(2)	(1),2
21	–	–	(1),2	1,(2)
Total no.	7	7	7	7
Heterozygosity	5	1	5	5
Allele loss	0	1	4	2

Homozygosity in the constitutional DNA (non-informative pattern) is indicated as a dash; where the normal tissue was informative the tumour genotype is shown in the table. Heterozygosity is indicated by 1,2. The continued presence of the larger allelic restriction fragment is indicated by '1' and '2' indicates continued presence of the smaller allelic fragment. Allele loss (deletion or reduction of intensity of a band) is indicated by ( ). 'nd' indicates no data.

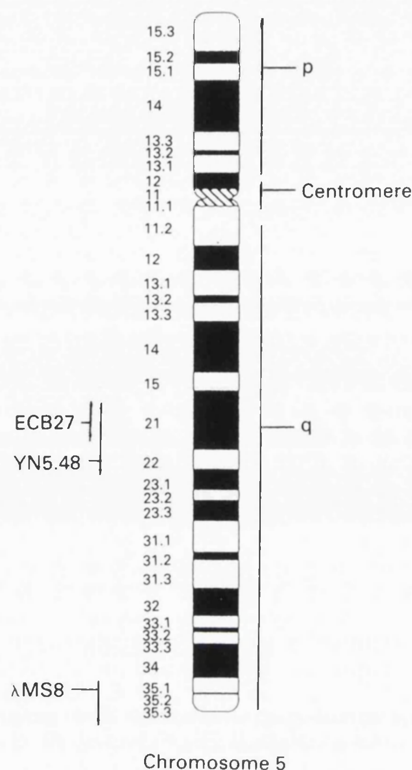

Figure 2 Localisation of some of the probes used for chromosome 5. ECB27 and YN5.48 screen 5q21–22 where APC gene locates, while λMS8 screens 5q35-qter where allele loss occurs in HCC.

rhotic patients with different aetiology. It is of interest that in some cases we have been able to compare DNA from lymphocytes with that from non-tumorous cirrhotic liver, but as yet no allele loss (pre-malignant loss) has been detected (data not shown).

Relatively few chromosome studies have been carried out on HCC, but investigations on HCC cell lines showed involvement of chromosome 5, regions p14 and q31–33 in rearrangement or deletion (Simon *et al.*, 1982; Simon & Knowles, 1986). Of particular relevance is a chromosome 5 (q34) rearrangment in direct preparations from an HCC arising in a patient without evidence of HBV infection (Simon *et al.*, 1990). In other studies (Buetow *et al.*, 1989; Zhang *et al.*, 1990) frequent allele losses were found on chromosomes 4 and 16 in both HBV positive and negative HCC. Tsuda *et al.* (Tsuda *et al.*, 1990) suggested that LOH on chromosome 16 represents tumour progression. Our own study did not show LOH on chromosomes 4q, 16p or 16q. This could reflect either the difference in probes used or a difference in the stage of the tumours studied. None of our patients had extrahepatic tumour spread and all underwent 'potentially curative' resection of the tumours. In agreement with other workers (Kiechle-Schwarz *et al.*, 1990), we have found no evidence for allele loss on 11p. A literature survey did not reveal previous screening of the terminal region of 5q in HCC. In a very recent study (Fujimori *et al.*, 1991) allelic loss was reported in HBV negative HCC in the region 5q21 (D5S84), but they did not mention the screening of 5q35-qter.

The comparison of the pattern of LOH on chromosome 5 between patients with non-cirrhotic HCC and patients with colorectal liver secondaries suggests that the loci are different in the two types of malignancies. The LOH in carcinoma of the colon peaks at the region 5q21–22 while the LOH in non-cirrhotic HCC is at 5q35 qter. A larger number of samples needs to be tested to confirm this preliminary finding. Future work will also aim to identify and characterise the gene associated with non-cirrhotic HCC.

In conclusion, this study suggests that one of the tumour suppressor genes in non-cirrhotic HCC could be located on chromosome 5 and appears to be distinct from the locus of the familial adenomatous polyposis coli (APC) gene.

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Loss of heterozygosity on chromosomes 1 and 11 in carcinoma of the pancreas

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Summary Little is known of the molecular-genetic changes in carcinoma of the pancreas (CaP). In order to investigate the allele loss, or loss of heterozygosity (LOH), in CaP, we studied 13 patients with exocrine CaP and two with endocrine CaP using restriction fragment length polymorphism analysis. Twenty probes assigned to chromosomes 1, 5, 7, 9, 11, 12, 13, 14, 16, 17 and 18 were used. The frequency of LOH, or fractional allele loss (FAL), was found in two endocrine tumours to be 0.333 and 0.455 respectively; and FAL in 13 oxocrine tumours ranged from 0 to 0.25. Allele loss was shown in both exocrine and endocrine tumours by the probes Lambda MS1 at 1p33-35, and pMS51 at 11q13. Probes for other chromosomes have as yet shown no consistent LOH. In conclusion, the study showed LOH on chromosomes 1 and 11 in both exocrine and endocrine CaP.

Carcinoma of the pancreas (CaP) is an increasingly common disease. The prognosis of CaP is poor with an overall mean survival of 3–4 months; only about 5% of patients survive for 2 years. Few tumours are amenable to resection with the chance of 'cure'. Neither radiotherapy nor cytotoxic drugs improve the prognosis significantly.

Much evidence has accumulated that loss of tumour suppressor genes is important in carcinogenesis (Stanbridge, 1990). A variety of tumours, including both inherited childhood and common adult malignancies, exhibit allele loss, or loss of heterozygosity (LOH), revealed by DNA restriction fragment length polymorphism (RFLP) analysis (Sager, 1989). Consistent loss of heterozygosity may represent tumour suppressor gene loss. Several such genes have been cloned, such as RB 1 (retinoblastoma) (Friend *et al.*, 1986; Lee *et al.*, 1987), DCC (deleted in colorectal cancer) (Fearon *et al.*, 1990), MCC (mutated in colorectal cancer) (Kinzler *et al.*, 1991*a*) and most recently, APC (adenomatous polyposis coli) (Kinzler *et al.*, 1991*b*; Groden *et al.*, 1991).

There are few reports about allele loss in CaP, in contrast to the comprehensive studies of other common malignancies, such as those in breast (Devillee *et al.*, 1989), colorectum (Vogelstein *et al.*, 1989), liver (Fujimori *et al.*, 1991) and lung (Kok *et al.*, 1987). Allele losses on chromosome 11 in both sporadic and familial pancreatic endocrine tumours, related to multiple endocrine neoplasia type 1 (MEN 1), have been reported (Bale *et al.*, 1991; Teh *et al.*, 1990). There have been preliminary reports of allele loss on 5q for exocrine CaP (Michelassi *et al.*, 1989; Westbrook *et al.*, 1990). It is of interest to know whether allele loss on chromosome 11 or other chromosomes also occurs in exocrine CaP, and whether there is any association between allele loss and clinical course in patients with CaP. Here we report a study of allele loss in CaP by screening with 20 RFLP markers, and the relationship between fractional allele loss and clinical parameters.

Materials and methods

Patients and biopsies

Fifteen patients with carcinoma of the pancreas were studied, including two with endocrine CaP and 13 with exocrine CaP. Of the 13 with exocrine CaP 12 had tumours of the head of pancreas while the remaining one had a tumour of the ampulla of vater. All underwent resection of their tumours (either by partial or total pancreatectomy) except one patient with peritoneal secondaries that had palliative bypass (hepaticojejunostomy and gastrojejunostomy). Of the 13 patients with exocrine CaP, four had their tumours localised to the pancreas while the other nine had metastases in local lymph nodes or extension of their tumours in adjacent portal vein. Judged by the operating surgeons, seven patients had small tumours that were resected radically while the remaining six had large tumours or late diseases such that their surgical procedures should be considered palliative. All patients, if applicable, were followed-up for detection of post-operation recurrence. The data were available until 1 year after tumour resection.

Surgical biopsies from the tumoral and non-tumoral pancreas tissues were snap frozen in liquid nitrogen at the time of operation. Lymphocytes from peripheral blood obtained pre-operatively were also used as a source of normal DNA. Tissue was stored at − 70°C until DNA extraction. None of the patients received chemotherapy or radiotherapy prior to surgery and tumour samples were examined histologically to confirm the type of tumour present and the degree of differentiation of tumour cells.

DNA extraction and analysis

DNA was prepared from blood and tissue samples by standard methods (Sambrook *et al.*, 1989). Southern analyses were done as previously described (Ding *et al.*, 1991). The 20 RFLP probes for chromosomes 1, 5, 7, 9, 11, 12, 13, 14, 16, 17 and 18 and the appropriate restriction enzymes are listed in Table I. If two alleles appeared as two separate bands in the resultant autoradiograph of the constitutional DNA, the patient was considered 'informative', or heterozygous, for the particular marker. Complete deletion or great loss of intensity of one band in the tumour DNA indicated an allele loss, or an LOH. The fractional allele loss (FAL) was defined in a tumour as the number of chromosomal arms on which allelic loss was observed divided by the number of chromosomal arms for which allelic markers were informative in the patient's normal cells (Vogelstein *et al.*, 1989).

Statistical analysis

The significance of the relationship between frequency of allele loss and clinical parameters was checked by the Fisher's exact test (Bland, 1987).

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Table I Loss of chromosomal heterozygosity in human carcinoma of the pancreas

Probe	Chromosomal region	Enzyme used	Exocrine CaP (n = 13)	Endocrine CaP (n = 2)
λMS1[a]	1p33-35	HinfI	3/12[b]	2/2
λMS32	1q42-43	AluI	0/11	2/2
cMS621	5p	HinfI	0/5	0/2
ECB27	5q21	BglII	0/4	0/1
YN5.48	5q21-22	MspI	0/4	1/1
λMS8	5q35-qter	HinfI	0/10	0/1
λMS31	7pter-q22	HinfI	0/8	0/2
pλg3	7q31.3-qter	HinfI	0/5	1/2
EFD126.3	9q34	PvuII	1/11	0/2
H-*ras*	11p15	BamHI	0/3	2/2
pMS51	11q13	HaeIII	2/7	1/1
λMS43	12q24.3-qter	HinfI	0/11	0/2
P3.8R	13q14.2	HindIII	0/8	0/2
cMS626	13q	HinfI	0/5	0/2
cMS627	14q	AluI	0/5	0/1
3'HVR	16p13.3	PvuII	0/10	1/1
pulB1148	16q22.1	TaqI	0/0	0/0
p144-D6	17p13	RsaI	0/9	0/2
pYNZ22	17p13	RsaI	0/6	0/2
cMS440	18q	HaeIII	0/5	0/1

[a]References for probes: λMS1, λMS32, λMS8, λMS31, pλg3 and λMS43: Wong *et al.*, 1987; cMS621, cMS626, cMS627 and cMS440: Armour *et al.*, 1990; ECB27: Varesco *et al.*, 1989; YN5.48: Nakamura *et al.*, 1988*a*; EFD126.3: Nakamura *et al.*, 1987; H-*ras*: Krontiris *et al.*, 1985; pMS51: Armour *et al.*, 1989; P3.8R: Friend *et al.*, 1986; 3'HVR: Higgs *et al.*, 1986; pulB1148: vd Straten *et al.*, 1983; p144-D6: Kondoleon *et al.*, 1987; pYNZ22: Nakamura *et al.*, 1988*b*. [b]No. with allele loss/No. of informative cases.

Results

Table I shows the overall allele loss in both exocrine and endocrine CaP; and the results of allele loss obtained in each tumour are shown in Table II. Overall, 171/252 Southern blots were informative (heterozygosity: 67.9%) and the overall LOH was 16/171 informative cases (9.4%). Figure 1 shows representative examples of allele loss.

Both tumours from the two patients with endocrine CaP had multiple allelic losses, with deletions on five chromosomal arms each (Tables I and II). The FAL was 0.333 and 0.455 for the two tumours respectively. The common regions deleted were at 1p33-35 (probe: Lambda MS1), 1q42-43 (Lambda MS32) and 11p15 (H-*ras*). One of the two patients (patient JJ) had allele loss at 11q13 (probe: pMS51), where the MEN 1 gene maps (Larsson *et al.*, 1988), while the other (HA) was non-informative for that marker. Patient HA showed LOH at 5q21-22, in the region of the adenomatous polyposis coli (APC) gene, but both of the two probes used for this region (ECB27 and YN5.48) showed a homozygous pattern for patient JJ and were hence uninformative.

As shown in Tables I and II, the 13 exocrine CaP had LOH in three out of 12 informative cases (25%) at the region 1p33-35, one of 11 (9%) at 9q34 and two of seven (28.6%) at 11q13, hecne both exocrine and endocrine tumours exhibited LOH at 1p32-33 and 11q13, the latter of which is close to the MEN 1 gene. The probe P3.8R for the RB1 gene at 13q14.2 showed no allele loss, nor did another probe, cMS626, screening 13q in either exocrine and endocrine tumours. For both groups, there was no allele loss found at 17p13 (where the p53 tumour suppressor gene maps), shown by the two probes used (p144-D6 and pYNZ22). The FAL in exocrine tumours ranged from 0 to 0.25 (Table II).

The possible relationship between allele loss and some clinical parameters in exocrine CaP was analysed (Table III). Of seven small tumours ($\leqslant 3$ cm), one had an allele loss, while out of six large tumours (>3 cm) five showed LOH ($P<0.05$). Allele loss was shown in four out of five tumours

Table II Allele loss in individual tumours

Patient name and age (year)	Chromosomal arms on which allelic markers were lost	Arms with no allele loss	FAL
Endocrine CaP (n = 2)			
JJ (32)	1p, 1q, 11p, 11q, 16q	5p, 5q, 7p, 7q, 9q, 12q, 13q, 14q, 17p, 18q	5/15 (0.333)
HA (47)	1p, 1q, 5q, 7q, 11p	5p, 7p, 9q, 12q, 13q, 17p	5/11 (0.455)
Exocrine CaP (n = 13)			
CJ (48)	9q	1p, 1q, 5p, 7p, 7q, 12q, 13q, 14q, 16p, 17p, 18q	1/12 (0.083)
LA (49)		1p, 1q, 5p, 5q, 7p, 7q, 9q, 11p, 13q, 14q, 16p, 17p	0/12 (0.000)
BA (68)	1p	1q, 5p, 5q, 7p, 9q, 11p, 13q, 16p, 17p, 18q	1/11 (0.091)
BE (52)	11q	1p, 1q, 5p, 5q, 7p, 7q, 9q, 12q, 13q, 14q, 16p, 17p, 18q	1/13 (0.077)
SD (55)		1q, 5p, 5q, 7p, 7q, 11p, 11q, 13q, 16p, 17p	0/11 (0.000)
KE (50)		1p, 1q, 5q, 7q, 11q, 12q, 13q, 16p	0/8 (0.000)
PD (60)		1p, 1q, 5q, 7p, 9q, 13q, 16p, 17p, 18q	0/9 (0.000)
GP (61)		1p, 1q, 5q, 7p, 9q, 11q, 13q, 16p, 17p, 18q	0/10 (0.000)
CV (51)	1p	1q, 5q, 7p, 9q, 13q, 16p, 17p	1/8 (0.125)
NW (67)		1p, 1q, 5q, 11q, 16p, 17p	0/7 (0.000)
MF (33)	11q	1p, 1q, 5q, 9q, 12q, 17p	1/6 (0.167)
PF (56)	1p	1q, 5q, 12q, 17p	1/4 (0.250)
KW (67)		1p, 9q, 11q, 12q, 17p	0/5 (0.000)

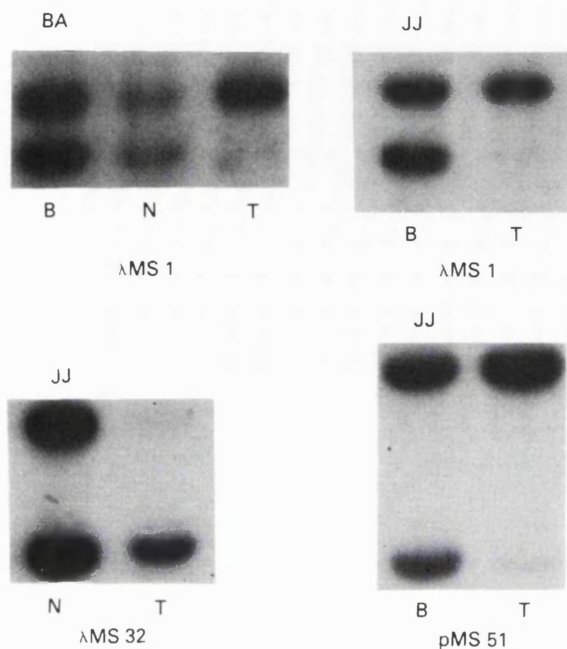

Figure 1 Representative autoradiographs of Southern hybridisation with Lambda MS1 (1p33-35), Lambda MS 32 (1q42-43) and pMS 51 (11q13). B = Blood lymphocyte DNA, N = Non-tumour tissue DNA, and T = Tumour tissue DNA. All show allelic losses in tumour DNA. Patient BA had exocrine CaP while Patient JJ had endocrine CaP.

Table III Association of allele loss with clinical course in human exocrine carcinoma of the pancreas

Tumour	*No. of cases*	*No. of allele loss*	*Significance*
Size[a]			
Small	7	1	$P<0.05$
Large	6	5	
Differentiation of tumour cells			
Well	2	0	
Moderate	5	3	N.S.[b]
Poor	2	1	
Unclassified	4	2	
Metastasis[c]			
Presence	9	5	N.S.
Absence	4	1	
Recurrence			
Presence	5	4	$P<0.05$
Absence	4	0	
Not applicable[d]	4	2	

[a]Size: $\leqslant 3$ cm = small, >3 cm = large. [b]N.S.: Not significant. [c]Metastasis: regional lymph nodes or liver deposits. [d]Two of these patients died from operative complication and the remaining two had very short follow-up.

from patients with recurrence, while none of the four tumours from the patients without recurrence had allele loss ($P<0.05$). There was a trend that tumours with poorer differentiation or with metastasis had more allelic losses, but the differences were not statistically significant (Table III).

Discussion

This study showed loss of heterozygosity on chromosomes 1p33-35 and 11q13 in both exocrine and endocrine carcinomas of the pancreas. Allele loss at 11q13 has been revealed in both sporadic and familial tumours arising in the endocrine pancreas (Bale *et al.*, 1991; Teh *et al.*, 1990). The informative patient with endocrine CaP in our study also had allele loss in this region. Interestingly, there was LOH shown by the marker at this region in two of seven informative cases of exocrine CaP, which has not been reported before. Whether the change in this region is involved in the development of exocrine CaP needs further study.

There are relatively few cytogenetic studies on CaP, but one study of particular interest showed deletion on chromosome 1p32 in one tumour and a translocation involving that breakpoint in a second (Johansson *et al.*, 1991). Allele loss at 1p33-35 was shown by the probe Lambda MS1 in this study in both exocrine (three out of 12 informative cases, Table I) and endocrine (2/2, Table I) CaP, which may indicate a possible tumour suppressor gene located there for both types of CaP, but as this region is frequently involved in advanced cancers of other types, its loss may be related to tumour progression (reviewed in Sager, 1989). More cases are needed to confirm the preliminary finding. It is of interest that allele loss also occurred on chromosome 1q in both endocrine cases, which may suggest that loss of genetic material in this region may be of importance for endocrine tumours.

Recently, loss or mutation of the p53 tumour suppressor gene at 17p13 has been seen at very high frequency in several common human malignancies (Stanbridge, 1990). A recent study in exocrine CaP also showed high frequency of overexpression of mutant forms of p53 by immunohistochemistry and of point mutations of the p53 gene by direct sequencing of genomic DNA (Barton *et al.*, 1991). Hence it was surprising to find that there was no allele loss shown by either probe (p144-D6 or pYNZ22) at 17p13 in either group of CaP in our study. This was in agreement with the finding of Westbrook *et al.* (1990), who did not find any LOH with pYNZ22 in seven informative pancreatic adenocarcinomas. It will be of interest to know if there is any overexpression of mutant p53 or point mutation of the p53 gene in our two groups of CaP.

Frequent rearrangement or loss of the prototype tumour suppressor gene, retinoblastoma (RB), also occurs in some other types of tumours (Horowitz *et al.*, 1990). No allele loss was shown by one of the cDNA probes from the RB gene in the two groups of CaP in this study.

Westbrook *et al.* (1990) reported allele loss in two out of seven informative exocrine CaP on chromosome 5 and suggested that the genetic changes associated with allele loss on that chromosome might be a common denominator in the development or progression of the gastrointestine cancers including those of colorectum and pancreas. In our study, the one informative endocrine CaP showed allele loss at 5q21-22, but four probes on chromosome 5 did not reveal LOH in the exocrine CaP group.

Vogelstein *et al.* (1989) reported that for colorectal carcinomas, patients with more LOH had a considerably worse prognosis than did the other patients. In this study we analysed the possible correlation between frequency of loss of heterozygosity and some clinical parameters within the group of exocrine CaP (Table III). There was a significant correlation found between the frequency of allele loss and the tumour size, and presence or absence of recurrence. The other data in Table III also showed a trend toward more aggressive behaviour in tumours with LOH. However it failed to reach statistical significance. A large study should be conducted in order to confirm the significance of these data.

In conclusion, the study showed LOH on chromosomes 1p33-35 and 11q13 in both exocrine and endocrine CaP. In the group of exocrine CaP, patients with larger tumours, or recurrence may have more allelic losses in their tumours.

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Differences in the tumour DNA analysis of a hepatocellular carcinoma and a synchronous fibrolamellar variant

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Abstract. A 60 year old man had two tumours resected from his liver which were shown histologically to be a well differentiated hepatocellular carcinoma and the fibrolamellar variant. Both are considered clinically distinct and of different pathogenesis and have not been previously reported occurring synchronously. DNA analysis revealed allele loss on chromosome 12 in the hepatocellular carcinoma which was not found in the fibrolamellar variant. This finding may suggest that hepatocellular carcinoma and fibrolamellar variant may have different molecular-genetic mechanisms for the development, in addition to their clinico-pathological difference.

Introduction

Fibrolamellar carcinoma (FLC) is a rare form of primary liver cell cancer which is clinically distinct from hepatocellular carcinoma(HCC). It occurs in younger patients (20-30 years) with an equal sex incidence. Cirrhosis and hepatitis B virus (HBV) infections are rarely seen in patients with FLC and it is thought that the tumour may arise from areas of focal nodular hyperplasia (1). Alpha-fetoprotein is not normally raised but, Vitamin B12 binding capacity and neurotensin levels have been found to be high in patients with FLC (2). The prognosis of patients with FLC is better than that of HCC with an average survival of 44 months compared to 6 months in HCC (3). It is these differences in clinico-pathological features which would suggest that FLC and HCC have a different pathogenesis. The difference in DNA analysis of a synchronous HCC and FLC has not been previously described.

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Key words: hepatocellular carcinoma, fibrolamellar carcinoma, allele loss

Patient and results

A 60 year old man presented with a four week history of right upper quadrant pain and was shown on ultrasound scan to have a tumour in the right lobe of the liver. He was referred to the hepatobiliary unit and an abdominal CT scan confirmed a mass in segment VI of the liver. Liver function tests were normal and HBV serology negative. The serum alpha-fetoprotein level was less than 20 mg/ml (normal range: less than 30 mg/ml). A needle biopsy of this lesion revealed a well differentiated hepatocellular carcinoma with adjacent liver which was interpreted as cirrhotic.

At laparotomy, two tumours were found within the right lobe of the liver. The tumour seen on the CT scan measured 3cm in diameter and was in segment VI. The second tumour was situated in segment VII and measured 1cm in diameter. Intra-operative ultrasonography excluded further lesions. Wide local resection of the two tumours was performed.

The larger tumour, which was yellow, and identical to the tumour detected preoperatively, was a well differentiated hepatocellular carcinoma with surrounding fibrosis (Fig. 1). The liver away from the tumour showed slight portal inflammation but no cirrhosis. The second smaller tumour which was found at the time of the operation was macroscopically and microscopically different from the former. It had a tan colour and was a fibrolamellar carcinoma with large polygonal cells and abundant eosinophilic cytoplasm. The tumour cells were separated into nests and sheets by lamellar bands of collagen (Fig. 2).

DNA analysis was performed on the patient's normal peripheral lymphocytes, both tumours and normal liver to look for chromosome allele loss which may represent tumour suppressor gene loss as previously described (4). Briefly, Southern analysis was performed with a panel of 19 probes recognising restriction fragment length polymorphism assigned to chromosomes 1, 2, 5, 7, 9, 11, 12, 13, 14, 16, 17 and 18. The pattern of DNA banding in the tumours was compared to that in blood lymphocyte DNA and normal liver cell DNA. The detection of chromosomal DNA loss was dependent on demonstrating a difference in restriction fragment length polymorphism between the tumour and the non- tumour DNA. When a probe can show 2 or more bands

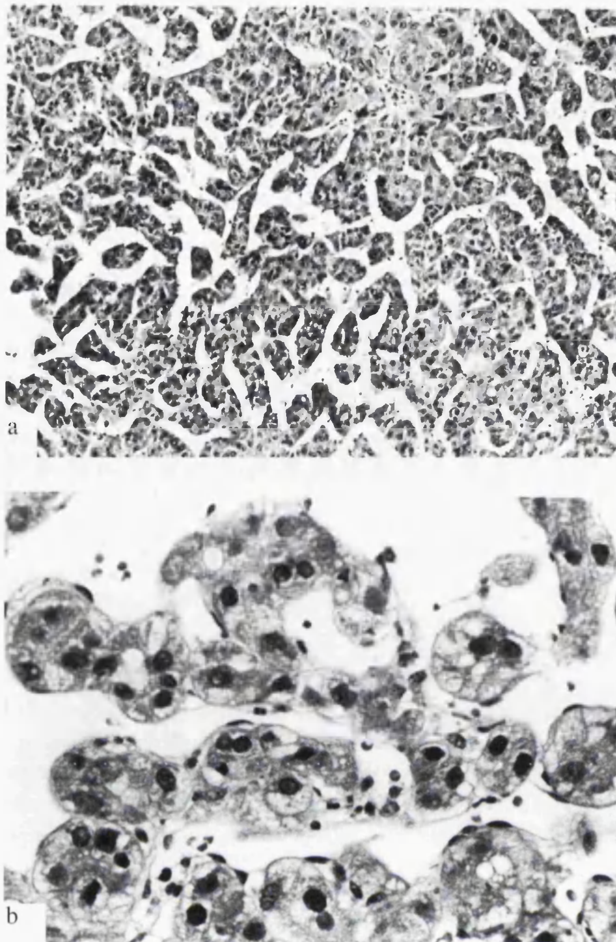

Figure 1. Microscopic Morphology of HCC. The larger tumour (3cm in diameter) resected is a well differentiated HCC. Haematoxylin and eosin, original magnification: a. x70; b. x300.

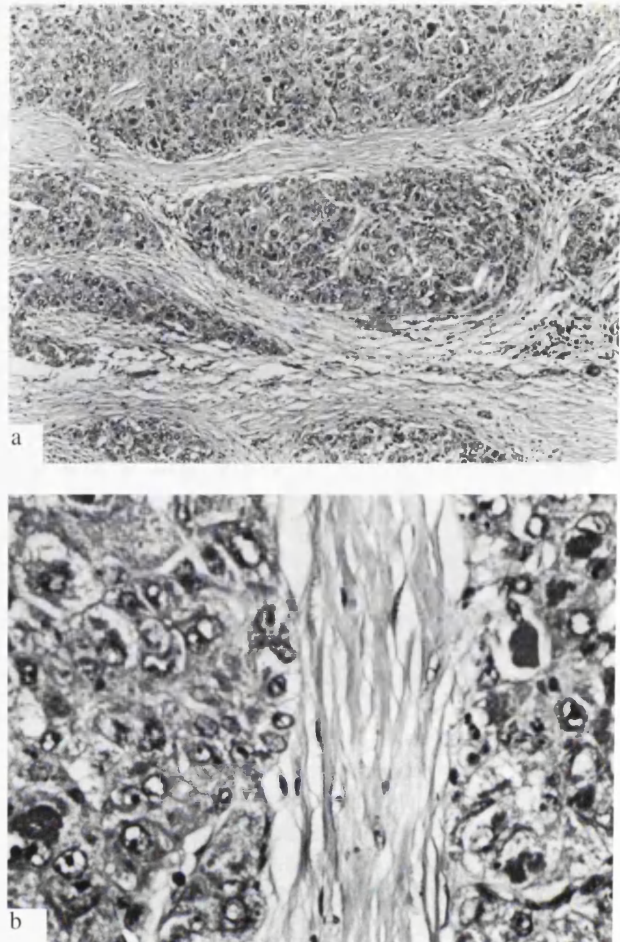

Figure 2. Microscopic Morphology of FLC. The smaller resected tumour (1cm diameter) consists of cells with abundant cytoplasm and a lamelated, fibrous stroma. It is an FLC and is quite different in appearance to the tumour in Figure 1. Haematoxylin and eosin, original magnification: a. x70; b. x300.

in autoradiographs of Southern hybridisation for normal DNA, the probe is called "informative". A deletion of one or more bands in tumour DNA can therefore be seen as allele loss. Some probes, however, only show one band in the constitutional DNA and are called "non-informative" because it is not possible to see deletion of bands in tumour DNA. The HCC of this patient showed allele loss on the long arm of chromosomes 12 with the probe λMS43 (12q24.3-qter), which was not found in the fibrolamellar tumour (Fig. 3). There were either no allele losses or non-informative patterns with probes in other chromosomes of the two tumours (Table I).

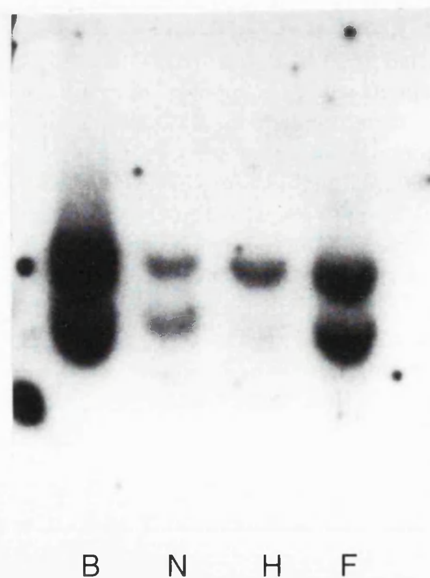

Figure 3. Autoradiograph of Southern Hybridisation with λMS43 (12q24.3-qter). B = Blood lymphocyte DNA; N = Non-tumour tissue DNA H = Hepatocellular carcinoma DNA; F = Fibrolamellar variant DNA. Note that the small allele is deleted in HCC DNA compared with lymphocyte and non-tumour DNA but, the allele is present in the FLC DNA.

Discussion

This is the first reported case of a hepatocellular carcinoma and a fibrolamellar carcinoma occurring in the same patient. The uniqueness of this is because of the markedly different pathogenesis of these tumours and has provided the opportunity for the first DNA analysis of these tumours arising synchronously.

HBV infection and cirrhosis are commonly found in patients with hepatocellular carcinoma, but neither of these factors can be implicated in the present case. It is thought

that HBV negative patients with HCC may have a different pathogenesis from those with HBV infection as the former have a better rate of resection and prognosis (5).

Table I. Allele status in the hepatocellular carcinoma and the synchronous fibrolamellar variant.

Probe Name	Chromosome Region	HCC	FLC
λMS1	1p33-35	1,2	1,2
λMS32	1q42-43	1,2	1,2
P5Gi	2q33-35	-	-
P2-96	2q35-37	1,2	1,2
pMS621	5p	1,2	1,2
ECB27	5q21	-	-
YN5.48	5q21-22	1,2	1,2
λMS8	5q35-qter	-	-
λMS31	17pter-q22	1,2	1,2
pλg3	7q31.3-qter	-	-
EFD126.3	9q34	-	-
pMS51	11q13	1,2	1,2
λMS43	12q24.3-qter	1,(2)	1,2
p3.8R	13q14.2	1,2	1,2
pMS627	14q	1,2	1,2
3'HVR	16p13.3	1,2	1,2
pulB1148	16q22.1	1,2	1,2
p144.D6	17p13	-	-
pMS440	18q	1,2	1,2

Non-informative pattern of normal DNA is indicated as a dash; where the normal DNA was informative the tumour genotype is shown in the Table as '1,2'. The continued presence of the larger allele is indicated by '1' and '2' indicates the continued presence of smaller allele. Allele loss is indicated by ( ).

The patient in this report is atypical in that FLC usually occurs in patients younger than 35 years of age (3). FLC typically occurs in non-cirrhotic livers and in those who are HBV negative. It may arise in an area of focal nodular hyperplasia (1) although there was no evidence of this in this case.

The hepatocellular carcinoma was unusual in the amount of surrounding fibrosis which led, on the pre-operative biopsy, to the mistaken assumption that the tumour was present within a cirrhotic liver. This has important implications for patient management as the size of liver resection which can safely be performed is dependent on the presence or absence of cirrhosis (6).

The molecular mechanisms involved in liver tumour carcinogenesis are not fully understood. HBV DNA integration, oncogene activation or mutation (7) and loss of tumour suppressor gene have all been implicated (4). There is increasing evidence that tumour suppressor gene loss is as important as oncogene activation in carcinogenesis and has been implicated in both childhood and adult common tumours (8). Recently allele loss has been found in all informative HCC without liver cirrhosis cases with the probe λMS8 (5q35-qter) and in majority of informative HCC patients with the probe p144.D6 (17p13), which may represent tumour suppressor gene losses in HCC (4). Unfortunately neither of the two probes is informative in the present patient (Table I), but allele loss on chromosome 12 found in the HCC which was not found in the FLC may provide further evidence for the concept that patients with greater allele loss have worse prognosis (9). To date, no tumour suppressor gene has been mapped on chromosome 12 yet. A recent study has shown allele loss on that chromosome in about 30% gastric carcinomas with the same probe (λMS43) (10) as we used in the present study. Further study is needed to establish whether there is a possible tumour suppressor gene located on chromosome 12.

The data of this study supports the view that FLC is a different clinico-pathological entity from HCC, and suggests that the former has a different molecular-genetic mechanism for the development.

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Different DNA changes in primary and recurrent hepatocellular carcinoma

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Abstract
DNA restriction fragment length polymorphism analysis was carried out on a primary and recurrent hepatocellular carcinoma in a hepatitis B virus negative patient. For the primary tumour, allele losses were found on the short arm of chromosome 17 (probe: p144-D6, 17p13) and the long arm of chromosome 5 with the probe Lambda MS8 (5q35-qter); other probes showed either no allele loss or a non-informative pattern. The recurrent cancer also showed allele loss with p144-D6, but not with Lambda MS8. In addition, the recurrent tumour had allele losses with Lambda MS43 (12q24.3-qter), pYNZ22 (17p13), and DNA rearrangement revealed by the probe Lambda MS32 (1q42-43), a pattern not seen in the primary lesion. These results indicate that the second hepatocellular carcinoma was of independent clonality and probably represents a de novo neoplasm rather than a recurrence.
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Hepatocellular carcinoma is one of the most lethal malignancies in the world. At present only surgical resection offers a chance for cure.[1] Intrahepatic tumour recurrence rates, after liver resection, however, can be as high as 50–82%.[2,3]

Information on the clonal origin of tumours is important for prevention of recurrence, as management strategies are formed based on the exact cause of this. It has been shown that, genetically, recurrence in hepatocellular carcinoma can either be monoclonal, with recurrent tumours originating from the primary lesion, or polyclonal, where recurrent tumours represent de novo neoplasms.[4] Detecting the integrated hepatitis B virus DNA in hepatocellular carcinoma genome is an effective means of determining the clonal origin of hepatitis B virus related hepatocellular carcinoma.[4,5] This approach is not possible for hepatitis B virus negative hepatocellular carcinoma, however, which is more common than hepatitis B virus related hepatocellular carcinoma in the West. Using DNA restriction fragment length polymorphism analysis, we report a case where a recurrent hepatocellular carcinoma showed a different clonality from the primary neoplasm.

Case report

A 74 year old man was noted to have a 10 cm mass in the left lobe of the liver on follow up ultrasonography for colorectal carcinoma. This was confirmed on computed tomography and biopsy showed it to be a well differentiated hepatocellular carcinoma. Laparotomy revealed a large lesion involving segments II, III, and IV of the liver, and a left hepatectomy was performed. Histology showed complete resection of tumour. A follow up computed tomography scan, performed 15 months later, revealed a 6 cm nodule in segment VI of the liver, with no extra hepatic

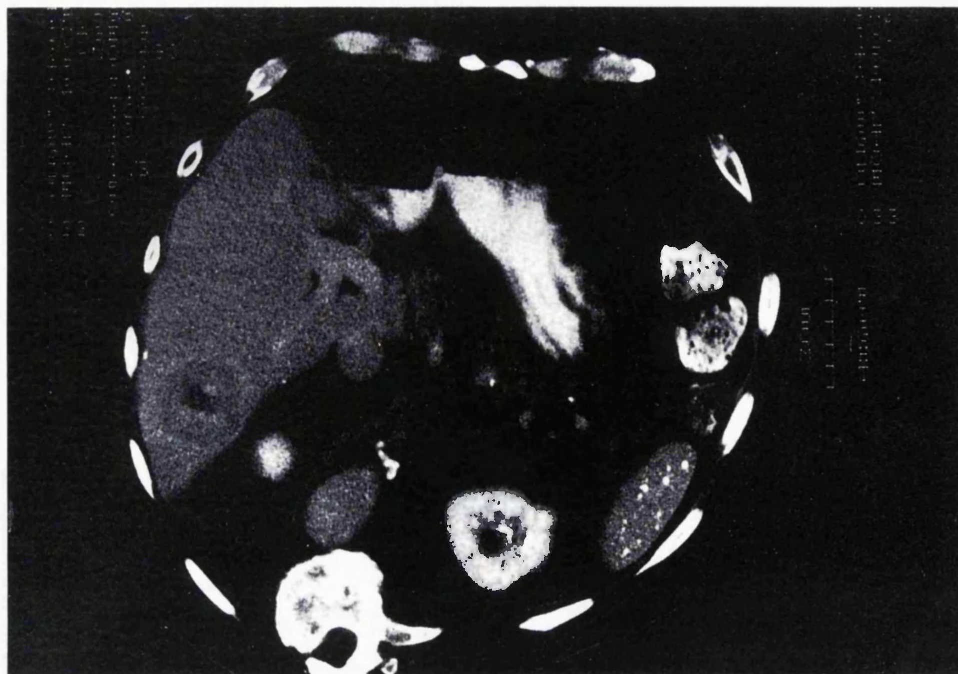

Figure 1: Computed tomography scan showing recurrent tumour in segment VI of liver.

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Pattern of DNA changes in the primary and recurrent hepatocellular carcinoma

Probe name	*Chromosome region*	*First HCC*	*Second HCC*	*Probe References*
Lambda MS1	1p33-35	1,2	1,2	8
Lambda MS32	1q42-43	–	R	8
Lambda MS8	5q35-qter	1,(2)	1,2	8
Lambda MS31	7pter-q22	1,2	1,2	8
p Lambda g3	7q31.3-qter	1,2	1,2	8
EFD126.3	9q34	1,2	1,2	9
pMS51	11q13	1,2	1,2	10
Lambda MS43	12q24.3-qter	1,2	(1),2	8
3'HVR	16p13.3	1,2	1,2	11
p144-D6	17p13	(1),2	(1),2	12
pYNZ22	17p13	1,2	(1),2	13

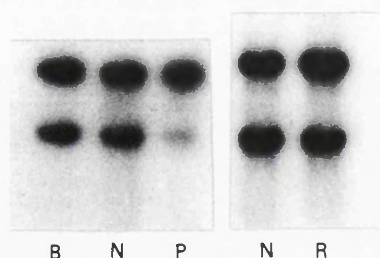
Homozygous pattern of normal DNA is indicated as a dash; where the normal DNA was heterozygous the tumour genotype is shown in the Table as '1,2.' The continued presence of the larger allele is indicated by '1,' and '2' indicates the continued presence of smaller allele. Allele loss is indicated by ( ). 'R' indicates DNA rearrangement. HCC = hepatocellular carcinoma.

metastasis (Fig 1). These findings were confirmed at laparotomy and local resection of segment VI was undertaken. Histology showed this to be a recurrent hepatocellular carcinoma. Serum neurotensin was normal throughout. Hepatitis B virus status, determined by blood assay and Southern analysis of hepatic tissue DNA, using the hepatitis B virus genome probe pEco63, was negative. At both operations, biopsies from the tumour and non-tumour liver tissue were collected and snap frozen in liquid nitrogen. Lymphocytes from peripheral blood, obtained before any blood transfusion, were also used as a source of constitutional (normal) DNA. Tissue and blood were stored at −70°C until DNA extraction.

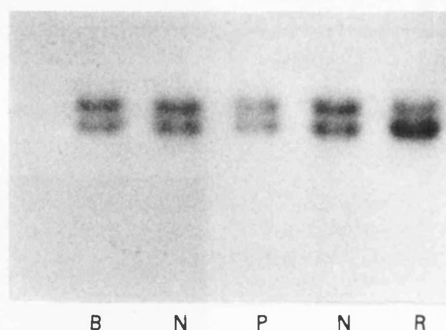
DNA was prepared from blood and tissue samples by standard phenol/chloroform methods.[6] Samples were digested with the appropriate restriction endonuclease and were size fractionated by electrophoresis through 0·9% agarose gels. The DNA was transferred to Hybond-N hybridisation filters (Amersham) according to the manufacturer's specifications. DNA probes recognising restriction fragment length polymorphisms were radiolabelled with alpha-^{32}P- dCTP (3000 Ci/mol) by the random hexanucleotide primer method to a high specific activity.[7] Hybridisations were performed at 65°C in 1% sodium dodecyl sulphate, 1 M NaCl and 5% dextran sulphate (W:V) for 16–24 h. Filters were washed to stringency of 2×standard saline citrate 1% sodium dodecyl sulphate (W:V) at 65°C and were autoradiographed at −70°C using Fuji: RX-L x-ray film.

DNA extracted from the tumours was compared with that from blood lymphocytes and normal liver. The detection of chromosomal DNA loss is dependent on the constitutional DNA being heterozygous for restriction fragment length polymorphisms at the various loci for which probes were available. Two bands are visible on the autoradiograph, representing different sized fragments of DNA. A deletion or loss of intensity of one of these bands in tumour DNA is indicative of loss of heterozygosity, or allele loss, in tumourigenesis. In some cases, rearrangement in tumour DNA can be shown with gain of one or more bands, compared with normal DNA. In this study, 11 probes assigned to chromosomes 1, 5, 7, 9, 11, 12, 16, and 17 were used (Table I).[8-13]

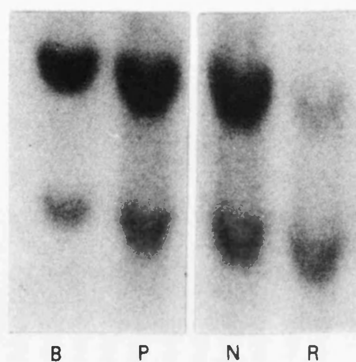
Of the 11 probes studied, three showed different patterns of loss of heterozygosity between the primary and recurrent hepatocellular carcino-



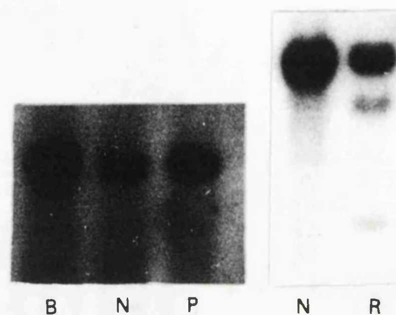
(A) Lambda MS8



(B) Lambda MS43



(C) pYNZ22



(D) Lambda MS32

Figure 2: Autoradiographs of Southern hybridisation with: (a) Lambda MS8 (5q35-qter); (b) Lambda MS43 (12q24.3-qter); (c) pYNZ22 (17p13); (d) Lambda MS32 (1q42-43); B=Blood lymphocyte DNA; N=Non-tumour tissue DNA; P=Primary hepatocellular carcinoma DNA; R=Recurrent hepatocellular carcinoma DNA.

mas (Table I, Fig 2). The primary tumour had allele loss on the long arm of chromosome 5 (probe: Lambda MS8, 5q35-qter) and the short arm of chromosome 17 shown by the probe p144-D6 (17p13) but not by the probe pYNZ22 (17p13), while the recurrent hepatocellular carcinoma had allele losses on chromosome 12 shown by Lambda 43 (12q24.3-qter) and chromosome 17 shown by both p144/D6 and pYNZ22, but no allele loss on chromosome 5 (Table I, Fig 2a–c). This patient was shown as homozygous with the probe Lambda MS32, assigned on the long arm of chromosome 1 (1q42-43), for his normal and primary tumour DNA. DNA from the recurrent tumour showed three bands, however, a gain of two, indicating a rearrangement (Fig 2d). Thus it was clear that the clonality was different between primary and recurrent tumours.

Discussion

DNA restriction fragment length polymorphism analysis in this patient showed differences in DNA changes between primary and recurrent hepatocellular carcinomas. The primary tumour had allele losses on chromosome 5 shown by Lambda MS8 and chromosome 17 shown by p144-D6, while the second tumour did not have allele loss on chromosome 5, but had additional allele losses on chromosomes 12 and 17 and rearrangement on chromosome 1. One of the possible explanations for the differences could be that a small subclone was not present initially in the sample of the first tumour and eventually grew up to form the recurrence, but as there were so many genetic changes present in the recurrence which were not initially there and the Lambda MS8 did not show allele loss, a balance of probabilities seemed that the latter tumour was not a progression from the first. More likely, both neoplasms were of different clonality.

Tumour recurrence can be the result of incomplete resection of the primary lesion, presence of satellite nodules at the time of primary resection, tumour seeding along needle biopsy track or genuine de novo tumour recurrence. DNA analysis in this patient allowed us to exclude the possibility of tumour seeding along the needle biopsy track of the original tumour. It also indicates that the first resection was indeed complete. It is of interest that this patient developed three independent primary neoplasms, which may indicate genetic predisposition.

It is imperative to study the clonal origin of the recurrent tumours, as strategies for preventing recurrence are dependent on causation. By detecting the integrated hepatitis B virus DNA in hepatocellular carcinoma genome, Chen *et al* reported five pairs of primary and recurrent hepatocellular carcinoma; two showing the same clonal origin, while the other three were of different clonalities.[4] For the first group, more careful diagnostic and treatment procedures, especially extent of surgical resection, are essential in preventing recurrence, while for the second group, one has to identify means of blocking the function of or deleting the effects of persisting carcinogenetic factors, such as hepatitis B virus infection. Although the techniques for these are not available at present, recent continuing advances in the field of molecular biology and tumour suppressor gene research might lead to some benefit with gene therapy in the future.[14]

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Infrequent chromosome allele loss in fibrolamellar carcinoma

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Summary As yet, there is no reported study of chromosome allele loss in fibrolamellar carcinoma (FLC), a distinct, rare variant of hepatocellular carcinoma (HCC). We searched for evidence of allele loss in FLC using 18 DNA probes for 10 chromosomes and compared the pattern of loss with our series of HCC. Two of the probes, λMS32 (1q42-43) and cMS621 (5p) showed allele losses in one tumour, while other probes showed no loss. The frequency of allele loss in FLC was much lower than in HCC, which may be associated with their different prognoses.

Fibrolamellar carcinoma (FLC) is a rare variant of hepatocellular carcinoma (HCC). It occurs in younger patients (20-30 years) with an equal sex incidence. Cirrhosis and hepatitis B virus (HBV) infections are rarely seen in patients with FLC and it is thought that the tumour may arise from areas of focal nodular hyperplasia (Vecchio *et al.*, 1984). The prognosis of patients with FLC is better than that of HCC with an average survival of 44 months compared to 6 months in HCC (Craig *et al.*, 1980). It is these differences in clinicopathological features which would suggest that FLC and HCC have a different pathogenesis.

Genes which are involved in tumorigenesis appear to belong to two classes, the cellular oncogenes and tumour suppressor genes. Normally, cell proliferation is controlled by a balance between growth-promoting proto-oncogenes and growth-limiting tumour suppressor genes. Malignant activation of the former occurs by point mutation, transposition or amplification, whereas loss of function in the latter group can be caused by complete gene deletion as well as by intragenic mechanisms (Aaronson, 1991; Weinberg, 1991). Where constitutional tissue is heterozygous at a particular gene locus, consistent reduction to homozygosity in tumorigenesis, caused by loss of genetic material, is taken as evidence for the presence of a tumour suppressor gene at or near that site. Chromosome allele loss, or loss of heterozygosity, occurs in all types of solid tumours analysed (Lasko *et al.*, 1991), and the frequency may be positively correlated with clinical prognosis, for example in colorectal cancer (Vogelstein *et al.*, 1989).

Recently, we and others have studied the pattern of chromosome allele loss (loss of genetic material) in HCC (Ding *et al.*, 1991; Zhang *et al.*, 1990; Fujimori *et al.*, 1991). In FLC, no such studies have been reported. Here we report the first study of chromosome allele loss in FLC with 18 DNA restriction fragment length polymorphism (RFLP) probes and compare the pattern of allele loss with that of HCC.

Materials and methods

Patients and biopsies

Due to the rarity of the condition, in the past 3 years we were able to study only five patients with fibrolamellar carcinoma who underwent surgical resection of their tumours at Hammersmith or the Royal Free Hospitals, despite the fact that both of these hospitals are national referral centres for liver cancers. Patients' clinical data are presented in Table I. None of the patients received chemotherapy or radiotherapy before surgery. Surgical biopsies from tumoral and non-tumoral liver tissues were snap frozen in liquid nitrogen at the time of operation. Lymphocytes from peripheral blood obtained pre-operatively were also used as a source of normal DNA. Tissue was stored at $-70°C$ until DNA extraction. A portion of each tumour sample was examined histologically to confirm the type of tumour present.

DNA extraction and analysis

DNA was prepared from blood and tissue samples by standard phenol/chloroform methods (Sambrook *et al.*, 1989). Southern analyses were done as previously described (Ding *et al.*, 1991). The 18 RFLP probes for chromosomes 1, 5, 7, 9, 11, 12, 13, 16, 17 and 18 and the appropriate restriction enzymes are listed in Table II. These 18 probes were those used in the previous study on HCC (Ding *et al.*, 1991), including probes screening regions near or flanking loci of most known tumour suppressor genes (Table II). If two alleles appeared as two separate bands in the resultant autoradiograph of the constitutional DNA, the patient was considered 'informative', or heterozygous, for the particular marker. Complete deletion or great loss of intensity of one band in the tumour DNA indicated an allele loss.

Statistical analysis

The significance of the difference in the frequency of allele loss was tested by a standard method for comparison of proportions (Bland, 1987).

Results

Table II shows the overall pattern of allele loss in fibrolamellar carcinoma. Overall, 55/78 Southern blots were infor-

Table I Clinical data of five patients with fibrolamellar carcinoma[a]

Case no.	*Sex*	*Age*	*HBV status*[b]	*Liver cirrhosis*	*FLC recurrence*	*No. of allele loss in FLC*
1	F	23	–	–	–	0
2	M	55	–	–	–	0
3	M	23	–	–	+	2
4[c]	M	60	–	–	–	0
5	F	19	–	–	–	0

[a]–: negative or absent; +: positive or present. [b]HBV status was determined by blood assay and Southern analysis of hepatic tissue DNA, using the HBV genome probe pEco63. [c]This patient had a synchronous HCC. Two tumours were resected together.

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Table II Chromosome allele loss in fibrolamellar carcinoma

Probe	*Chromosomal region*	*Enzyme used*	*Allele loss*[a]
λMS1[b]	1p33-35	HinfI	0/3
λMS32	1q42-43	AluI	1/3
cMS621	5p	HinfI	1/4
ECB27[c]	5q21	BglII	0/0
YN5.48[c]	5q22	MspI	0/3
λMS8	5q35-qter	HinfI	0/2
λMS31	7pter-q22	HinfI	0/4
pλg3	7p31.3-qter	HinfI	0/3
EFD126.3	9q34	PvuII	0/2
H-ras	11p15	BamHI	0/3
pMS51	11q13	HaeIII	0/4
λMS43	12q24.3-qter	HinfI	0/5
cMS626[d]	13q	AluI	0/4
3'HVR	16p13.3	PvuII	0/4
pulB1148	16q22.1	TaqI	0/1
p144-D6[e]	17p13	RsaI	0/2
pYNZ.22[e]	17p13	RsaI	0/5
cMS440	18q	HaeIII	0/3

[a]No. with allele loss/No. of informative cases. [b]References for probes: See Table I in Ding *et al.* (1991). [c]These two probes screen the region flanking the MCC (mutated in colorectal cancer) (Kinzler *et al.*, 1991*b*) and APC (familial adenomatous polyposis coli) genes (Kinzler *et al.*, 1991*a*; Groden *et al.*, 1991). [d]This probe was assigned to the chromosome arm where the RB (retinoblastoma) tumour suppressor gene locates. [e]These two probes screen the regions near the locus of the p53 tumour suppressor gene.

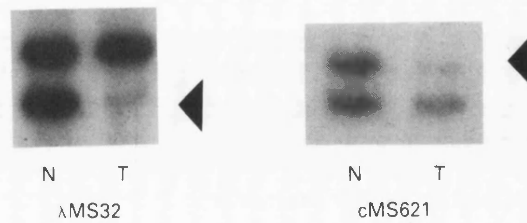

Figure 1 Autoradiographs of Southern hybridisations of Patient No. 3's DNA with λMS32 (1q42-43) and cMS621 (5p). N = non-tumour tissue DNA; T = tumour tissue DNA. Both show allelic losses in tumour DNA (indicated by arrows).

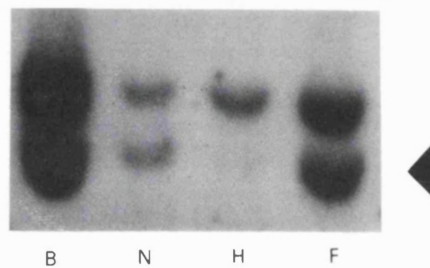

Figure 2 Autoradiograph of Southern hybridisation of Patient No. 4's DNA with λMS43 (12q24.3-qter). B = Blood lymphocyte DNA; N = Non-tumour tissue DNA; H = Hepatocellular carcinoma DNA; F = Fibrolammellar variant DNA. Note that the small allele is deleted in HCC DNA compared with lymphocyte and non-tumour DNA but, the allele is present in the FLC DNA (indicated by the arrow).

mative (heterozygosity: 70.5%) and the overall allele loss was only two out of 55 informative cases (3.6%). The frequency of allele loss in FLC is significantly lower than that in HCC (30/186, 16.1%, Ding *et al.*, 1991) [$P = 0.03$, SE $(P_1 - P_2) = 2.7\%$]. Figure 1 shows the two allelic losses, both of which occurred in a single patient (No. 3). Only that patient had a recurrent FLC (Table I). The chromosomal regions deleted in his tumour were 1q42-43 detected by the probe λMS32 and 5p by cMS621. These two probes also showed a high frequency of allele loss in HCC with liver cirrhosis, as previously reported (Ding *et al.*, 1991).

Patient No. 4 had a synchronous HCC. The HCC of this patient had an allele loss detected by the probe λMS 43 (12q24.3-qter), but his FLC had no similar allele loss (Figure 2). The probe was informative also in all the other four patients but showed allele loss in none of them (Table II).

Discussion

This study showed that the frequency of allele loss in fibrolamellar carcinoma was very low (2/55, 3.6%). With the same method, we found a much higher frequency of allele loss in HCC (30/186, 16.1%) (Ding *et al.*, 1991). For colorectal carcinomas, patients with a higher frequency of allelic losses had a considerably worse prognosis than did the other patients (Vogelstein *et al.*, 1989). A similar correlation was observed in carcinomas of the pancreas (Ding *et al.*, 1992). Thus this study showing a much lower frequency of allele loss in FLC than in HCC is in agreement with the above observations since FLC has a much better prognosis than HCC (Craig *et al.*, 1980). Of the five patients with FLC in this study, the FLC with two allelic losses recurred while the others did not (Table I).

Previously, we reported that in HCC with liver cirrhosis the highest frequency of allele loss occurred in chromosomal regions 1q42-43, 5p and 17p13, and in HCC without cirrhosis, in 5q35-qter and 17p13 (Ding *et al.*, 1991). The probes used for the region 17p13, i.e. p144-D6 and pYNZ.22, were near the locus of the p53 tumour suppressor gene. The high frequency of allele loss shown by these probes in HCC might represent the p53 gene loss in the tumour. The specific mutation of codon 249 of the p53 gene has been reported in the HCC from patients with high exposure to aflatoxin B_1 (Hsu *et al.*, 1991; Bressac *et al.*, 1991). All the HCC cases in our study were patients from Europe and Egypt, the areas with a low exposure to aflatoxin B_1. No mutation at codon 249 of the p53 gene has been found (Ding *et al.*, unpublished data).

None of the informative FLC had allele loss in 5q35-qter and 17p13 (Table II), the chromosomal regions where our HCC series showed a high frequency of allele loss. It is of interest to note that the two allelic losses in the FLC occurred in 1q42-43 and 5p. A larger study is needed to determine whether the loss is characteristic of this type of tumour or due to chance (Lasko *et al.*, 1991). In Patient No. 4 who had a synchronous HCC and FLC, the HCC showed allelic loss in the region 12q24.3-qter, but not the FLC.

These results may for the first time show the differences in genetic background in these two primary liver cancers, in addition to their clinico-pathological differences.

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Loss of constitutional heterozygosity on chromosomes 5 and 17 in cholangiocarcinoma

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Summary It has been established that loss of tumour suppressor genes is crucial in carcinogenesis. There has been no reported study on searching for tumour suppressor genes in cholangiocarcinomas as yet. In order to investigate the loss of heterozygosity (LOH), which may represent such gene loss, in cholangiocarcinoma, we studied 14 patients with this tumour using restriction fragment length polymorphism analysis. Twenty-two probes assigned to chromosomes 1, 5, 7, 9, 11, 12, 13, 14, 16, 17 and 18 were used. Allelic losses were found in chromosomal regions 5q35-qter and 17p13. Loss of genetic material in these regions in cholangiocarcinoma was shared with hepatocellular carcinoma. Probes for other chromosomes have as yet shown no consistent LOH. In conclusion, this study for the first time showed LOH on chromosomes 5 and 17 in cholangiocarcinoma.

Cholangiocarcinoma, the intrahepatic bile duct carcinoma, is thought to arise from the same stem cell as hepatocellular carcinoma (HCC) (Sell & Dunsford, 1989). Cholangiocarcinoma is reported as occurring less frequently than HCC in most parts of the world. The prognosis of cholangiocarcinoma is poor, with the majority of patients dying 6-12 months after diagnosis. The overall survival rate in treated cases at 5 years is below 9% (Czerniak & Blumgart, 1989).

There is a growing realisation that cancer is a set of fundamentally genetic diseases (Lasko *et al.*, 1991). Muliple genetic alterations including the activation of oncogenes and the inactivation of tumour suppressor genes are important in carcinogenesis. Tumour suppressor genes are normal cellular genes whose products are thought to be inhibitors of the uncontrolled cellular proliferation characteristic of cancer. Several tumour suppressor genes have been cloned, including the RB1 (Friend *et al.*, 1986), p53 (Oren *et al.*, 1981), WT1 (Call *et al.*, 1990), NF1 (Wallace *et al.*, 1990; Viskochil *et al.*, 1990; Cawthon *et al.*, 1990) and APC (Kinzler *et al.*, 1991*a*; Groden *et al.*, 1991) genes. DCC was clones and could prove to be a candidate suppressor gene (Fearon *et al.*, 1990). Introduction of a normal tumour suppressor gene, for example the RB1 gene, into tumour cells can inhibit tumorigenesis (Bookstein *et al.*, 1990).

Inactivation of tumour suppressor genes can occur via a variety of mechanisms including allele loss and mutation. One of the most widely used techniques for detection of tumour suppressor gene loss is the demonstration of consistent allele loss or loss of heterozygosity (LOH), in tumour cells. This is achieved by using a battery of restriction fragment length polymorphism (RFLP) probes to analyse DNAs from paired samples of non-tumour and tumour tissues (Lasko *et al.*, 1991). A variety of tumours, including both childhood and common adult malignancies, exhibit LOH (Lasko *et al.*, 1991).

Expression of oncogenes, including ras, myc and erbB-2, and point mutations at K-ras codons 12 and 61 have been reported in a high proportion of cholangiocarcinomas (Voravud *et al.*, 1989; Tada *et al.*, 1990). Cytogenetic studies on two cholangiocarcinoma cell lines revealed several chromosomal abnormalities (Storto *et al.*, 1990). To our knowledge, however, there has been no reported study of loss of heterozygosity in cholangiocarcinomas as yet. Here we report the first study of LOH in cholangiocarcinoma with 22 RFLP probes assigned to 11 chromosomes.

Materials and methods

Patients and biopsies

Fourteen patients with cholangiocarcinoma were studied. All underwent resection of their tumours. None of the patients received chemotherapy or radiotherapy before surgery. Surgical biopsies from tumoral and non-tumoral liver tissues were snap frozen in liquid nitrogen at the time of operation. Lymphocytes from peripheral blood obtained pre-operatively were also used as a source of normal DNA. Tissue was stored at − 70°C until DNA extraction. A portion of each tumour sample was examined histologically to confirm the type of tumour present.

DNA extraction and analysis

DNA was prepared from blood and tissue samples by standard phenol/chloroform methods (Sambrook *et al.*, 1989). Southern analyses were done as previously described (Ding *et al.*, 1991). The 22 RFLP probes for chromosomes 1, 5, 7, 9, 11, 12, 13, 14, 16, 17 and 18 and the appropriate restriction enzymes are listed in Table I. If two alleles appeared as two separate bands in the resultant autoradiograph of the constitutional DNA, the patient was considered 'informative', or heterozygous, for the particular marker. Complete deletion or loss of intensity of one band in the tumour DNA indicated an allele loss, or an LOH. The loss of band intensity was confirmed by examination of the autoradiographs with densitometry. A cutoff level of 50% or more of allele intensity was considered as evidence of LOH.

Results

Table I shows the overall pattern of allele loss in cholangiocarcinoma. Overall, 164/229 Southern blots were informative (heterozygosity: 71.6%) and the overall LOH was 17 out of 164 informative cases (10.4%). Figure 1 shows representative examples of allele loss.

As shown in Table I, the 14 cholangiocarcinomas had a higher rate of LOH on chromosomes 5 and 17 than on other chromosomes. Allelic losses were shown in two out of 14 informative cases (14.3%) for the region of the short arm of chromosome 1 (1p33-35) detected by the probe λMS1, three

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Table I Loss of constitutional heterozygosity in human cholangiocarcinoma

Probe	Chromosomal region	Enzyme used	LOH[a]
λMS1[b]	1p33-35	HinfI	2/14
λMS32	1q42-43	AluI	3/13
cMS621	5p	HinfI	0/4
ECB27	5q21	BglII	0/5
L5-71	5q21	MspI	0/7
54-D	5q21	MspI	0/6
YN5.48	5q21-22	MspI	0/4
λMS8	5q35-qter	HinfI	3/10
λMS31	7pter-q22	HinfI	1/13
pλg3	7q31.3-qter	HinfI	0/12
EFD126.3	9q34	PvuII	1/11
H-ras	11p15	BamHI	0/3
pMS51	11q13	HaeIII	0/7
λMS43	12q24.3-qter	HinfI	1/11
p3.8R	13q14.2	HindIII	0/7
cMS626	13q	AluI	0/5
cMS627	14q	AluI	0/5
3'HVR	16p13.3	PvuII	0/8
pulB1148	16q22.1	TaqI	0/3
p144-D6	17p13	RsaI	4/9
pYNZ.22	17p13	RsaI	2/5
cMS440	18q	HaeIII	0/2

[a]No. with LOH/No. of informative cases. [b]References for probes: λMS1; λMS32, λMS8, λMS31, pλg3 and λMS43: Wong *et al.*, 1987; cMS621, cMS627 and cMS440: Armour *et al.*, 1990; ECB27: Varesco *et al.*, 1989; L5-71: Kinzler *et al.*, 1991*b*; 54-D: Kinzler *et al.*, 1991*a*; YN5.48: Nakamura *et al.*, 1988*a*; EFD126.3: Nakamura *et al.*, 1987; H-ras: Krontiris *et al.*, 1985; pMS51: Armour *et al.*, 1989; P3.8R: Friend *et al.*, 1986; 3'HVR: Higgs *et al.*, 1986; pulB1148: van der Straten *et al.*, 1983; p144-D6; Kondoleon *et al.*, 1987; pYNZ22: Nakamura *et al.*, 1988*b*.

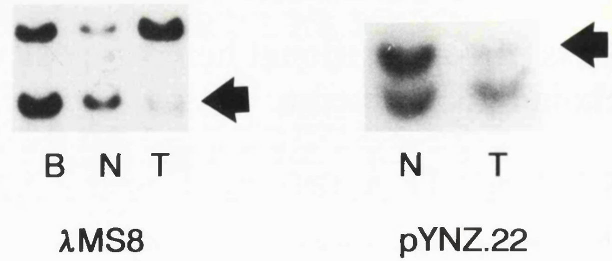

Figure 1 Representative autoradiographs of Southern hybridisations with λMS8 (5q35-qter) and pYNZ.22 (17p13). B = blood lymphocyte DNA; N = non-tumour tissue DNA; T = tumour tissue DNA. The autoradiographs show allele losses in tumour DNA (indicated by arrows).

out of 13 (23.1%) for the region of the long arm of chromosome 1 (1q42-43) by λMS32, three out of 10 (30%) for 5q35-qter by λMS8, four out of nine (44.4%) at 17p13 by p144-D6 and two out of five (40%) also at 17p13 by pYNZ22. No consistent allele loss was revealed by any other probes used in this study.

Previous work on tumours of the colon and rectum has shown that the chromosome 5 region (5q21-22) encompassing the familial adenomatous polyposis coli (APC) gene and the mutated in colorectal cancer (MCC) gene is deleted in inherited and sporadic colorectal cancer (Miyaki *et al.*, 1990; Ashton-Rickardt *et al.*, 1991). For this reason we compared the pattern of allele loss in cholangiocarcinoma with that of secondary liver tumours from colorectal origin using various

Table II Allele loss on chromosome 5 in cholangiocarcinomas and colonic metastases in liver

	Probes and regions or genes					
Patients	*cMS621 (5p)*	*ECB27 (5q21)*	*L5-71 (MCC)*	*54-D (APC)*	*YN5.48 (5q21-22)*	*λMS8 (5q35-qter)*
Cholangiocarcinoma						
1	1,2	–	1,2	1,2	1,2	(1),2
2	1,2	1,2	–	1,2	1,2	1,2
3	–	1,2	1,2	–	–	1,2
4	1,2	–	1,2	–	1,2	1,2
5	–	1,2	–	1,2	1,2	–
6	1,2	nd	1,2	1,2	–	1,(2)
7	nd	1,2	–	–	nd	1,2
8	nd	1,2	1,2	–	nd	1,(2)
9	nd	nd	1,2	1,2	–	–
10	nd	nd	1,2	1,2	nd	1,2
11	nd	nd	nd	–	nd	1,2
12	nd	nd	nd	–	nd	–
13	nd	nd	nd	nd	nd	–
14	nd	nd	nd	nd	nd	1,2
Total no	6	7	10	12	7	14
Heterozygosity	4	5	7	6	4	10
Allele loss	0	0	0	0	0	3
Colonic metastasis						
15	1,2	–	1,2	1,(2)	1,2	1,2
16	1,2	–	–	(1),2	(1),2	–
17	1,2	–	(1),2	–	–	1,2
18	1,2	(1),2	–	1,(2)	1,(2)	–
19	–	–	–	1,2	–	1,2
20	1,2	–	(1),2	–	1,(2)	(1),2
21	–	–	–	–	(1),2	1,(2)
Total no	7	7	7	7	7	7
Heterozygosity	5	1	3	4	5	5
Allele loss	0	1	2	3	4	2

Homozygosity in the constitutional DNA (non-informative pattern (is indicated as a dash; where the normal tissue was informative the tumour genotype is shown in the table. Heterozygosity is indicated by 1,2. The continued presence of the larger allelic restriction fragment is indicated by '1' and '2' indicates continued presence of the smaller allelic fragment. Allele loss (deletion or reduction of intensity of a band) is indicated by (). 'nd' indicates no data.

probes for chromosome 5q, including a genomic probe L5-71-3 for MCC and a cDNA probe, 54-D, for APC. Table II shows that patients with cholangiocarcinoma had no allele loss when screened with probes mapped to regions of the chromosome other than 5q35-qter. On the other hand the majority of patients with hepatic metastases from colorectal cancers showed allele loss with probes from 5q21-22, the region of the chromosome associated with colorectal cancer.

Discussion

This is the first study on loss of heterozygosity in cholangiocarcinomas. Three out of 22 probes revealed a relatively high rate of LOH in two chromosomal regions, namely, 5q35-qter (30%) and 17p13 (44.4% and 40%). There were also allelic losses at 1p33-35 (2/14, 14.3%), 1q42-43 (23.1%), 7pter-q22 (1/13, 7.7%), 9q34 (1/11, 9.1%) and 12q24.3-qter (1/11, 9.1%), but these lower values might represent random losses since rapid division of malignant cells can produce loss of heterozygosity at a certain region by chance (Lasko *et al.*, 1991).

We have previously reported allelic losses at 1q42-43 and 17p13 in hepatocellular carcinoma with liver cirrhosis and at 5q35-qter and 17p13 in HCC without liver cirrhosis (Ding *et al.*, 1991). Hence it is of interest to find LOH at 5q35-qter and 17p13 in cholangiocarcinoma in this study. It has been proposed that HCC and cholangiocarcinoma arise from the same pluripotent liver stem cell (Sell & Dunsford, 1989). These two types of primary liver malignancies, therefore, may share similar genetic changes. Allele loss on chromosome 17p is shared with other tumours and may be involved in 'tumour progression' (Sager, 1989; Lasko *et al.*, 1991). Loss of heterozygosity at 5q35-qter in both HCC and cholangiocarcinoma thus might represent a common genetic change in the development of the two tumours. Further study is needed to confirm this finding. This investigation reports the results of 14 patients collected simultaneously from two active liver centres over 3 years. The scarcity of this material highlights the difficulty of surgical resection of intrahepatic cholangiocarcinoma. Most patients present usually at such an advanced stage that precludes surgical resection. The familial adenomatous polyposis coli (APC) gene is located at 5q21 and the gene has been cloned (Kinzler *et al.*, 1991*a*; Groden *et al.*, 1991). We previously compared the pattern of allele loss in non-cirrhotic HCC with that of hepatic metastases from colorectal cancers using various probe for chromosome 5q (Ding *et al.*, 1991). The majority of LOH in hepatic metastases from colorectal cancers was found at the region 5q21-22 while the LOH in non cirrhotic HCC was at 5q35-qter. In the present study on cholangiocarcinomas allele loss also occurred at 5q35-qter. However, probes from 5q21-22, including a cDNA probe from APC gene, did not show any allele loss in cholangiocarcinoma (Tables I and II). The possible common region involved in both HCC and cholangiocarcinoma appears to be distinct from that encompassing APC. This is supported by the finding that the three patients exhibiting allele loss at 5q35-qter with the probe λMS8 have shown no allele loss with the probe 54-D from the APC gene.

There has been no reported direct cytogenetic study as yet on cholangiocarcinoma tissue. Chromosome study on two cholangiocarcinoma cell lines showed a number of abnormalities (Storto *et al.*, 1990). It is of particular interest that chromosome 5 was among the most commonly involved chromosomes in structural abnormalities in both cell lines. This finding and the results of RFLP analysis in this study suggest that mutation or deletion of a possible tumour suppressor gene located on chromosome 5, distal to 5q21-22, may play a role in the development of cholangiocarcinoma.

Recently, loss or mutation of the p53 tumour suppressor gene at chromosome 17p has been seen at a very high frequency in a variety of human malignancies (Weinberg, 1991). Loss of heterozygosity occurred in four out of nine cholangiocarcinoma shown by p144-D6, and in two of five shown by pYNZ22, in this study. Both probes are assigned to the region of 17p13, near the locus of the p53 tumour suppressor gene. This finding makes it likely that loss of the p53 gene is also involved in the development of cholangiocarcinoma. It will be of interest to know if there is any overexpression of mutant p53 or point mutation of the p53 gene in cholangiocarcinoma.

In conclusion, this study showed allelic losses on chromosomes 5q35-qter and 17p13 in cholangiocarcinoma. These losses are shared with HCC.

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Lack of demonstrable chromosome allele loss in hepatocellular adenoma

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Abstract. Several studies of loss of constitutional genetic heterozygosity in the development of hepatocellular carcinoma have been carried out with the aim of determining the location of relevant tumour suppressor genes, but there is as yet no report of a similar investigation in hepatocellular adenomas. In this study we analyzed six such patients searching for evidence of chromosome allele loss, or loss of heterozygosity, with 25 DNA probes recognising restriction fragment length polymorphisms. The 25 probes have been assigned to 21 chromosome arms. None of those probes detected allele loss in any of the 6 tumours. One of the patients had a synchronous hepatocellular carcinoma. Chromosome 17p allele loss was detected in her hepatocellular carcinoma but not in the adenoma. The study suggests that chromosome allele loss is not a frequent genetic change in the development of hepatocellular adenoma.

Introduction

Hepatocellular adenoma is a rare liver tumour, but the incidence has increased significantly following the introduction of oral contraceptives in the last 20 years (1). Ninety percent of patients with hepatocellular adenoma are young women, of whom another ninety percent have used an oral contraceptive (2). The prognosis of this benign tumour is good (2).

Carcinogenesis is a multi-step process (3). In an animal model of liver tumour induction by the HBx gene of hepatitis B virus, the sequential process from multifocal areas of altered hepatocytes, to benign adenomas and eventually to malignant carcinomas was observed (4). Although oral contraceptive-associated hepatocellular adenomas may not be premalignant and may undergo reversible change after withdrawal of oral contraceptives, foci or areas of liver cell dysplasia within adenomas are premalignant and may transform into hepatocellular carcinoma (5). In the colorectum most, if not all, malignant tumours (carcinomas) arise from pre-existing benign tumours (adenomas) and a genetic model has been proposed for this process (3). There are fewer genetic changes in adenomas compared with those in carcinomas, but changes like chromosome allele loss, or loss of heterozygosity, do occur in colorectal adenomas, though at a much lower frequency (3,6). Allele loss in one thyroid adenoma and one parathyroid adenoma has also been reported (7,8).

Detection of chromosome allele loss, or loss of heterozygosity (LOH), has been widely used in searching for the sites of tumour suppressor genes (9). Among liver tumours, LOH at consistent chromosomal locations in hepatocellular carcinoma (HCC) has been well documented (10-12). The aim of our study was to find out whether LOH also occurs in the counterpart adenoma. To our knowledge no such studies have hitherto been reported.

Materials and methods

Patients and biopsies. Six patients with hepatocellular adenoma were studied. All were female and ages ranged from 16 to 51 years old. Four of them had been on oral contraceptives. All underwent resection of their tumours. One of the patients (No. 1, aged: 51) had synchronous hepatocellular carcinoma and adenoma and both tumours were resected. She had never used any oral contraceptives. Surgical biopsies from tumoral and non-tumoral liver tissues were snap frozen in liquid nitrogen at the time of operation. Lymphocytes from peripheral blood obtained pre-operatively were also used as a source of normal DNA. Tissue was stored at -70°C until DNA extraction. A portion of each tumour sample was examined histologically to confirm the type of tumour present. There were no foci or areas of liver cell dysplasia within any of the adenomas. All non-tumorous liver tissues were non-cirrhotic.

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Key words: hepatocellular adenoma, hepatocellular carcinoma, loss of heterozygosity, chromosome 17

DNA extraction and analysis. DNA preparation and Southern analyses were performed as previously described (10). The 25 RFLP probes for 21 chromosome arms, ie, 1p, 1q, 2q, 3p, 3q, 4q, 5p, 5q, 7p, 7q, 9q, 11p, 11q, 12q, 13q, 14q, 16p, 16q, 17p, 18q and 20q, and the appropriate restriction enzymes are listed in Table I. These 25 probes were those used in the previous study on HCC (10). If the two alleles recognised by a particular probe were of different sizes and appeared as two separate bands in the resultant auto-radiograph of the constitutional DNA, the patient was heterozygous for the locus detected by that probe and hence informative. Complete deletion or great loss of intensity of one band in the tumour DNA indicated an allele loss, due to deletion of one copy of the DNA sequence at that locus.

Results

The results obtained from the 25 probes are shown in Table I. No allele loss was detected in any of the 6 adenomas. For each chromosome locus detected by the probes, at least two patients were heterozygous, but none showed any LOH. Fig. 1 shows some examples of autoradiographs. For patient No. 1, her HCC had an allele loss detected by the probe pYNZ.22. This probe has been assigned to the region of the short arm of chromosome 17 (17p13) (13), near the p53 tumour suppressor gene (14). The patient's hepatocellular adenoma, resected together with her HCC, did not show such loss (Fig. 2).

Discussion

This study showed no evidence for chromosome allele loss in hepatocellular adenoma. In hepatocellular carcinoma without liver cirrhosis, we found a high frequency of LOH at 5q35-qter and 17p13 (10) but none of the heterozygous adenoma in this study had such loss (Table I, Fig. 1). It is known that in the sequential process of carcinogenesis, early stage tumours have few genetic changes (3). Chromosome allele losses have been detected in colorectal adenoma, a premalignant tumour, but the frequency is very low (3,6). This is to be expected as the initiating event in carcinogenesis is likely to be a point mutation or small molecular deletion (15). Therefore it is not surprising that none of the liver adenomas in this study exhibited LOH since none of them contained foci or areas of liver cell dysplasia, a sign of premalignant status (5).

Allele loss at 17p13 may represent loss of the p53 tumour suppressor gene, which is normally seen in carcinomas, but rarely in adenomas, such as those in the colorectum (3), and ovary (16). This study and our previous one (10) show that this is also the case in liver primary tumours. Patient No. 1 in this study provided a unique opportunity to observe this phenomenon in a single patient, and it is of interest to note that allele loss at 17p13 detected by pYNZ.22 was found in her HCC DNA, but not in her hepatic adenoma DNA. She was homozygous for another probe at 17p13, p144-D6.

In conclusion, this study suggests that if LOH occurs in hepatocellular adenoma it is a rare event.

Table I. DNA probes used and number of heterozygotes at each locus in hepatocellular adenoma.

Probe[a]	Chromosome location	Enzyme digest	No. of allele losses/ No. of heterozygotes
λMS1	1p33-35	HinfI	0/5
PB3	1p21-pter	MspI	0/2
λMS32	1q42-43	AluI	0/6
P5Gi	2q33-35	TaqI	0/2
H3H2	3p21	HindIII	0/3
HS-3	3q12	HindIII	0/2
VC63	4q	TaqI	0/3
pMS621	5p	HinfI	0/4
ECB27	5q21	BglII	0/2
YN5.48	5q21-22	MspI	0/3
λMS8	5q35-qter	HinfI	0/3
λMS31	7pter-q22	HinfI	0/5
pλg3	7q31.3-qter	HinfI	0/6
EFD126.3	9q34	PvuII	0/4
H-*ras*	11p15	BamHI	0/2
pMS51	11q13	HaeIII	0/5
λMS43	12q24.3-qter	HinfI	0/5
pMS626	13q	HinfI	0/4
pMS627	14q	AluI	0/3
3'HVR	16p13.3	PvuII	0/3
pulB1148	16q22.1	TaqI	0/2
p144.D6	17p13	RsaI	0/3
pYNZ.22	17p13	RsaI	0/4
pMS440	18q	HaeIII	0/3
pMS617	20q	AluI	0/4

[a]References for probes, see reference (10).

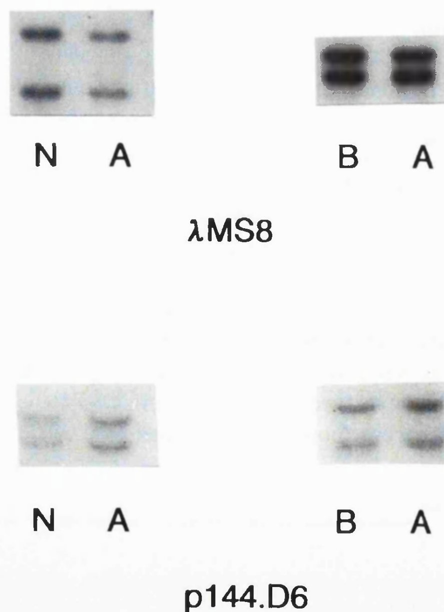

Figure 1. Representative autoradiographs of Southern hybridisations. B = Blood lymphocyte DNA; N = Non-tumour tissue DNA; A = Adenoma tissue DNA. None shows allele loss.

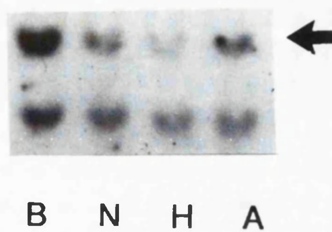

Figure 2. Autoradiograph of Southern hybridisation of DNA from patient No. 1 with pYNZ.22 (17p13). B = Blood lymphocyte DNA; N = Non-tumour tissue DNA; H = Hepatocellular carcinoma DNA; A = Adenoma DNA. Note that the intensity of larger allele is greatly reduced in HCC DNA compared with lymphocyte and non-tumour DNA but, the allele is present in the adenoma DNA (indicated by the arrow).

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Chromosome 17 allele loss in hepatocellular carcinoma but not in synchronous liver adenoma

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A 51-year-old female underwent resection of two synchronous liver tumours, a hepatocellular carcinoma and an adenoma. DNA analysis revealed allele loss on chromosome 17 (17p13, near the locus of p53 tumour suppressor gene) in the hepatocellular carcinoma but not in the adenoma. This finding may support the view that loss of p53 tumour suppressor gene is associated with tumour progression.

Key words: hepatocellular carcinoma; adenoma; allele loss; p53 tumour suppressor gene.

Introduction

Tumour suppressor gene loss may be important in the activation of carcinogenesis.[1] Recent work has suggested that this phenomenon is involved in the adenoma-carcinoma sequence in colorectal cancer.[2] However, there has been no study to date describing such changes in liver tumours. The occurrence of synchronous hepatocellular carcinoma (HCC) and liver cell adenoma in a single patient has provided an opportunity for DNA analysis in this situation.

Case report

A 51-year-old woman presented with a two-month history of upper abdominal discomfort. Five years previously, she underwent a right hepatectomy for a large haemangioma. She was never on the contraceptive pill and there was no evidence of cirrhosis. Blood investigations, including liver function tests, hepatitis B serology and serum neurotensin were normal. Computed tomography (CT) showed two 3 cm low attenuation areas in the remnant left liver—one in the quadrate lobe and the other involving the lateral segments (Fig. 1).

Laparotomy revealed a hypertrophied non-cirrhotic left liver with two nodular lesions, as identified on CT scanning. Intraoperative ultrasound did not demonstrate any further abnormality. Non-anatomic wedge excisions of these lesions were performed. Histology of the quadrate lobe lesion was reported as a well-differentiated hepatocellular carcinoma, while the other lesion proved to be a liver-cell adenoma.

DNA analysis was performed on the patient's normal peripheral lymphocytes, both tumours and normal liver, to identify chromosome allele loss which may represent tumour suppressor gene loss as previously described.[3] Briefly, Southern analysis was performed with a panel of 14 probes recognizing restriction fragment length polymorphisms (RFLPs) assigned to chromosomes 1, 5, 7, 9, 11, 12, 13, 16 and 17. The pattern of DNA banding in the tumours was compared to that in blood lymphocyte DNA and normal liver cell DNA. The

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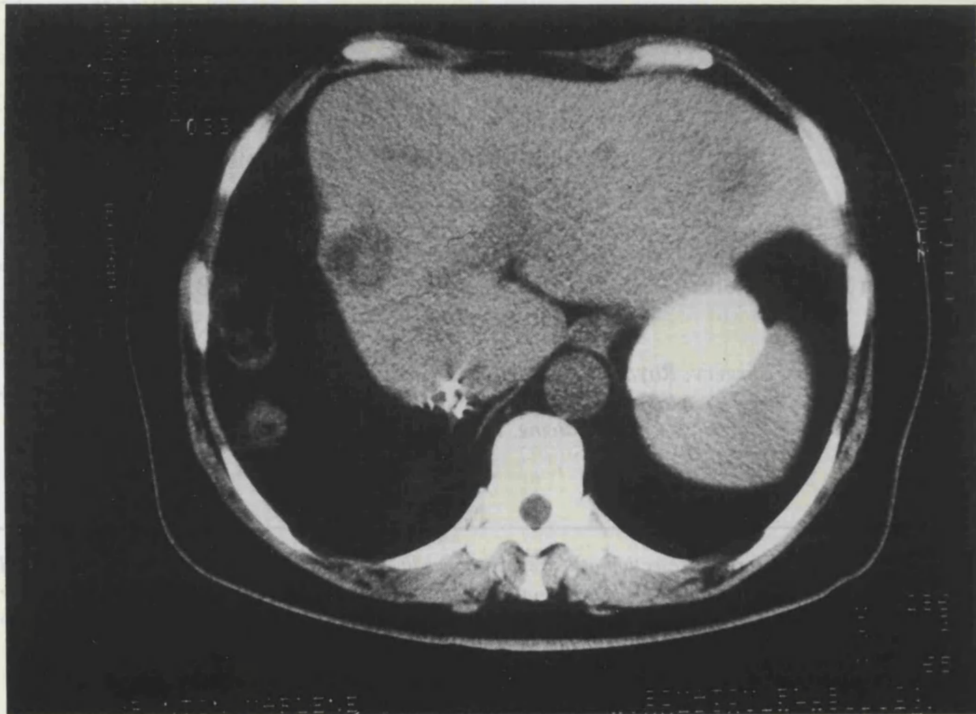

Fig. 1. CT scan showing synchronous hepatocellular carcinoma (left) and liver adenoma (right).

detection of chromosomal DNA loss was dependent on demonstrating a difference in RFLPs between tumour and non-tumour DNA. When a probe showed two or more bands in autoradiographs of Southern hybridization for normal DNA, the probe was called 'informative'. A deletion or great loss of density of one or more bands in tumour DNA could, therefore, be seen as allele loss. Some probes, however, showed only one band in the constitutional (normal) DNA and were called 'non-informative' because it was then not possible to identify deletion of bands in tumour DNA. The HCC of this patient showed allele loss on the short arm of chromosomes 17 with the probe pYNZ.22 (17p13), which was not found in the adenoma (Fig. 2). There were either no allele loss or non-informative patterns with probes in the other chromosomes studied (Table 1).

Discussion

Molecular mechanisms involved in liver tumour carcinogenesis are not fully understood. Hepatitis B virus DNA integration, oncogene activation or

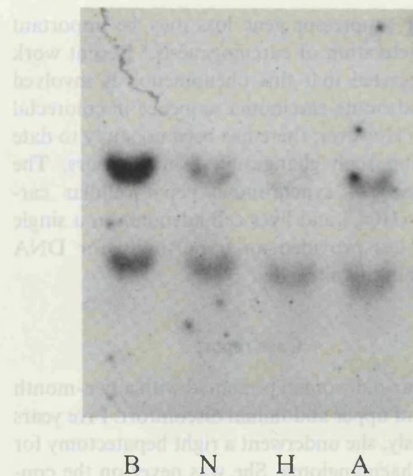

Fig. 2. Autoradiograph of Southern hybridization with pYNZ.22 (17p13). B = blood lymphocyte DNA; N = non-tumour tissue DNA; H = hepatocellular carcinoma DNA; A = adenoma DNA. Note that the intensity of the big allele is greatly reduced in HCC DNA but not in adenoma DNA, as compared with lymphocyte and non-tumour tissue DNA.

Table 1. Allele loss in hepatocellular carcinoma and synchronous adenoma

Probe name	Chromosome region	HCC	Adenoma
Lambda MS1	1p33–35	1,2	1,2
Lambda MS32	1q42–43	—	—
ECB27	5q21	1,2	1,2
Lambda MS8	5q35-qter	—	—
Lambda MS31	7pter-q22	1,2	1,2
p Lambda g3	7q31.3-qter	1,2	1,2
EFD126.3	9q34	1,2	1,2
pMS51	11q13	—	—
Lambda MS43	12q24.3-qter	1,2	1,2
p3.8R	13q14.2	1,2	1,2
3′HVR	16p13.3	1,2	1,2
pulB1148	16q22.1	—	—
p144.D6	17p13	—	—
pYNZ.22	17p13	(1),2	1,2

Non-informative pattern of normal DNA is indicated as a dash; where the normal DNA was informative the tumour genotype is shown in the table as 1,2. The continued presence of the larger allele is indicated by '1' and '2' indicates the continued presence of the smaller allele. Allele loss is indicated by ( ).

mutation[4] and loss of tumour suppressor genes[3] have all been implicated. There is increasing evidence that tumour suppressor gene loss is as important as oncogene activation in carcinogenesis.[1] Recently, mutations of p53 tumour suppressor gene have been found in HCC from China and southern Africa.[5,6] Our previous study has shown allele loss on chromosome 17p13, the region encompassing p53 tumour suppressor gene, in most HCC.[3] Chromosome 17p13 allele loss and/or p53 tumour suppressor gene mutation have been found in diverse human tumour types and seem to be associated with tumour progression.[1,7] In the sequential process of colorectal tumorigenesis, chromosome 17p13 allele loss has been seen in the majority of colorectal carcinomas, but such loss is relatively infrequent in large bowel adenomas of any differentiation.[2] Such differences in liver tumorigenesis have not been reported. The uniqueness of this patient has provided the opportunity for the first study into the genetic comparison of HCC and adenoma arising synchronously. Chromosome 17p13 allele loss found in the patient's HCC was not, however, found in her adenoma. This may support the view that 17p13 allele loss (loss of p53 tumour suppressor gene) is associated with tumour progression and provides further evidence that loss of p53 tumour suppressor gene may play a role in the development of HCC.

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Malignant eccrine poroma with lymph node metastasis followed by multiple basal cell carcinomas in a young female patient

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A case of metastasizing malignant eccrine poroma is described. This case is unique because a relatively young patient presented later on with multiple basal cell carcinomas. Our observation is compared with a review of the literature.

Key words: malignant eccrine poroma; basal cell carcinoma.

Introduction

Since Pinkus *et al.* (1956) first described a benign tumour of the intraepidermal eccrine sweat duct (acrosyringium)[1] and seven years later first reported about the malignant eccrine poroma,[2] there have been only a few reports and reviews on this rare tumour.[3–17]

We present an additional case which is unique because of its combination with multiple basal cell carcinomas in a young female patient.

Case report

In April 1986 a 34-year-old healthy woman, teacher by profession, presented with a firm, elevated, nodular and painless cutaneous tumour on the occipital scalp. The lesion measured 1.5 cm in diameter and was excised. Histopathological diagnosis was malignant eccrine poroma. Because the lesion was not completely excised, three weeks later reexcision was performed. Histologic sections showed some small rest tumour localization, but now the tumour was completely removed. In March 1987 in both sides of the neck a palpable node measuring 1.7 cm and 1.0 cm in diameter was felt. The largest node was biopsied and revealed a lymph node metastasis histologically identical with the primary malignant eccrine poroma. A bilateral posterolateral lymph node dissection was performed. Histological sections showed one more lymph node to contain metastatic tumour with extranodular growth.

In 1989 she presented with two cutaneous lesions at the upper lip and abdominal wall, respectively 0.8 and 2.0 cm in diameter. The lesions were excised and on histopathological examination showed two basal cell carcinomas that were completely removed. Additionally three other cutaneous lesions were excised (abdominal wall: 0.3 cm; right breast: 0.8 cm, and back: 0.4 cm in diameter). The abdominal lesion showed to be one more basal cell carcinoma while the two others were verrucae seborrhoicae of the acanthotic type.

Until September 1991 no further cutaneous lesions were discovered, nor signs of local recurrence or distant metastases and until then the patient remained free of disease.

Histopathology

April 1986

Microscopical studies revealed in the dermis a tumour composed of interconnected nests of polymorphic round or polygonal atypical cells with

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The Putative Tumor Suppressor Gene on Chromosome 5q for Hepatocellular Carcinoma Is Distinct From the *MCC* and *APC* Genes

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ABSTRACT: We have previously shown that the tumor suppressor gene for hepatocellular carcinoma (HCC) without cirrhosis may be located on chromosome 5q35-qter.[1] In this study, we analyzed nine cases of primary HCC without cirrhosis using probes from the *MCC* and *APC* genes, which are in the region 5q21–22. None of the informative cases had allele loss detected by these probes, whereas the probe λMS8 for the region 5q35-qter showed allele loss in six out of six informative cases. The results confirm that the putative tumor suppressor gene for HCC without cirrhosis on chromosome 5q is distinct from the *MCC* and *APC* genes.

KEY WORDS: hepatocellular carcinoma, loss of heterozygosity, tumor suppressor gene, adenomatous polyposis coli.

I. INTRODUCTION

We previously reported that a possible tumor suppressor gene for hepatocellular carcinoma (HCC) without cirrhosis is located on the terminal region of the long arm of chromosome 5 (5q35-qter) and appears to be distinct from the locus of the adenomatous polyposis coli (APC) gene.[1] Recently, two tumor suppressor genes, *MCC* (mutated in colorectal cancer) and *APC* for colorectal tumors on the long arm of chromosome 5 (5q21–22) were cloned, and probes made available.[2–4] These two genes are frequently lost or mutated in colorectal cancers.[5,6] In this study, we compared the patterns of allele loss, which may represent tumor suppressor gene loss, in HCC without cirrhosis and colorectal-liver metastases (CLM) on chromosome 5q using DNA probes from *MCC* and *APC*.

II. MATERIALS AND METHODS

A. Patients and Biopsies

Nine patients with primary HCC without liver cirrhosis and seven patients with CLM were studied. Among the nine patients with HCC, seven were hepatitis B virus (HBV) negative and the remaining two were positive (Table I). All underwent surgical resection. None of the patients received chemotherapy or radiotherapy before surgery. Surgical biopsies from tumoral and nontumoral liver tissues were snap frozen in liquid nitrogen at the time of surgery. Lymphocytes from peripheral blood obtained preoperatively also were used as a source of normal DNA. Tissue was stored at –70°C until DNA extraction. A portion of each tumor sample was examined histologically to confirm the type of tumor present.

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TABLE I
Allele Loss on Chromosome 5q in HCC without Cirrhosis and Colorectal-Liver Metastases

		DNA probes and loci[a]				
Patients	HBV status[b]	ECB27 (5q21) D5S98	L5–71.3 (5q21) *(MCC)*	FB54-D (5q21) *(APC)*	YN5.48 (5q21-22) D5S81	λMS8 (5q35-qter) D5S43
HCC without Cirrhosis						
1	Negative	1,2[c]	1,2	1,2	—	1,(2)
2	Negative	1,2	—	1,2	—	1,(2)
3	Negative	—	—	1,2	—	1,(2)
4	Positive	1,2	1,2	—	1,2	(1),2
5	Negative	—	—	1,2	1,2	—
6	Negative	1,2	1,2	—	1,2	—
7	Negative	1,2	—	1,2	—	1,(2)
8	Negative	nd	1,2	—	nd	—
9	Positive	nd	1,2	1,2	nd	1,(2)
Total No.		**7**	**9**	**9**	**7**	**9**
Heterozygosity		**5**	**5**	**6**	**3**	**6**
Allele loss		**0**	**0**	**0**	**0**	**6**
CLM						
10		—	1,2	1,(2)	1,2	1,2
11		—	—	(1),2	(1),2	—
12		—	(1),2	—	—	1,2
13		(1),2	—	1,(2)	1,(2)	—
14		—	—	1,2	—	1,2
15		—	(1),2	—	1,(2)	(1),2
16		—	—	—	(1),2	1,(2)
Total No.		**7**	**7**	**7**	**7**	**7**
Heterozygosity		**1**	**3**	**4**	**5**	**5**
Allele loss		**1**	**2**	**3**	**4**	**2**

a References for probes: ECB 27;[7] L5–71.3;[2] FB54-D;[3] YN5.48;[8] λMS8.[9]

b HBV status was determined by blood assay and Southern analysis of hepatic tissue DNA, using the HBV genome probe pEco63.

c Homozygosity in the constitutional DNA (noninformative pattern) is indicated as a dash; where the normal tissue was informative, the tumor genotype is shown in the table. Heterozygosity is indicated by 1,2. The continued presence of the larger allelic restriction fragment is indicated by "1", and "2" indicates continued presence of the smaller allelic fragment. Allele loss (deletion or reduction of intensity of a band) is indicated by ( ). "nd" indicates no data.

B. DNA Extraction and Analysis

DNA extraction and Southern analyses were done as previously described.[1] The probes used are shown in Figure 1 and Table I, including a genomic DNA probe, L5–71.3, from *MCC* and a cDNA probe, FB54-D, from *APC*. If two alleles appeared as two separate bands in the resultant autoradiograph of the constitutional DNA, the patient was considered "informative," or heterozygous, for the particular probe. Complete deletion or obvious loss of intensity of one band in the tumor DNA indicated an allele loss, or loss of heterozygosity (LOH).

III. RESULTS

As shown in Table I, in nine cases of primary HCC without liver cirrhosis, five were informative with L5–71.3, six with FB54-D, but none of them had allele loss. This is in agreement with the previous results for ECB27 and YN5.48, which flank *MCC* and *APC* (Table I). Of six informative cases (HBV negative: 4; HBV positive: 2) with the probe λMS8 (5q35-qter), all had allele loss. In contrast, in seven cases of CLM, allele loss was found in only two out of five informative cases with λMS8, whereas most of the informative cases with probes from 5q21–22, including the *MCC* and *APC* probes, had allele loss (Table I). Figure 2 shows representative autoradiographs.

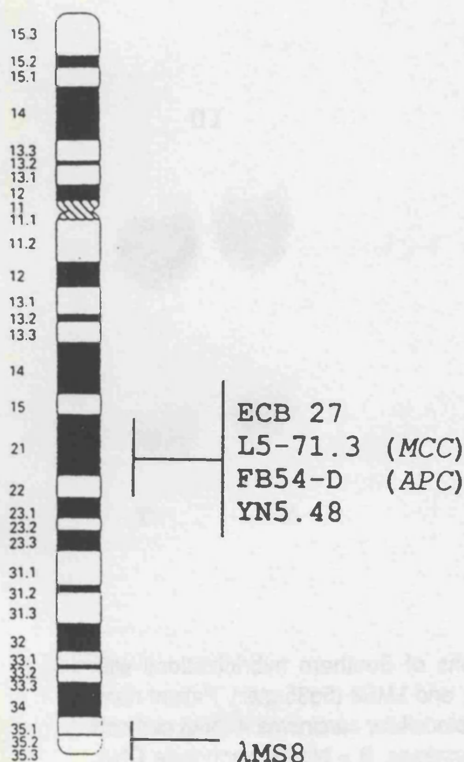

FIGURE 1. Localization of the probes used for chromosome 5.

IV. DISCUSSION

Inactivation of tumor suppressor genes plays an important role in carcinogenesis.[10] Detection of LOH can be used in searching for tumor suppressor gene loss.[11] Using this method, we showed previously that a possible tumor suppressor gene for HCC without cirrhosis may be located on chromosome 5q35-qter.[1] Because the two recently cloned tumor suppressor genes *MCC* and *APC*[2–4] are located on 5q, it is of interest to eliminate these gene loci from playing a role in HCC without cirrhosis. In the present study, we showed that none of the informative cases of HCC without liver cirrhosis had allele loss detected by the *MCC* and *APC* probes, whereas all six informative cases with the probe λMS8 (5q35-qter) exhibited LOH. On the other hand, probes from 5q21–22 detected a high frequency of allele loss in CLM. In conclusion, the results confirm that the putative tumor suppressor gene for HCC on 5q is distinct from the *MCC* and *APC* genes.

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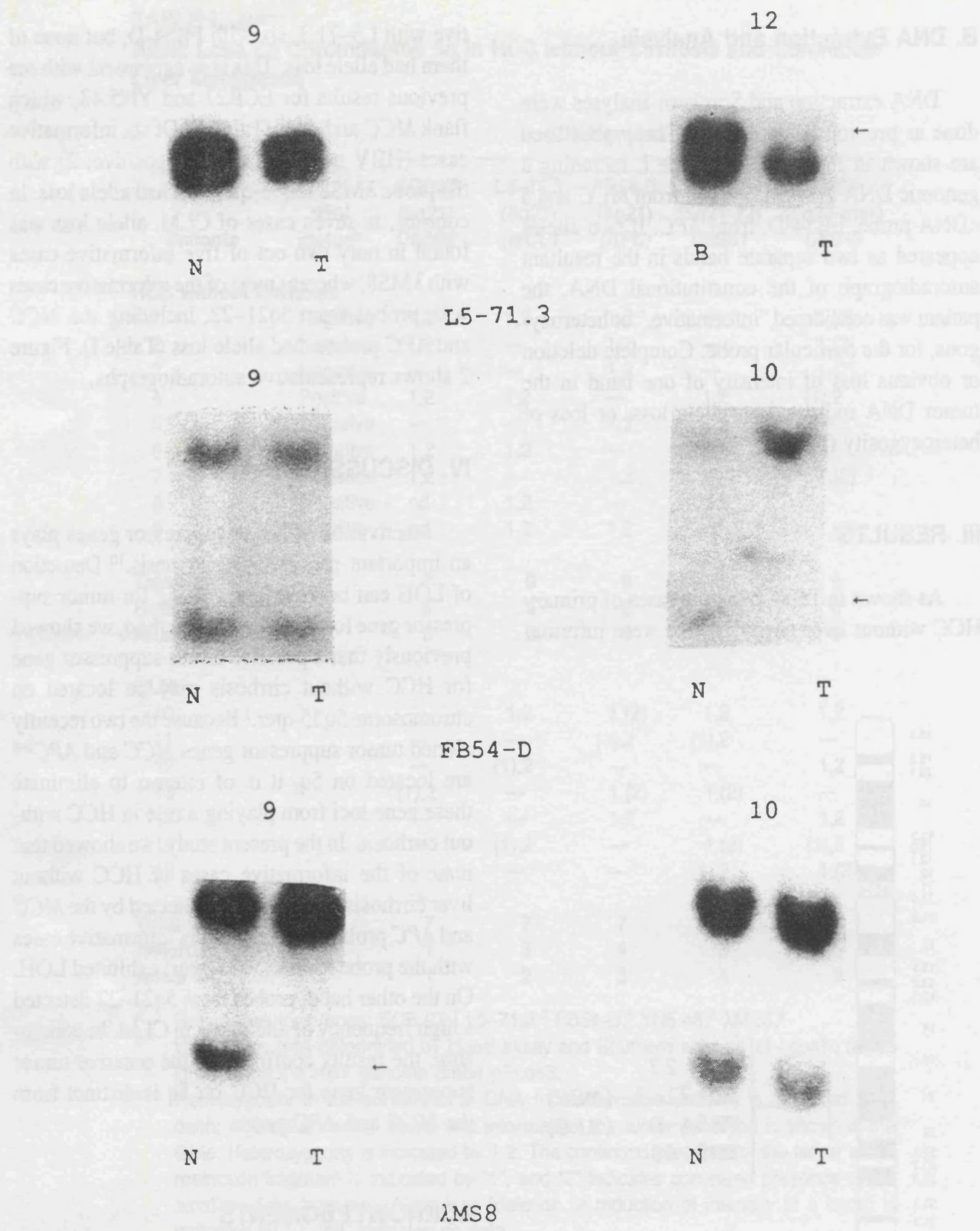

FIGURE 2. Representative autoradiographs of Southern hybridizations with L5-71.3 (*MCC*, 5q21), FB54-D (*APC*, 5q21), and λMS8 (5q35-qter). Patient numbers are indicated above tracks. No. 9 is hepatocellular carcinoma without cirrhosis and Nos. 10 and 12 are colorectal-liver metastases. B = blood lymphocyte DNA; N = nontumor tissue DNA; T = tumor tissue DNA. Allelic losses in tumor DNA are indicated by arrows.

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Multiple allelic losses in sarcomatoid liver carcinoma

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Sarcomatoid carcinoma is an uncommon variant of carcinoma. There has been no molecular-genetic study on this type of tumour as yet. Here we report the first study of chromosome allele loss in two patients with sarcomatoid liver carcinoma. Twenty-five DNA restriction fragment length polymorphism probes were used, of which sixteen revealed informative patterns in each of the patient's normal DNA. One of the patients had allelic losses shown by ten probes and a rearrangement detected by one probe in her tumour DNA. The second patient had eight allelic losses in her tumour. In summary, both tumours revealed multiple genetic changes.

Key words: Carcinoma; Tumour; Sarcomatoid; DNA; Molecular genetics; Liver

Sarcomatoid liver carcinoma is an uncommon form of liver tumour and is thought to arise from the sarcomatoid transformation of hepatocellular carcinoma (HCC) [1]. The incidence of sarcomatoid changes in primary liver cancers is 2.2–3.9% [1,2]. Sarcomatoid changes also occur in carcinomas of other organs, such as those in lung and prostate, and often constitute a more aggressive variant [3,4].

Recent advances in understanding the molecular genetics of cancer have indicated that multiple genetic alterations are important in carcinogenesis. These changes include oncogene activation and tumour suppressor gene inactivation. Inactivation of tumour suppressor genes can be caused by complete gene deletion as well as by intragenic mechanisms [5,6]. The gene deletion can be detected by the demonstration of a difference in the patient's normal and tumour DNA by the use of restriction fragment length polymorphisms (RFLPs). Thus, if a patient's constitutional DNA is heterozygous at a particular locus the deletion can be seen as a band loss in tumour DNA after Southern hybridisation with an RFLP probe specific for that locus. Such loss, designated as chromosome allele loss or loss of heterozygosity, occurs in both common and uncommon tumours [6]. In certain tumours multiple allelic losses have been correlated with a poor clinical prognosis [7].

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Recently chromosome allele loss in HCC has been quite comprehensively studied [8–12], but no such studies have been reported in sarcomatoid liver carcinoma, nor in any other sarcomatoid variants of carcinomas. Here we report the first study of allele loss in two sarcomatoid liver carcinomas with 25 RFLP probes.

Materials and Methods

Clinical details for the two patients are given in Table 1. In addition, both patients had high levels of α fetal protein (greater than 6000 kU/l) and there were no sign of lung or other distant metastases before operation. Pathological and immunohistochemical studies of the biopsies showed that both tumours were sarcomatoid liver carcinomas. Follow-up investigation found local recurrence and lung metastases in both patients (Table 1).

Both tumour and non-tumour liver tissues from the two patients were collected at operation and snap frozen in liquid nitrogen. The tissues were stored at −80°C until DNA extraction. Peripheral blood was obtained in EDTA tubes before any blood transfusion. Blood lymphocytes were used as another source of control DNA.

DNA extraction and Southern analyses were done as previously described [12]. The 25 RFLP probes for chromosomes 1, 2, 3, 4, 5, 7, 9, 11, 12, 13, 14, 16, 17, 18 and 20 and the appropriate restriction enzymes are listed in Table 2. These 25 probes were those used in the previous study on HCC and those showing allele loss in other tumours [12]. If the two alleles recognised by a particular probe were of different sizes and appeared as two separate bands in the resultant autoradiograph of the constitutional DNA, the patient was considered 'informative', or heterozygous, for the locus detected by that probe. Complete deletion or great loss of intensity of one band in the tumour DNA indicated an allele loss, due to deletion of one copy of the DNA sequence at that locus.

TABLE 1

Clinical details of two patients with sarcomatoid liver carcinoma

	Patient No. 1	Patient No. 2
Age (years)	62	63
Sex	female	female
HBV infection*	negative	negative
Liver cirrhosis	no	no
Tumour site	right lobe of the liver, segments V and VI	right lobe of the liver, segments V and VI
Local invasion	abdominal wall, right kidney, right side of colon	invasion of the right kidney capsule
Operation	bisegmentectomy of V and VI of the liver, right hemicolectomy, right nephrectomy, right adrenalectomy	bisegmentectomy of V and VI of the liver, excision of Gerota's fascia over right kidney
Tumour size	14 cm in diameter	10.5 cm in diameter
Tumour differentiation	undifferentiated	undifferentiated
Recurrence	local recurrence and multiple lung metastases	multiple local recurrences and multiple lung metastases
Death from the disease	5 months after operation	6 months after operation

*HBV = hepatitis B virus.

Results

Table 2 shows the allele status in the two sarcomatoid liver carcinomas. Out of 25 probes used, 16 showed informative patterns in each of the patients' normal liver DNA (heterozygosity: 64%). In patient No. 1 allele loss was found in ten out of the 16 informative Southern hybridisations (fraction of allele loss: 62.5%). Furthermore, one probe, p144-D6, showed a gain of one novel band in tumour DNA, indicating a rearrangement. This probe has been assigned to the short arm of chromosome 17 (17p13), near the locus of the p53 tumour suppressor gene. For patient No. 2, eight out of 16 informative probes showed allelic losses (50%). Altogether, the percentage of allele loss in the two tumours was 56.2%. The common losses for both patients were those on chromosomes 1, 12, 14, 16 and 20. The probe p144-D6, which showed a rearrangement in patient No. 1, revealed an allele loss in patient No. 2. Figure 1 shows the examples of allelic losses. Figure 2 shows the rearrangement found in patient No. 1.

TABLE 2

Allele status in the two sarcomatoid liver carcinomas*

Probe**	Chromosome location	Enzyme digest	Patient No. 1	Patient No. 2
λMS1	1p33-35	*Hin*fI	(1),2	1,(2)
PB3	1p21-pter	*Msp*I	1,(2)	1,2
λMS32	1q42-43	*Alu*I	1,2	1,2
P5G1	2q33-35	*Taq*I	-	1,2
H3H2	3p21	*Hind*III	-	1,2
HS-3	3q12	*Hind*III	-	-
VC63	4q	*Taq*I	1,(2)	-
pMS621	5p	*Hin*fI	1,2	(1),2
ECB27	5q21	*Bgl*II	-	-
YN5.48	5q21-22	*Msp*I	1,(2)	1,2
λMS8	5q35-qter	*Hin*fI	-	-
λMS31	7pter-q22	*Hin*fI	1,2	1,2
pλg3	7q31.3-qter	*Hin*fI	1,2	1,2
EFD126.3	9q34	*Pvu*II	-	-
H-*ras*	11p15	*Bam*HI	-	1,(2)
pMS51	11q13	*Hae*III	1,(2)	-
λMS43	12q24.3-qter	*Hin*fI	(1),2	1,(2)
pMS626	13q	*Hin*fI	1,2	-
pMS627	14q	*Alu*I	(1),2	1,(2)
3'HVR	16p13.3	*Pvu*II	(1),2	(1),2
pULB1148	16q22.1	*Taq*I	1,(2)	-
p144.D6	17p13	*Rsa*I	R	1,(2)
pYNZ.22	17p13	*Rsa*I	-	-
pMS440	18q	*Hae*III	-	1,2
pMS617	20q	*Alu*I	(1),2	(1),2
No. of allelic losses and rearrangement/number of informative probes			11/16	8/16

*Non-informative pattern of normal DNA is indicated as a dash; where the normal DNA was informative the tumour genotype is shown as '1,2'. The continued presence of the larger allele is indicated by '1' and '2' indicates the continued presence of smaller allele. Allele loss is indicated by ( ). R indicates DNA rearrangement.
**References for probes, see Ding et al. [12].

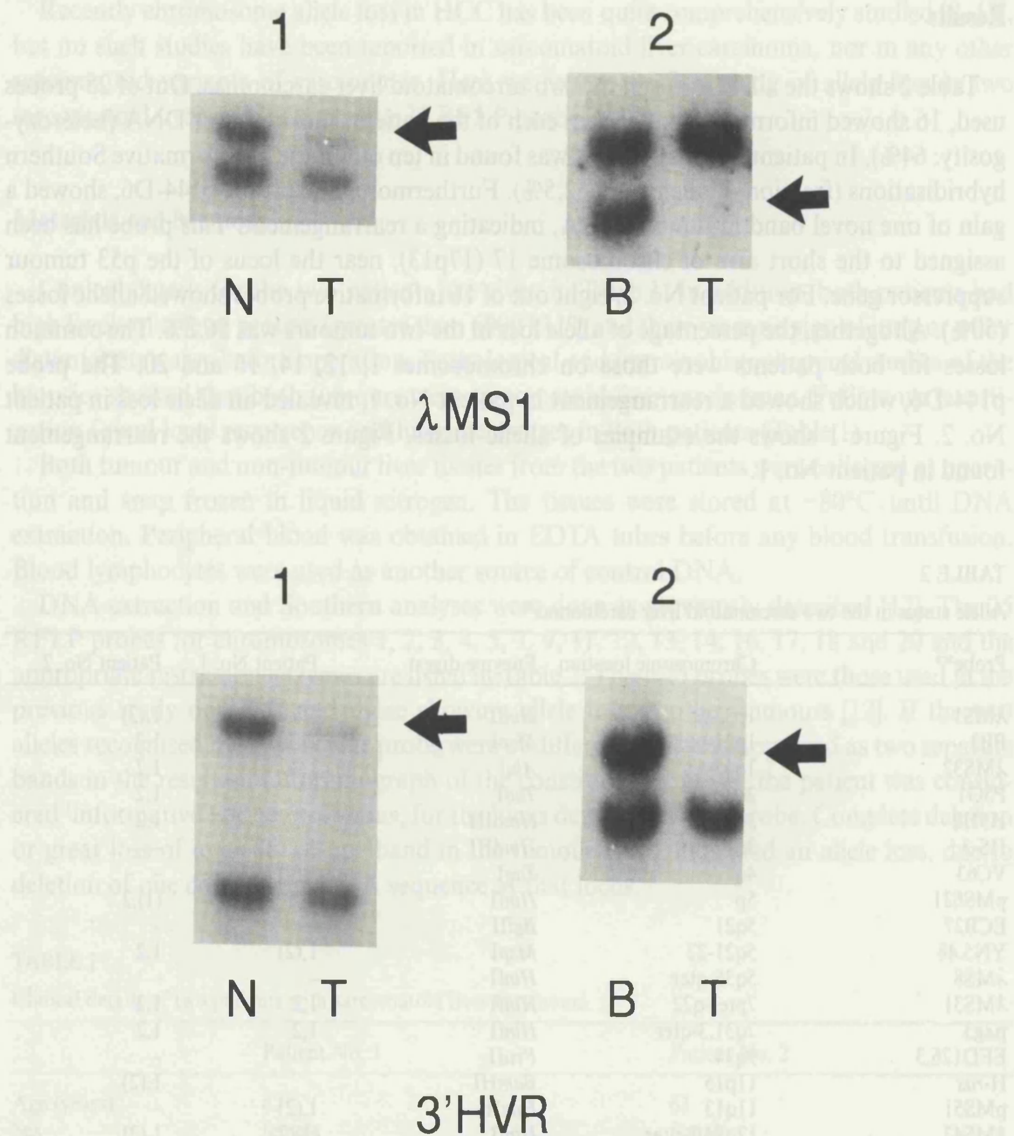

Fig. 1. Allelic losses detected by probes λMS1 (1p33–35) and 3'HVR (16p13.3). B = blood lymphocyte DNA; N = non-tumour liver tissue DNA; T = tumour tissue DNA. Patient numbers are above the tracks. Allelic losses are indicated by arrows.

Discussion

This study of allele loss has shown multiple allelic losses in two sarcomatoid liver carcinomas. Consistent allele loss in a certain type of tumour in a particular region of genome may represent loss of a tumour suppressor gene. Although data from only two patients may not necessarily represent such loss, the results obtained in this study may still be consistent with the concept that cancer is fundamentally a genetic disease [6], since there were multiple

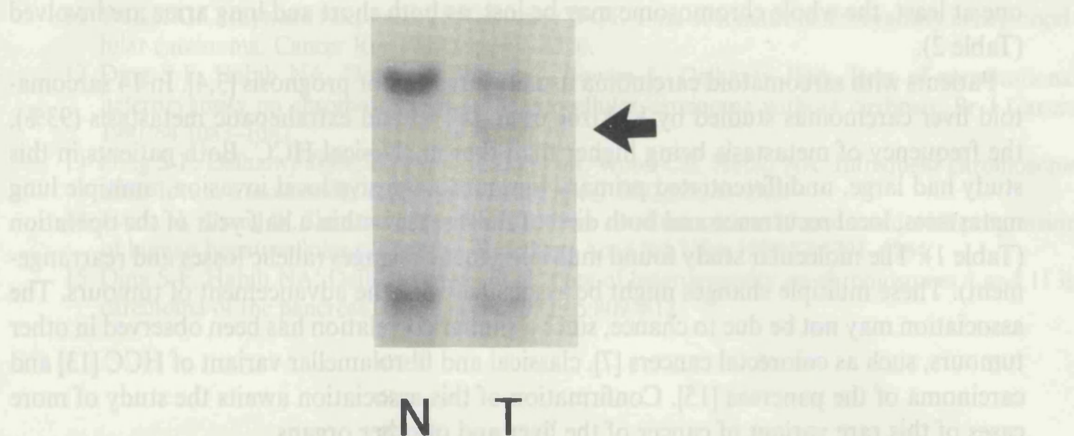

Fig. 2. DNA rearrangement revealed by the probe p144-D6 (17p13) in patient No. 1. N = non-tumour liver tissue DNA; T = tumour tissue DNA. The arrow indicates the extra band.

genetic alterations in both tumours. Using the same method as in this study, we found a much lower frequency of allele loss in 'classical' hepatocellular carcinoma (30/186, 16.1%, [12]) and an even lower frequency in fibrolamellar carcinoma of the liver (2/55, 3.6%, [13]). Work from other groups showed that the percentage of allele loss was 13–20% in HCC (Table 3).

In HCC without liver cirrhosis we found a consistent allele loss in the chromosome region 5q35-qter detected by the probe λMS8 and a frequent loss in the region 17p13 (near the locus of the p53 tumour suppressor gene) with the probes p144-D6 and pYNZ.22 [12]. The probes λMS8 and pYNZ.22 showed non-informative (homozygous) patterns in both patients in this study (Table 2), but interestingly the probe p144-D6 revealed a rearrangement in one patient and a loss in another. Since abnormalities of the p53 tumour suppressor gene have been found in most, if not all, human malignancies [5], the findings here may reflect alterations of that gene in the two sarcomatoid carcinoma. Loss of heterozygosity on chromosome 16 in HCC has been associated with advanced stage disease [14]; the involvement of this chromosome in both cases in the present study confirm this association. In case

TABLE 3

Comparison of frequency of allele loss in primary liver tumours

Patient origin	Tumour*	No. of allele losses/ no. of heterozygotes	% Allele loss	Ref.
American	HCC	18/104	17.3	Buetow et al. [8]
Japanese	HCC	47/361	13.0	Zhang et al. [9]
Japanese	HCC	111/792	14.0	Fujimori et al. [10]
Australian	HCC	23/112	20.5	Walker et al. [11]
European	HCC	30/186	16.1	Ding et al. [12]
European	FLC	2/55	3.6	Ding et al. [13]
European	SLC	18/32	56.2	this study

*HCC = hepatocellular carcinoma; FLC = fibrolamellar carcinoma; SLC = sarcomatoid liver carcinoma.

one at least, the whole chromosome may be lost, as both short and long arms are involved (Table 2).

Patients with sarcomatoid carcinoma usually have a poor prognosis [3,4]. In 14 sarcomatoid liver carcinomas studied by Kakizoe et al. [1], 13 had extrahepatic metastasis (93%), the frequency of metastasis being higher than that in classical HCC. Both patients in this study had large, undifferentiated primary tumours, extensive local invasion, multiple lung metastases, local recurrence and both died of the disease within a half year of the operation (Table 1). The molecular study found multiple genetic changes (allelic losses and rearrangement). These multiple changes might be associated with the advancement of tumours. The association may not be due to chance, since a similar correlation has been observed in other tumours, such as colorectal cancers [7], classical and fibrolamellar variant of HCC [13] and carcinoma of the pancreas [15]. Confirmation of this association awaits the study of more cases of this rare variant of cancer of the liver and of other organs.

In conclusion, this study found multiple allelic losses and rearrangement in two sarcomatoid liver carcinomas and these multiple genetic changes might be associated with the advancement of the tumours.

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