GENETIC ABERRATIONS ASSOCIATED WITH METASTASIS IN COLORECTAL CANCER: AN INSIGHT INTO TUMOUR HETEROGENEITY

Dr. Fahd Al-Mulla, B.Sc., MB., Ch.B.

This thesis is submitted in part fulfilment of the degree of Doctor of Philosophy in the University of Glasgow

Beatson Institute for Cancer Research
CRC Beatson Laboratories,
Bearsden, Glasgow

Faculty of Medicine
University of Glasgow
Glasgow

May 1999

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"In the laws of averages
Chance favors the prepared mind"

Pasteur

To my wife
Zsofia
And my teacher
Professor George D. Birnie

ABSTRACT

The purpose of this thesis was to obtain some insight into the role of genetic changes in progression of human colorectal cancer. Two approaches were adopted: (i), determination of frequency and nature of mutations in Ki-*ras* and (ii), determination of the nature and extent of genetic aberrations, at chromosomal level, in advanced colorectal cancer. The main theme of the work was the use of paired tumour samples from the same patients in an attempt to measure the extent of genetic heterogeneity and clonal diversity between primary tumours and their synchronous metastases.

For Ki-*ras* gene, I have determined the point mutations occurring in codons 12 and 13 of exon 1 of the Ki-*ras* gene in 78 patients with colorectal carcinoma (31 Dukes' A and B, 21 Dukes' C, 26 Dukes' D) by allele specific oligonucleotide hybridisation and sequencing. Duplicate samples of invasive primary carcinoma, adjacent normal tissue and available lymph node and liver metastases from the same patients were microdissected from paraffin sections. There were no differences in incidence of Ki-*ras* mutations between primary carcinomas and secondary deposits: 26 of 78 (33%) primary carcinomas, 10 of 32 (31%) lymph node metastases, and 10 of 26 (38%) liver metastases. Multiple sampling revealed frequent heterogeneity within carcinomas: 9 of 26 primaries with Ki-*ras* mutations also contained areas of carcinoma with only the wild-type gene, implying that Ki-*ras* mutation, even when present in a colonic carcinoma, may not have been necessary for establishing the malignant phenotype. Also, 2 of 26 (8%) Dukes' D patients had a mutation in their primary carcinoma but none in liver metastases and 6 of 47 (13%) Dukes' C and D patients had mutations in liver or lymph-node metastases but none in the primary carcinoma. Mutation of codon 12 from GGT (glycine) to GTT (valine) was more prevalent in primary and metastatic deposits of Dukes' C/D carcinomas ($p=0.01$) than in primary carcinomas from Dukes' A/B patients. Mutations of codon 12 to GAT, AGT, GCT and codon 13 GGC to GAC were also found, but no correlation with carcinoma aggressiveness was apparent. Follow-up of 71/78 patients (up to 12 years) revealed decreased overall survival ($P=0.001$) in patients with the GGT to GTT transversion in codon 12, even when the analysis was restricted to Dukes' D cases, supporting the suggestion that this mutation may confer a more aggressive phenotype in colorectal carcinoma.

I then wanted to determine whether the correlation between valine-12 mutant Ki-RAS and tumour aggressiveness observed *in vivo* could be reflected *in vitro.* For this, Rat-1 fibroblasts transfected with valine-12 or aspartate-12 mutant or wild-type Ki-*ras* gene were assessed in terms of transformation, VEGF production and *in vitro* invasion. Both mutants demonstrated equal abilities to transform Rat-1 cells and induce VEGF production while cells expressing the wild-type Ki-Ras protein failed to do so. Clones of Rat-1 cells expressing valine-12 mutant Ki-Ras protein demonstrated increased ability to invade matrigel and to stimulate stromelysin-1 production compared to cells expressing aspartate-12 mutant Ki-Ras.

A central question in the role of mutant Ras protein in tumour biology is why should one particular mutation be associated with aggressive behaviour while the other is not? To address this question, I have used molecular modelling on the wild type, valine-12 and aspartate-12 Ras molecules and shown there to be a significant conformational differences in the GTP-bound state between the two mutants involving the effector

binding domain of Ras. These 'topographic' differences could be responsible for the different biological activities between the two mutants.

It is arguable, based on both *in vivo* and *in vitro* work, that the biological effects of the two different Ki-*ras* mutations cannot be assumed to be the same and the use of valine-12 mutant Ki-*ras* as a clinical marker for worse prognosis should be considered.

To determine the extent of heterogeneity between primary tumours and their synchronous metastases and to ascertain the role of genetic aberrations, other than Ki-*ras* mutations, in the metastatic process, I have analysed 26 tumours from 12 patients with metastatic colorectal adenocarcinoma by Comparative Genomic Hybridisation (CGH). Primary tumours and their lymph node metastases from five Dukes' C patients and primary tumours and their liver metastases from seven Dukes' D patients were used to assess the extent of genetic differences between primary and secondary colorectal carcinomas from the same patients, to assess the degree of clonal divergence and genetic heterogeneity in metastatic colorectal cancer, and to determine differences in genetic imbalances between Dukes' C and D stage tumours. The results show that the same genetic aberrations were frequently found in the primary tumours and their metastases. However, metastases frequently contained genetic aberrations not found in the corresponding primary tumours. The comparison of Dukes' stages C and D revealed genetic aberrations common to both. However, reduced copy number of chromosome arm 17p (5/5 versus 0/7; p=0.001) was significantly associated with Dukes' stage C and lymph node metastases, while increased copy number of chromosome arms 6p (6/7 versus 0/5; p=0.007), 8q(6/7 versus 1/5; p>0.05), and 17q (5/7 versus 0/5; p=0.027) were more associated with Dukes' stage D and liver metastases. These results establish a repertoire of chromosomal alterations associated with metastatic colorectal cancer, and suggest that Dukes' C (lymph node metastasis) tumours are not always simply an earlier stage of Dukes' D (liver metastasis) tumours and, thus, in some instances at least, they are distinct forms of the disease.

ACKNOWLEDGEMENTS

There are many people I want to thank for making this work possible. I would like to thank the Dean of the Faculty of Medicine of Kuwait Dr. Abdullatif A. Al-Bader for his support to obtain a Ph.D. studentship from the university of Kuwait and his continuous support during my studies. Many thanks for the staff of the Cultural Affairs Office at the Embassy of the State of Kuwait for their help and support. I would like to express my sincere gratitude to my teacher and supervisor Professor George D. Birnie for his unlimited support, help, and encouragement during the course of this work. George taught me "good" science and inspired me to look at things not only differently but also simply. My special gratitude goes for my adviser Dr. Donald Black for his continuous support and guidance. I am grateful for having two distinguished scientists as my supervisor and adviser because they not only allowed me enough room to explore my ideas but because they also bestowed on me enough freedom to do things "my way".

I would like to thank my collaborators Dr. James J. Going for his help with microdissection and for answering my pleas for tumour sample and Dr. Nicol Keith, his staff, and Peter McHardy for their help with CGH. Special thanks for Dr. James Milner-White for the help with Rasmol and for Elaine MacKenzie for her help and contribution towards this thesis.

Many thanks for my colleagues Dr. Pierre Schembri-Wismayer, Dr. Eltahir, Dr. Katsanakis and Endom Ismail for their help and support during the past three years. Believe me during the last three years, I have spent more time with Pierre than I have done with my wife. To her I apologise. Many thanks to all other staff at the Beatson, Robert McFarlane for help with the automated sequencing, to Bill Simpson in the stores for helping me to carry things to my bench and Don who engineered a trolley specially for me to carry my plates on. It is not easy to do science with your crutches on! Without their help it would not have been possible for me to complete this work.

Thanks to Professor John Wyke for his special welcome during my first days in the Beatson and his continuous help and hospitality throughout my stay.

I wish to thank my parents, brothers, sisters and all other members of my family for their love and support throughout my 12 years away from them in Glasgow. Last, but certainly not least, my heartfelt thanks for my wife for her continuous love, sacrifice, help and immortal patience with me during the many nights and weekends I spent away from her in the lab. Thank you for being such a good friend. Thank you Jaber "my son" for being such a content baby and for helping me in typing or " un-typing" parts of the thesis. Perhaps when you grow older you will see how irrational our science was! But also I hope you will come to realise how hard we are working for you! As I now realise how hard others have worked to make this thesis possible!

I am sorry to all of you that I have sometimes used the words "I have shown", it should have been "we have shown" since you were all part of it. Thank you all.

Declaration: The work presented in this thesis is my own work unless otherwise stated.

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ABBREVIATIONS

AP-1:	Activator Protein-1
APC:	Adenomatous Poliposis Coli
ASO:	Allele Specific Oligonucleotide Hybridisation
ATF-2:	Activating Transcription Factor 2
BAD:	BCL2 Antagonist of cell Death
BCIP:	5-Bromo-4-chloro-3 indolylphosphate p-toluidine salt
BM:	Basement Membrane
bp:	Base pair
BrdU:	Bromodeoxy uridine
BSA:	Bovine Serum Albumin
CDC42:	Cell Division Cycle 42
CEA:	CarcinoEmbryonic Antigen
CGH:	Comparative Genomic Hybridisation
cm:	Centimeters
Ci:	Curie
Cip1:	CDK-interacting protein 1
CREB2:	Cyclic AMP Response Element-Binding protein 2
DAPI:	4', 6-Diamidino-2-phenylindole dihydrochloride
DCC:	Deleted in Colorectal Cancer
dH_2O:	Di-ionized water
DMEM:	Dulbecco's Modified Eagle's Medium
DNA:	Deoxyribonucleic acid
dNTP:	3' deoxyribonucleoside 5' triphosphate
E. Coli:	Escherichia coli
ECL:	Enhanced chemilluminesence
ECM:	Extracellular matrix
EDTA:	Ethylenediamine tetra-acetic acid
EGF:	Epidermal Growth Factor
EGFR:	Epidermal Growth Factor Receptor
ELK:	Member of ETS oncogene family
ERK1:	Extracellular Regulated Kinase 1
ERK2:	Extracellular Regulated Kinase 2
FACS:	Fluorescent Activated Cell Sorter
FAK:	Focal Adhesion Kinase
FITC:	Fluorescein-isothiocyanate
FLK1:	Foetal Liver Kinase 1
FAP:	Familial Adenomatous Polyposis
g:	Gram
G418:	Geneticin, G418-sulphate
GAP:	GTPase Activating Protein
GDP:	Guanosine diphosphate
GEF:	Guanine nucleotide exchange factor
G-protein:	GTP-binding protein
GRB2:	Growth factor Receptor-Bound protein 2
GTP:	Guanosine triphosphate
h:	Hours
HBSS:	Hanks Balanced Salt Solution

HGF:	Hepatic Growth Factor
H-Ras:	Harvey Ras
HNPCC:	Hereditary non-polyposis colonic cancer
IGF-1:	Insulin-like growth factor 1
ILK:	Integrin linked kinase
INS:	Insuline
i.v:	Intravenous
IVVM:	Intravital Video Microscopy
JNK:	Jun N-terminal Kinase
k:	Kilo
kbp:	Kilo base pairs
kD:	Kilodalton
KDR:	Kinase insert Domain Receptor
Ki:	Kirsten
l:	Litre
LPA:	Lysophosphatidic acid
M:	Molar
mg:	Milligram
$MgCl_2$:	Magnesium chloride
ml:	Millilitre
µ:	Micro
MAPK:	Mitogen Activated Protein Kinase
MAPKK:	Mitogen Activated Protein Kinase Kinase (MEK)
MAPKKK:	Mitogen Activated Protein Kinase Kinase Kinase (Raf)
MEKK:	MEK Kinase
min:	Minute
mM:	Millimolar
mm:	Millimetre
MMP:	Matrix Metalloprotease
mRNA:	Messenger ribonucleic acid
MT-MMP:	Membrane-type Matrix Metalloprotease
n:	Nano
NBT:	Nitroblue tetrazolium chloride
neo:	Neomycin
NF-κB:	Nuclear Factor-kappa B
ng:	Nanogram
NGF:	Nerve Growth Factor
p:	P value
PAGE:	Polyacrylamide Gel Electrophoresis
PAK1:	P21/CDC42/Rac1-Activated Kinase 1
PBS:	Dulbecco's Phosphate Buffered Saline
PCR:	Polymerase Chain Reaction
PDGF:	Platelet Derived Growth Factor
PI-3K:	Phosphatidylinositol 3-Kinase
PIP2:	Phosphatidylinositol 4,5-bisphosphate
PIP-5K:	Phosphatidylinositol 4 phosphate 5-Kinase
PKA:	Protein Kinase A
PKB:	Protein Kinase B
PKC:	Protein Kinase C

PLC:	Phospholipase
Por1:	Partner of Rac1
RDA:	Representational Differential Analysis
rev/min:	Revolutions per minute
RFLP:	Restriction Fragment Length Polymorphism
RNA:	Ribonucleic acid
RNase:	Ribonuclease
RTK:	Receptor Tyrosine Kinase
RT-PCR:	Reverse Trancriptase-Polymerase Chain Reaction
SAPK:	Stress Activated Protein Kinase
SDS:	Sodium Dodecyl Sulphate
Sec:	Second
SEK:	SAPK/ERK Kinase
SoS:	Son of Sevenless
TEMED:	N, N, N', N'-tetramethylethylenediamine
TKR:	Tyrosine kinase receptor
TMAC:	Tetramethyl Ammonium Chloride
TPA:	Tetradecanoyl-Phorbol-12-myristate-13 Acetate
Tris:	Tris (hydroxymethyl) aminomethane
Tween 20:	Polyoxyethylene sorbitan monolaurate
UV:	Ultraviolet
v/v:	Volume per unit volume
VEGF:	Vascular Endothelial Growth Factor
w/v:	Weight per unit volume
WAF1:	Wild type p53-Activated Fragment 1
WASP:	Wiskott-Aldrich Syndrome Protein
WT:	Wild Type

μg:	Microgram
μl:	Microlitre
μM:	Micromolar
%:	Percentage

Single letter amino acid code

Alanine	Ala (A)
Argenine	Arg (R)
Asparagine	Asn (N)
Aspartic acid	Asp (D)
Cysteine	Cys (C)
Glutamic acid	Glu (E)
Glutamine	Gln (Q)
Glycine	Gly (G)
Histidine	His (H)
Isoleucine	Ile (I)
Leucine	Leu (L)
Lysine	Lys (K)
Methionine	Met (M)
Phenylalanine	Phe (F)

Proline	Pro (P)
Serine	Ser (S)
Threonine	Thr (T)
Tryptophan	Trp (W)
Tyrosine	Tyr (Y)
Valine	Val (V)

INTRODUCTION

CHAPTER 1. COLORECTAL CANCER

1.1. Epidemiology

Colorectal carcinoma is a common malignancy. It is the second most common malignancy in women and the third most common in men in the West of Scotland (Coleman et al., 1993). 31000 new cases of colorectal cancer and 18000 deaths are registered in the United Kingdom per annum. The peak incidence of colorectal cancer is 60-80 years. Colonic primary tumours outnumber rectal tumours by almost 2 to 1. For colon cancer, there is a female-to-male predominance of 4:3, while for rectal cancer there is a male-to-female predominance of 4:3. Colorectal cancer is predominantly a disease of the developed countries, and is rare in Africa and Asia. However, immigrants from low incidence countries to countries with high incidence of the disease acquire the risk of the indigenous population, which stress the importance of environmental factors in the aetiology of the disease. Diet may account for the marked geographical variation in incidence. This is presumed to be due to changes in the bowel flora which produce carcinogen from ingested food, the effect being exacerbated by the slow bowel transit time seen in people taking a low-fibre diet.

In the majority of patients with colorectal cancer, the disease arises sporadically from pre-malignant adenomas, while in 1-15 % of patients there seems to be a hereditary predisposition to colorectal cancer. Familial adenomatous polyposis (FAP) is inherited in an autosomal dominant fashion and has been shown to involve germline mutations and deletions of both *APC* alleles located on chromosome 5q21-q22 (Powell et al., 1993), which almost always result in a

truncated APC protein (Nagase and Nakamura, 1993). Individuals with FAP develop numerous pre-neoplastic polyps in the colon and rectum at a relatively young age of which a subset invariably progresses to malignant cancer if not surgically removed. Patients with FAP may also present with tumours in other regions of the gastrointestinal tract, brain, thyroid, and elsewhere (Burt, 1991).

Hereditary non-polyposis coli (HNPCC) is another autosomal dominant hereditary disease. Individuals with the disease are prone to develop right-sided colorectal cancer at a young age but without the formation of adenomas (Aaltonen et al., 1993). Interestingly, HNPCC is also associated with development of carcinoma of the endometrium, ovary, breast, stomach and urinary tract (Lin et al., 1998). Mutation or deletion of mismatch repair genes *MLH1* and *MSH2* have been associated with development of HNPCC (Leach et al., 1993; Liu et al., 1996).

Studies of these familial syndromes have provided unique insights into both inherited and sporadic forms of human tumours and stress the important contribution of genetic aberrations in the development of colorectal and other cancers. Indeed, more than 80 % of sporadic colorectal cancers and the adenomas from which these cancers are derived carry genetic alterations in the *APC* gene (Jen et al., 1994; Smith et al., 1993). Also, more than 13 % of sporadic colorectal cancers harbour defective mismatch repair genes *MSH2* and *MLH1* (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993). Interestingly, like HNPCC, the mismatch repair deficient tumours involve mainly the right or ascending colon and have diploid to near diploid chromosomal content.

1.2. Dukes' classification

In 1932 Cuthbert Dukes described a system for staging rectal cancer. The staging system associated the clinical-pathological behaviour of rectal tumours with prognosis (Figure 1). When the tumour is confined entirely within the wall of the bowel (Dukes' A) more than 90 % of patients can be cured by surgery alone. Penetration through the *muscularis propria* (Dukes' B) worsens the prognosis, and when there is metastasis to the lymph nodes (Dukes' C) the outlook deteriorates further so that two-thirds of the affected patients die of the disease within five years. When distant metastases to the liver are present (Dukes' D) most patients will die by the end of the first year (Dukes, 1932).

In recent years, Dukes' classification has undergone a number of modifications including the incorporation of colon cancer (Hermanek, 1995). The most widely used version is the Astler-Coller modification. In this system, stage A tumours are confined to the mucosa. Stage B is subdivided into B1 and B2 depending on whether or not the outer muscle layer, the muscularis propria has been breached. Stage C is spread to the lymph nodes with subdivision into C1 and C2 depending on whether or not the bowel wall has been breached or whether the apical lymph node is involved (C2). Stage D is spread of the tumour to distant organs. The Gunderson and Sosin modification includes a B3 and C3 category depending on whether or not the tumour or lymph nodes involves other organs or structures, which are still removable *en bloc* at operation or if the tumour has perforated the serosa (B3). The TNM classification and the UICC are other staging systems (Table 1).

Table 1: Dukes', Astler-Coller, TNM and UICC staging systems for colorectal cancer

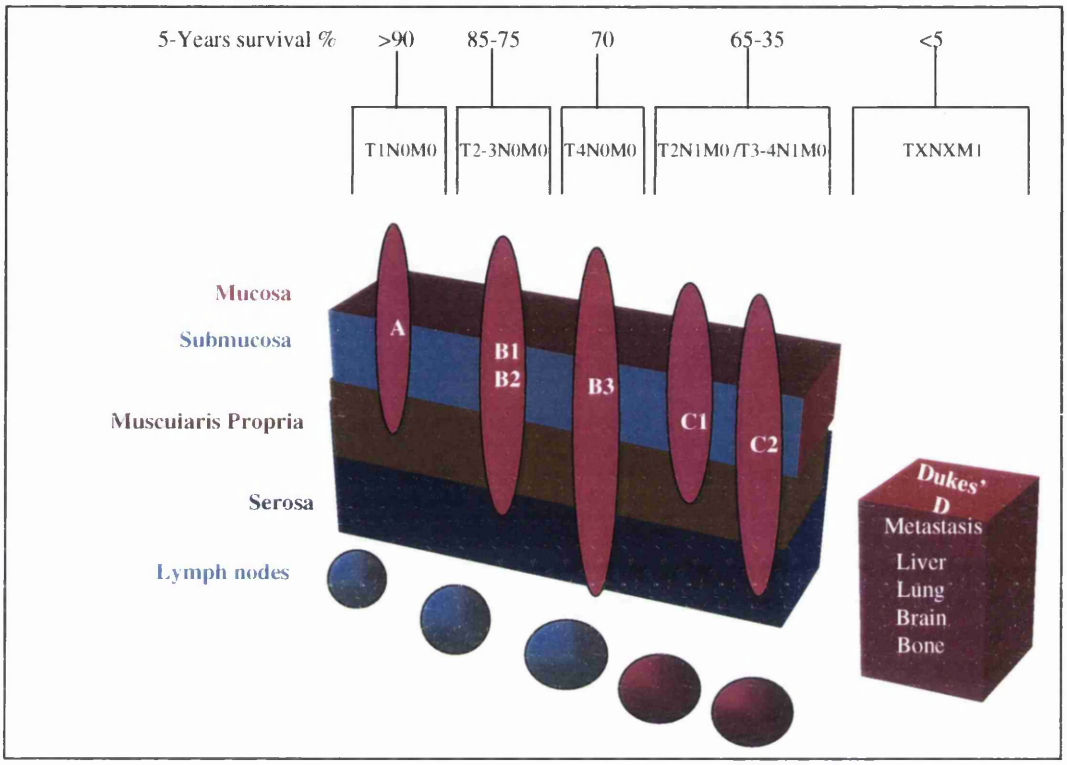
Dukes	Astler-Coller	TNM	UICC
A	A	T1N0M0	Stage I
B	B1	T2N0M0	
	B2	T3N0M0	Stage II
	B3[a]	T4N0M0	
C	C1	T1-2N1-3M0	Stage III
	C2	T3N1-3M0	
	C3 [a]	T4N1-3M0	
D	D	T any, N any, M1	Stage IV

[a] B3 and C3 according to the Gunderson and Sosin modification.

The notion that colorectal cancer progresses in defined stages makes it an appealing tumour for correlating the genetic alterations with a particular Dukes' stage. However, it is important to note that there is considerable variation in the clinical course among patients with apparently equivalent stages of the disease. For example, 20-50% of patients without clinical evidence of metastasis die within five years from recurrent disease (Kune et al., 1990; Ovaska et al., 1990) because either they have been miss-classified as harbouring early tumours (Caplin et al., 1998) or the patients have micrometastasis (occult metastasis) that are undetectable using current routine pathological methods. In addition, these classification systems fail to explain why some early staged colorectal cancers reoccur and/or metastasise. Thus, the identification of markers of aggressiveness and metastases will not only have significant benefits in identifying patients at high risk of metastases but could also aid our understanding of the molecular bases of the metastatic process.

Figure 1: *Modified Dukes' staging*

Colorectal cancer staging is classified according to the extent of infiltration of the submucosa, muscularis propria and serosa (T1-4) and the presence (N1) or absence of involved regional lymph nodes (N0) and distant metastatic deposits (M 0-1). Staging is significant in determining 5-year survival. The letters A, B, C and D indicate Dukes' stage. The tumour, lymph nodes and liver metastasis are coloured purple.



1.3. Genetic alterations associated with progression of adenoma to carcinoma

Tumour progression implies the gradual transition of a localised, slow growing tumour to an invasive, metastatic cancer. Colorectal carcinomas arise usually from adenomas. The patterns and frequencies of four different genetic abnormalities (losses in 5q, 17p, 18q and mutations of *ras* genes) were analysed in a study by Vogelstein et al. (1988). In this study, adenomas with different sizes and forms (class I-III) were compared with carcinomas from different patients in an attempt to delineate genetic alterations associated with progression of adenomas to carcinomas. Deletions involving 5q chromosome were found in 29% of class II adenomas and 35% of carcinomas, indicating early involvement of genes on this chromosome arm in tumour progression. A specific region of chromosome 18q was found to be deleted in 47% of late adenomas (class III) and 73% of carcinomas, indicating that this genetic aberration occurred late in the progression sequence. In addition, deletions involving 17p were most frequent in carcinomas (75%) and were rarely found in early adenomas (although present in 24% of late adenomas). Mutant *ras* genes were found in 12% of class I adenomas, 42%-57% of class II-III adenomas respectively and in 47% of carcinomas, indicating that *ras* mutation occurs early in the progression sequence. The authors concluded that the four molecular alterations accumulated in a fashion that paralleled the clinical progression of tumours and that there appears to be a preferred sequence of genetic changes that leads to progression in colorectal cancer (Figure 2). However, it was also evident from analysis of separate regions of adenomatous and carcinomatous tissue from the same patients that loss of 17p

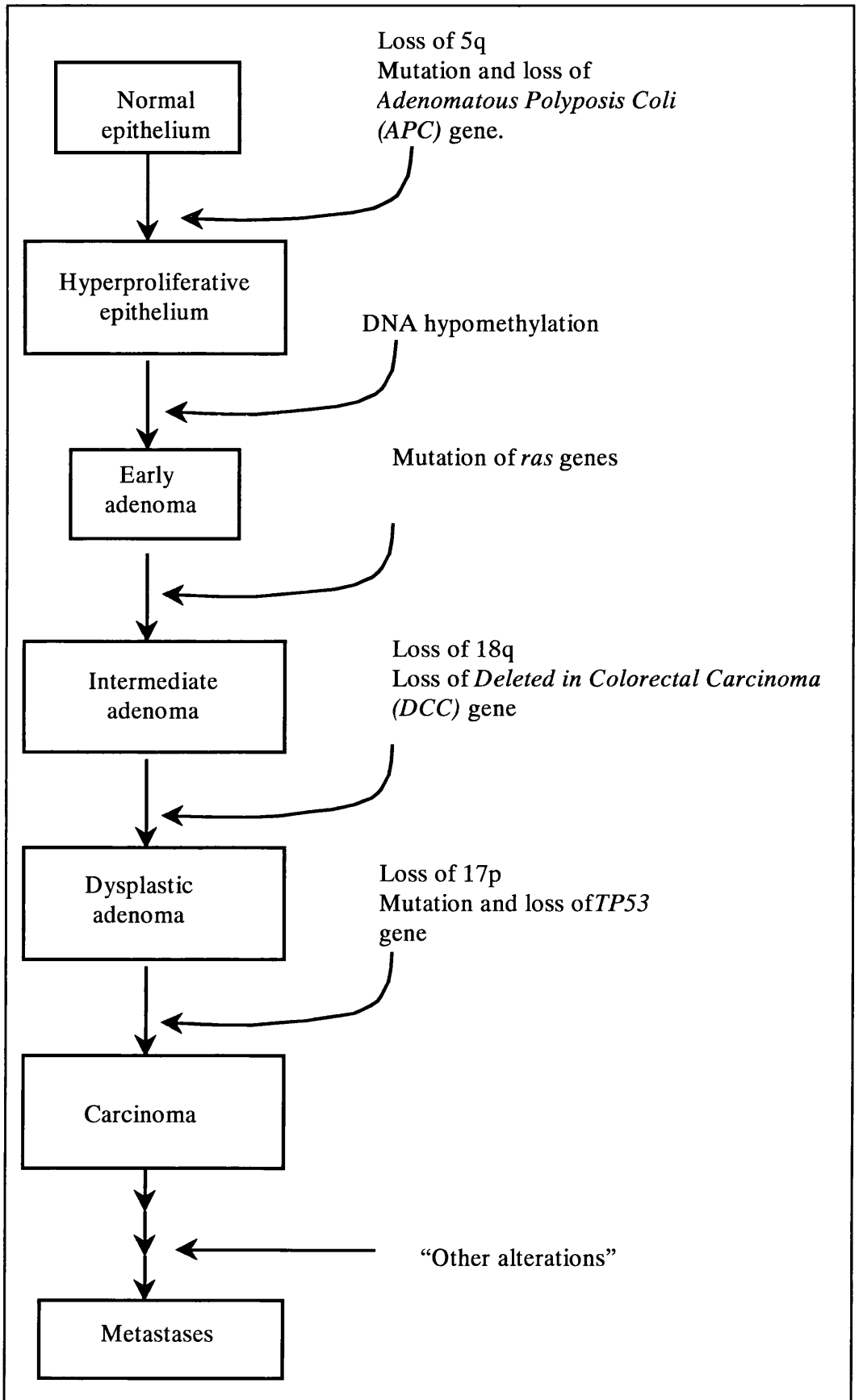
could occur early, while in another patient *ras* mutation appeared late in this sequence, which led the authors to conclude that it is not the sequential genetic aberration of particular genes in any given order that is of prime importance, rather it is the overall accumulation of genetic aberrations (Fearon and Vogelstein, 1990). Thus, while only 9% of class I adenomas accumulated more than two of the four genetic aberrations, 40% of carcinomas have accumulated more than two genetic aberrations. However, no single aberration was found in all tumours, regardless of stage. Moreover, all four genetic aberrations were only found in 10% of carcinomas. Therefore, the represented model of colorectal cancer progression is misleading in that there is no rigid requirement for each genetic step to occur at a precisely pre-determined step in tumour progression.

The accumulation of various genetic aberrations during progression from adenoma to carcinoma has been confirmed by various methodologies, including CGH (Ried et al., 1996), chromosome banding analysis (Bardi et al., 1993, 1995; Muleris et al., 1994), DNA content and interphase cytogenetics (Herbergs et al., 1994).

Recently, Boland et al. (1995) analysed multiple microdissected adenomatous and carcinomatous regions from the same tumours and have shown that deletions involving 5q occurred very early in the progression sequence and that 18q deletions were found in all stages of the progression sequence. Also, 17p deletions were frequent in grade III adenomas (Figure 3b). Thus, the genetic aberrations involved in the evolutionary sequence occurred at a somewhat earlier histologic stage than in Vogelstein's study (Boland et al., 1995). More important

Figure 2: *Multistep model of colon cancer progression*

Progression of colorectal cancer involves several genetic and epigenetic alterations, which have been associated with specific steps along the progression sequence. See section 1.3 for details.



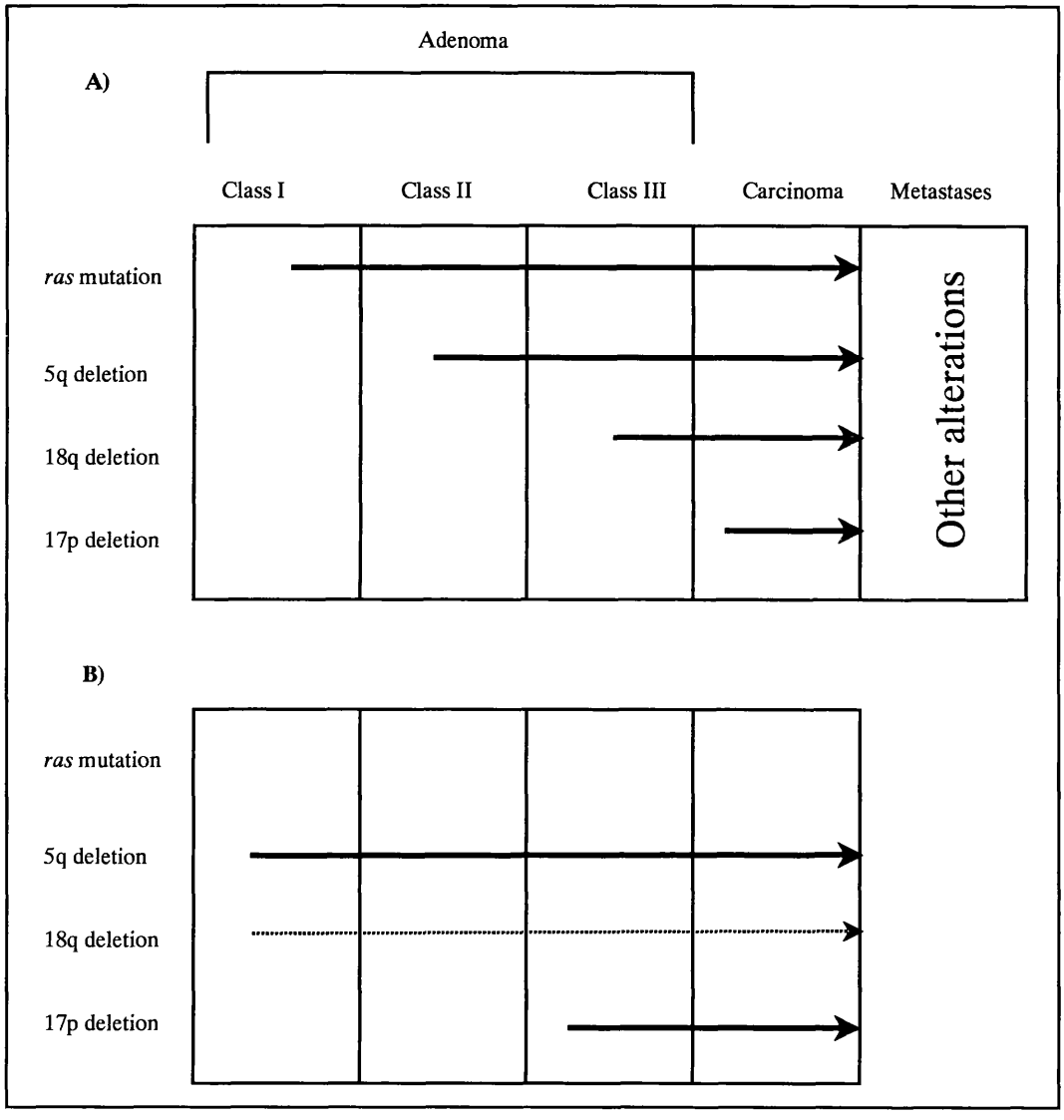
is the evidence that high-grade adenomas were heterogenous with respect to the LOH on the three chromosomes analysed, whereas carcinomas were homogenous (Figure 3). Thus, if adenomas are made up of a mixture of subclones (as the authors have shown), each with different patterns of genetic imbalances, this could make it practically difficult to detect the subclone that progressed to carcinoma (because it could be a minority population). Also, the fact that not all adenomas progress to carcinomas and adenomas that do not progress might have a different genetic imbalances to those that do progress to carcinoma (Giaretti, 1994; Giaretti and Santi, 1990; Morris et al., 1996), when such adenomas are included in cancer progression studies they could cause a significant dilutional or masking effects on the overall genetic aberration involved in cancer progression. Taken together, it is possible that the genetic aberrations involved in carcinoma progression could be present in adenomas but are not amenable to detection bescause of the reasons stated above. Indeed, not only has the study of Boland et al. (1995) shown that LOH involving 17p occurred earlier in the progression sequence, but another study has shown that a considerable number of dysplastic adenomas have chromosomal rearrangements leading to the appearance of aberrations previously have been found frequent in carcinomas, including gains in 8q, 13q and loss of 17p (Bomme et al., 1994). Nevertheless, the tumour progression model does provide a concept upon which to found further studies on the process of carcinogenesis. More attention, however, should be paid to the choice of methodologies and standardisation of tumour materials in future studies.

To date, the genetic alterations associated with progression of *in situ* carcinomas to invasive and metastatic carcinomas are not well understood. In Vogelstein's model, these aberrations were labelled as "other alterations". Many

studies have concentrated on few genes associated with the metastatic phenotype because there appear to be a lack of guidance as to "where to look" in the genome for genetic events associated with the metastatic process. Moreover, studies designed to show the role of single or few genes in the metastatic process are bound to fail in showing such an association. For example, this is evident from the contradictory findings in the literature regarding, for example, the role of single genes in the metastatic process. Although single gene aberrations could be playing a significant part in the metastatic process, when studied in isolation their significance to the metastatic process become obscure because metastasis is a multi-step process involving the interaction of many genetic and epigenetic phenomenon. Therefore, the development of wide-scanning genome methodologies such as CGH and RDA could offer a great insight in narrowing down areas of the genome and in elucidating the combination of genetic events involved in the multi-step process of metastasis.

Figure 3: *Comparison of the genetic alterations in the multistep progression model as viewed by Vogelstein et al. (A) with those found by Boland et al. (B)*

Solid arrows indicate the point at which the specific aberration has occurred in the progression sequence and the direction indicates increased frequency of the aberration. The dotted arrow indicates that the genetic aberration was found in all stages of the progression sequence with similar frequency. See section 1.3 for details.



CHAPTER 2. THE METASTATIC PROCESS

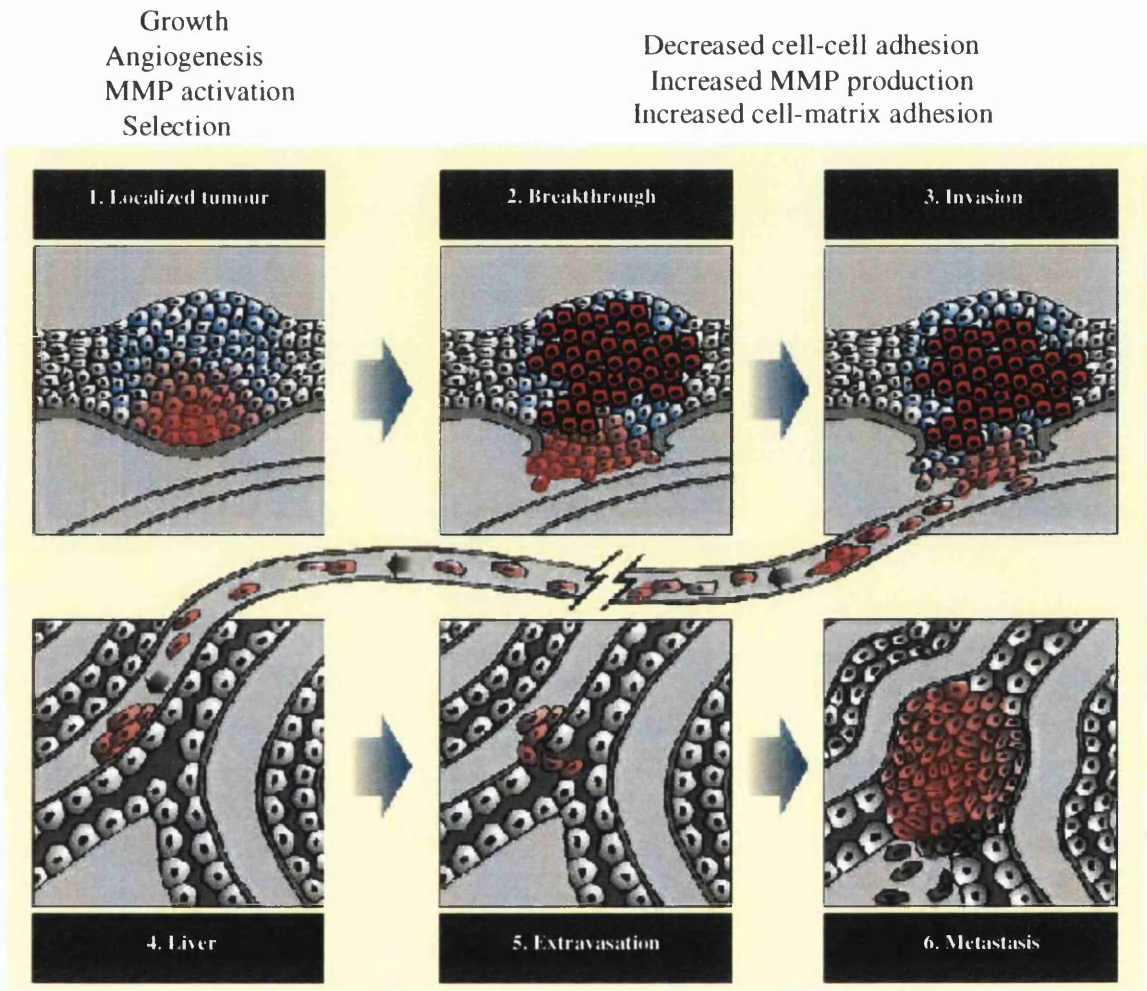
2.1. An overview of the metastatic process

Metastasis is defined as the ability of primary tumours to form tumour deposits distant to their site of origin. It is this process that distinguishes benign from malignant tumours. Metastasis is not a unique phenomenon to tumours. A closer look at foetal development, the immune system surveillance, and placental development reveals the ability of these systems to invade and metastasise. Physiologically, this process is strictly controlled. In tumours, however, metastasis is under no such physiological controls.

Metastasis is a multi-step process (Figure 4). A selected clone of cells growing in the primary tumour has to detach from the tumour mass and invade through a thick layer of connective tissue known as the basement membrane (BM). This process involves the production of a collection of proteolytic enzymes capable of destroying collagen type IV and other proteins and carbohydrate polymers, which are the main constituents of the BM. This process is discussed in section 2.3.2. The metastatic cells then reach blood or lymphatic vessels by which they are transported to distant sites. The tumour cells then extravasate from the vessels to colonize distant organs as micrometastases. Micrometastases have been substantially documented in early stages of colorectal cancer (Adell et al., 1996; Greenson et al., 1994; Hayashi et al., 1995; Jeffers et al., 1994; Nakamori et al., 1997). Although only 1% of micrometastases can eventually give rise to clinically detectable tumours (Luzzi et al., 1998), a recent study has shown that the five-year survival rate decreased from 91 % to 50 % in patients with colorectal cancer

Figure 4: *Sequential steps involved in the metastatic process*

Blue cells represent the original founding clone. The light red cells represent the emergence of a clone or clones of cells with invasive and metastatic potential, which later dominate the tumour mass (dark red cells). The biological processes important to each step are also noted.



lymph node micrometastases, while patients without lymph node micrometastases had a significantly better prognosis (Liefers et al., 1998).

Metastasis is therefore a complex process involving multiple steps and requiring the expression of multiple unrelated genes. A successful metastatic cell should have a complete set of abnormally expressed genes where each gene or a combination of genes is required to complete single steps in the metastatic cascade. The process of blood vessel formation or angiogenesis and adhesion of tumour cells to distant vessels are described in sections 2.6 and 2.2.3 respectively.

Metastasis is an inefficient process. Metastatic tumour cells are typically cleared from the host biphasically. The initial phase (6-24 hours), represents an exponential decline of cell numbers due to mechanical trauma, oxygen toxicity and clearance by the immune system. The second more gradual decline phase represents cell death at colonized sites (Weiss, 1990). However, recent evidence from intravital videomicroscopy, have strongly proposed that the major cause of metastasis inefficiency is failure of cells to grow in distant sites post-extravasation (Chambers et al., 1995; Koop et al., 1996).

The liver represents the major site for metastasis in colorectal cancer. However, metastasis to the lung, brain and bone is not uncommon. Haemodynamic considerations could explain the involvement of the liver in the majority of metastases, since the liver represents the first capillary bed encountered by metastatic colorectal cancer cells. Similarly, tumours in the lower rectum are more likely to metastasize to the lung since the lower rectum principally drains into the systemic circulation via the inferior vena cava. Although haemodynamic distribution contributes to the formation of metastases by mechanical trapping, it fails to account for liver, instead of the expected lung,

colonization after intravenous injections of tumour cells in mice. The preference to metastasize to specific organs is well documented. Indeed, this 'soil phenomenon' was first described by Pagets as far as a century ago.

Tumour cells "the seeds" also posses genetic or epigenetic characteristics that allow them to colonize distant organs. This has been elegantly shown by Kuo et al., (1995). The authors have demonstrated that cells from metastatic colorectal tumours were able to colonize the liver after directly injecting or implanting the tumours on it, while non-metastatic tumours failed to colonize the liver. The authors argued that the genetic characteristics of tumour cells were of prime importance in successful colonization of distant sites. Indeed, this thesis is devoted to pinpointing genetic alterations in colorectal cancer associated with the metastatic process.

2.2. The role of cell adhesion molecules in metastasis

2.2.1. Cadherin

The cadherins comprise a family of calcium-dependent adhesion receptors that are usually employed in homotypic cell-cell adhesion (Suzuki, 1997). Cadherins superfamily can be divided into two subgroups based on their association with cytoskeletal components (Arnaout, 1993).

The first group includes the N, P, R, B, and E cadherins. This group localises in adherence-type junctions, and can form linkage to the actin part of the cytoskeleton. The second comprise a group of desmosome-associated cadherins known as desmogleins and desmocollins that can form linkages to intermediate filaments (Mechanic et al., 1991). The best characterised cadherins are E-cadherin (Uvomorulin) found commonly on epithelial cells, N-cadherin found on neural cells (Ozawa and Kemler, 1992), and P-cadherin of placental cells (Nose et al., 1987).

The structure of a typical cadherin molecule consists of an amino-terminal external domain having five tandem repeats that mediate homotypic interaction with another cadherin molecule on adjacent cell via calcium ions, a single transmembrane segment, and a cytoplasmic carboxy-terminal domain of about 150 amino acids of variable length. The carboxy-terminal domain plays a crucial part in attaching and stabilising cadherins to the actin filament indirectly via intracellular proteins known as catenins (Gumbiner and McCrea, 1993).

The catenins are members of the armadillo family of proteins. β-catenin binds direcly to a specific 72 amino acid region in the cytoplasmic domain of

cadherins and serves as a bridge for α-catenin association with cadherins. α-catenin links cadherins to the actin cytoskeleton, while γ-catenin appears to weakly associate on the periphery of this complex (Aberle et al., 1996). The linkage to the actin cytoskeleton is essential for the adhesion function of cadherins (Aberle et al., 1996). In addition to its function in adhesion, β-catenin serves as a signal transduction molecule by forming complexes with the Tcf-Lef family of trascription factors (Behrens et al., 1996). The β-catenin-Tcf complex transactivates downstream genes that inhibit apoptosis and increase cell proliferation (Peifer, 1997; Rubinfeld et al., 1997).

APC interacts with β-catenin and serves in its degradation, thus decreasing the cytoplasmic pool of β-catenin. Recently, it has been shown that the APC protein down regulates the transcriptional activity of β-catenin-Tcf complex in colorectal tumours (Korinek et al., 1997; Morin et al., 1997). Moreover, ectopic expression of wild-type APC eliminates excess β-catenin from APC-defective melanoma cells (Rubinfeld et al., 1997). These results indicate that APC plays an essential role in clearance of β-catenin from the cytoplasm and that β-catenin acquires oncogenic activity when it is mutated or when it is upregulated by inactivation of APC as seen in patients with familial and sporadic colorectal cancer. This is in keeping with the gatekeeper function of the APC protein.

The phosphorylation status of cadherins and β-catenin may influence adhesive function of cadherins as well. For example, v-Src tyrosine phosphorylation of these proteins have been demonstrated to disrupt E- and N-cadherins mediated adhesion (Behrens et al., 1993; Takeda et al., 1995). Also, β-catenin can directly complex with the proto-oncogene *c-erbB-2* and the EGF

receptor, the disruption of this complex has been shown to suppress the invasion and metastasis of cancer cells (Shibata et al., 1996). Moreover, activation of the EGF and HGF receptor tyrosine kinases induces tyrosine phosphorylation of β-catenin, and correlates with decreased cell-cell adhesion and increased cell migration (Shibamoto et al., 1994).

There is mounting *in vivo* and *in vitro* evidence to support the involvement of cadherin mediated adhesion in invasion and metastasis. *In vitro* studies, utilising either the collagen matrix gel assays or the chick heart fragment invasion assays, have shown that transfection of invading cells with E-cadherin renders them non-invasive, while transfection of anti-sense E-cadherin into E-cadherin expressing cells or neutralising E-cadherin by antibodies renders them invasive (Vleminckx et al., 1991). Studies of tumours from patients presenting with breast, gastric, or head and neck squamous cell cancers indicate that loss or reduced E-cadherin expression correlates with invasive tumours (Oka et al., 1993; Schipper et al., 1991). Similarly, loss of E-cadherin and inceased stability of β-catenin has been shown to be frequent in colorectal cancers (Dorudi et al., 1993; Hiscox and Jiang, 1997). Moreover, the loss of E-cadherin has been shown to correlate with Dukes' progression (Mohri, 1997).

In the cadherin-catenin system, cadherins seem to localise β-catenin to the cytosol of the cell. Increased nuclear and decreased cytosolic β-catenin is associated with colorectal carcinoma and increasing degree of dysplasia in adenomas (Hao et al., 1997). Indeed, this is in line with the metastatic suppressor function of cadherins, since inactivation of this system induces dissociation of cell-cell adhesion and increases the metastatic potential of cancer cells. Therefore, the amount of cytoplasmic β-catenin is likely to be regulated by its binding to

APC or to the adhesion apparatus. In tumour cells, inactivation of the *APC* gene through mutation or deletion, and alterations of the adhesion apparatus by increased tyrosine phosphorylation could serve to stabilise β-catenin. Recently, β-catenin has been shown to activate transcription from the cyclin D1 promoter, which contains sequences related to TCF/LEF (Tetsu and McCormick 1999). The authors hypothesise that β-catenin accumulation may contribute to neoplastic transformation by causing accumulation of cyclin D1.

2.2.2. Integrins

Integrins are a large family of heterodimeric integral plasma membrane cell surface receptors that mediate cell-matrix and cell-cell interaction (Hynes, 1992). Such interactions are involved in a wide variety of physiological processes including regulation of cellular function during embryonic development, wound healing, inflammation, cell growth, and metastasis (Giancotti and Mainiero, 1994).

Each integrin heterodimer is composed of an α and a β subunit, of size ranges 120-180KD and 90-110KD respectively. To date, there are 16 distinct α subunits and 8 β subunits which variously combine to form 22 receptor types, For example, β_1 subunit can form heterodimers with many types of α subunits each characterised by a distinct, although largely overlapping, ligand-binding specificity. Similarly, the α subunit can dimerize with many types of β subunits (Hynes, 1992). This property is useful for a motile cell because some integrins can functionally substitute for others when the cell encounters different matrix proteins along its journey. A further level of complexity is introduced by

alternative splicing of some α subunits (Hogervorst et al., 1991) and β subunits (Altruda et al., 1990).

Both α and β subunits are transmembrane glycoproteins with large globular amino-terminal extracellular domains that bind principally RGD or other sequences in the extracellular matrix or ICAMS on other cells (Pierschbacher and Ruoslahti, 1984; Pierschbacher and Ruoslahti, 1984). Each subunit provides a thin leg that transverse the plasma membrane and end in a relatively small cytoplasmic tail of less than 60 amino acids (Tamura et al., 1990). β_4 integrin subunit is an exception to this general rule because it has a cytoplasmic domain of about 1000 amino acids (Hogervorst et al., 1990). The cytoplasmic domain of the β subunit interacts with talin which in turn interacts with vinculin and thereby paxillin (Lewis and Schwartz, 1995). Vinculin and paxillin interact with the actin cytoskeleton thus attaching the extracellular matrix via integrin heterodimer to the cytoskeleton.

Integrins differ from other cell adhesion molecules in that they bind their ligand with low affinity. However, clustering integrins increases the strength of binding to their ligand as seen in focal adhesions. This is a useful characteristic for motile cells, because in this way cells can bind simultaneously but weakly to large number of matrix molecules and still have the opportunity to explore their environment without losing all attachment to it by building or breaking down focal contacts. Strong binding of the receptors to their ligand would reduce or abolish cell motility (Palecek et al., 1996). This problem is avoided when the attachment depends on multiple weak adhesions.

It is now becoming clear that integrins have functions other than merely attaching cells to the extracellular matrix. Integrins play a crucial part in

modulating cellular responses to growth factors and extracellular matrix by transmitting signals, a process known as "outside-in" integrin signalling. Upon binding to the extracellular matrix, integrins are activated by conformational change " affinity modulation" or through integrin clustering " avidity modulation". The mechanistic details of the down stream signalling is still scanty, but involves the recruitment of a distinct quarternary signalling molecules (ILK, FAK, C-SRC, $p130^{CAS}$ and paxillin) to focal adhesions whose assembly is normally initiated by autophosphorylation of FAK. This results in activation of the MAPK pathway via RAS (Clark and Hynes, 1996; Wary et al., 1996), or RAS independent pathway directly via RAF-1 (Chen et al., 1996), or via PI3K-PKC-RAF-1 pathway (Clark and Hynes, 1996).

Cell growth requires both growth factors and anchorage to the extracellular matrix. It is becoming evident that integrin-dependent adhesions often have a major role in potentiating the efficiency of growth factor signalling through RTKs and MAPK cascade "collaborative signalling". For example, by comparing suspension cells with integrin-anchored cells, the former was found to have impaired MEK and MAPK kinases (Lin et al., 1997). Also, both integrin clustering and stimulation of growth factor receptors enhanced auto phosphorylation of the corresponding growth factor receptor (Schneller et al., 1997; Yamada, 1997). Thus, it is not surprising that cells would use both positional information via integrin, as well as information about the availability of growth factors, to determine the appropriate time to enter the cell cycle. However, tumours activated by various co-operating oncogenes could circumvent the need for integrin anchorage-dependant growth by constitutively activating any of the kinases involved in the integrin pathway. For example, recent data have indicated

that a constitutively active form of FAK can promote anchorage-independent survival and growth in epithelial cells (Frisch et al., 1996). Also, FAK expression was found to be elevated in highly malignant tumours compared to normal tissue (Weiner et al., 1993). Moreover, transformation of cells by *v-src* overrides the requirement of attachment to the extracellular matrix leading to anchorage-independent growth by constitutively activating FAK or by phosphorylating its substrates (Guan and Shalloway, 1992).

Integrins affinity to the extracellular matrix could also be modulated from within the cell in a process known as "inside-out" integrin signalling. This phenomenon is important since it allows the cell to modulate its adhesion in response to intracellular signals, as in cell division, where the cell loses its attachment to the extracellular matrix (see section 3.5.1 for details).

*2.3. **The role of cell invasion and motility in metastasis***

2.3.1. Extracellular Matrix Degrading Enzymes

The ECM defines boundaries between tissue compartments and organ parenchyma. It is principally composed of two forms: basement membrane and the underlying interstitial stroma. The basement membrane separates epithelial and endothelial cells from mesenchymal tissue. Glycoproteins, such as laminin, collagens (mainly type IV), entactin, fibronectin, and proteoglycans such as heparan sulfate and chondroitin sulfate are the main constituents of basement membranes. These components interact with one another to create non-fibrillar continuous network. Underlying this layer is the interstitial stroma, which is composed of glycosaminoglycans, collagen (type I, II and III), glycoproteins and elastin. Composition of the ECM varies from one organ to the other to guide specialised functions for the normal physiology of the organ.

The ECM molecules form a continuous impermeable layer around tissues that becomes focally permeable to cell movement during selective physiological processes such as tissue remodelling and wound healing (Timpl and Dziadek, 1986). The function of the ECM is not merely to provide strength and support for overlying tissue, but proteins of the ECM also can act as ligand for cellular receptors (integrins), creating specialised cell-matrix links called focal contacts (Burridge et al., 1988), which are critical for cellular motility. The ECM also sequesters and protects growth factors and cytokines through interactions with a variety of molecules such as collagen and heparan sulfate (Taipale and Keski-Oja, 1997). For example, heparan sulphate interacts with both basic fibroblast growth

factor FGF-2 and acidic fibroblast growth factor FGF-1, which are potent angiogenic factors. This interaction not only serves to provide a rich source of growth factors that are protected from degradation but also can be presented specifically to FGF receptors by heparan sulfate present on the cell surface (Miao et al., 1996).

The basement membrane is a critical barrier to tumour cell dissemination. However, tumour cells selectively reorganise and degrade their local basement membrane so that they can disseminate to other location. The interaction of tumour cells, growth factors and proteolytic enzymes with the ECM are critical during the invasion process. These concepts will be emphasised in the following sections.

2.3.2. Matrix Metalloproteases

Matrix metalloproteases are a family of transmembrane or secreted proteins that are capable of degrading ECM components. Currently, 16 family members have been identified (Table 2). They share a catalytic domain (VAAHEXGHXXGXXH) with the underlined motif responsible for zinc binding. MMPs are also characterised by possessing a distinctive PRCGVPD sequence in the amino-terminal prodomain that is responsible for maintaining latency of the enzymes (Birkedal-Hansen et al., 1993). The carboxy-terminal region of the MMPs possesses a haemopexin/vitronectin-like domain that is important in substrate and inhibitor bindings (Sanchez-Lopez et al., 1993). Matrilysin (MMP-7) is the only member that does not possess such a domain.

There are five major subclasses of MMPs based on their substrate preferences (Table 2): collagenases degrade fibrillar collagen, stromelysins

degrade proteoglycans and glycoproteins, gelatinases are particularly potent in degrading non-fibrillar and denatured collagens or gelatin (Birkedal-Hansen et al., 1993; Matrisian, 1992), metalloelastases are potent in elastin degradation and the membrane type MT-MMPs, which possess a transmembrane domain that localises their activity to the plasma membrane and are important activators of the pro-form of MMP-2 or gelatinase A (Sato et al., 1994).

Table 2: Matrix metalloproteases nomenclature and substrate specificities

Enzyme	Alternative names EC designation	Substrate
Matrilysin	MMP-7, pump-1 EC 3.4.24.23	Proteoglycans, Laminin, Fibronectin, Gelatin, Collagen IV, Elastin, Entactin, Tenascin
Stromelysin-1	Transin, MMP-3 EC 3.44.24.17	Proteoglycans,Laminin, Fibronectin, Collagen III, IV, V, Gelatin
Stromelysin-2	Transin-2, MMP-10 EC 3.4.24.22	Proteoglycans,Fibronectin, Collagen III, VI, V, Gelatins
Stromelysin-3	MMP-11	Laminin, Fibronectin (weak)
Metalloelastase	MMP-12 EC 3.4.24.65	Elastin
Interstitial collagenase	Fibroblast Collagenase, MMP-1 EC 3.4.24.7	Collagens I, II, III, VII, X, Gelatins
Neurophil collagenase	MMP-8 EC 3.4.24.34	Collagens I, II, III,
Collagenase-3	MMP-13	Collagen I
MT1-MMP MT2-MMP MT3-MMP MT4-MMP	MMP-14 MMP-15 MMP-16 MMP-17	Activation of GelatinaseA Unknown Gelatinase A Unknown
Gelatinase A	72 kDa gelatinase, MMP-2 EC 3.4.24.24	Gelatins, Collagens IV, V, VII, X, Elastin, Fibronectin
Gelatinase B	92 kDa gelatinase, MMP-9 EC 3.4.24.35	Gelatins, Collagens IV, V, Elastin

(Abstracted and modified from (MacDougall and Matrisian, 1995))

The MMPs are regulated at a number of levels, including transcriptional regulation (induce the expression of MMPs) by growth factors and cytokines, post-transcriptional regulation at the level of mRNA stability, post-translational regulation by the activation of the secreted latent form, and inhibition by the general serum proteinase inhibitor α2-macroglobulin and specifically by a family of endogenous tissue inhibitors known as the tissue inhibitors of metalloproteases (TIMPS) (Stetler-Stevenson et al., 1993). The balance between the levels of activated MMPs and free TIMPs determines the net MMP activity, altering this equilibrium affects the process of invasion. To date, there are three well-defined members of the TIMP family. These are termed TIMP-1, TIMP-2 and TIMP-3. Recently, a murine TIMP-4 has been isolated, although a homologue of this new member is yet to be identified in humans (Leco et al., 1997).

It has been known for sometime that MMPs are associated with malignant tumours (Liotta et al., 1982). Since that time, extensive literature demonstrating a close association between MMPs family members and tumour progression has evolved (MacDougall and Matrisian, 1995)

There are two important observations made recently regarding the role of MMPs in the metastatic process. First, MMPs are not only expressed by tumours but are also expressed by stromal cells surrounding the tumours. For example, stromelysin-3 mRNA expression has been shown to be specifically localised to the stroma of colon, breast, and head and neck cancers (Powell and Matrisian, 1996). Also, growth factors such as PDGF, secreted by tumours have been shown to stimulate collagenase expression by human fibroblasts (Circolo et al., 1991). In addition, several *in vitro and in vivo* tumour cells have been shown to possess a collagenase stimulatory factor termed EMMPRIN (a member of the

immunoglobulin superfamily) on their cell surface. This factor has been shown to stimulate the production of interstitial collagenase, stromelysin-1 and MMP-2 in human fibroblast (Guo et al., 1997; Kataoka et al., 1993). Thus, MMP production in the surrounding stroma could enhance the ability of tumour cells to degrade the local ECM. Secondly, the ability of MMPs to enhance tumour invasion and metastasis has been "assumed" to be due to facilitating extravasation and increasing ECM degradation. However, recent evidence from IVVM has shown that not only do normal fibroblasts extravasate as well as malignant cells but that only malignant cells expressing high levels of MMPs could colonize distant organs (Koop et al., 1996; Morris et al., 1994). Also, B16F10 mouse melanoma cells engineered to overexpress TIMP-1 extravasated as well as cells expressing normal levels of TIMP-1. These results were surprising in that it was expected that cells overexpressing TIMP-1 would be defective in extravasation. However, the reduced metastatic ability of cells overexpressing TIMP-1 was due to failure to colonize distant organs i.e. post-extravasation growth (Koop et al., 1994; Soloway et al., 1996). In light of these results, the role of MMPs in the metastatic process should be re-evaluated. It is possible that MMPs enhance the growth of metastatic cells by increasing growth factor availability and by enhancing tumour angiogenesis. This is particularly important in early stages of colonization as will be discussed in section (2.5). In support of this, it has been shown that matrilysin is expressed in 88 % of adenomas arising in *min*-mice, which are transgenic mice bearing a nonsense mutation in the *APC* gene giving rise to multiple intestinal neoplasia. Matrilysin knockout in the *min* mouse demonstrated that matrilysin-deficient mice developed 60 % fewer intestinal adenomas (Wilson et al., 1997). Similarly, stromelysin-3 deficient mice demonstrated impaired tumour formation

in response to chemical mutagenesis (Masson et al., 1998). These results argue for a role of MMPs in tumour initiation and growth beside their role in tumour metastasis.

2.3.3. Serine Proteases

Serine proteases are enzymes involved in activation of plasminogen and are therefore termed plasminogen activators. There are two types of plasminogen activators, the tissue-type (t-PA) and the urokinase-type (u-PA). Both convert the inactive plasminogen to the active proteases plasmin by cleavage of the arginine 560-valine 561 bond. There is general agreement that t-PA generates plasmin for thrombolysis and that its activity is enhanced by the presence of fibrin, while u-PA generates plasmin for the degradation of the ECM and does not require fibrin for its function. u-PA can also activate the plasmin like HGF (Naldini et al., 1992). For these reasons, u-PA has been widely studied in relation to tissue remodelling and tumour metastasis. u-PA binds a cell surface receptor termed u-PAR. The receptor was first described by Vassalli et al. (1985). Later, it has been shown that u-PAR is attached to the cell membrane by a glycosyl-phosphotidyl-inositol anchor (Ploug et al., 1991). u-PAR has been shown to bind a somatomedin B domain (an RGD integrin-binding sequence) on vitronectin (Deng et al., 1996). This binding not only stabilises the u-PA/-uPAR on the ECM for activation of the membrane bound plasminogen and subsequent degradation of the ECM but is important in juxtaposition of the u-PA/u-PAR system to integrins ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$, $\alpha_8\beta_1$), which also bind the RGD sequences on vitronectin. Moreover, β_2-integrin is frequently co-localised and purified along with u-PAR

(Bohuslav et al., 1995) and binds to u-PAR in a purified system (Wei et al., 1996). In addition, u-PAR can be co-immunoprecipitated with caveolin and β_1-integrins, and has been found to inhibit binding of β_1-integrins to fibronectin (Wei et al., 1996). It is therefore not surprising to find u-PA/u-PAR concentrated at focal adhesions. This localisation of the plasminogen activators to focal contacts is not only important for facilitating cellular motion through the ECM but could also alter the capacity of integrins to mediate intracellular signalling (Chapman, 1997).

There are two main inhibitors of the plasminogen activators, PAI-1 and PAI-2, while plasmin is inhibited by α_2-anti-plasmin. Binding of PAI-1 or PAI-2 to u-PA/u-PAR complex (PAI can bind and compete with u-PAR for vitronectin binding) results in binding of the complex to the α_2-macroglobulin/lipoprotein-receptor related protein, which is an endocytic receptor, and rapid clearance of the complex from the cell surface (Conese et al., 1995).

There is now mounting evidence supporting the involvement of plasminogen activators in the process of cancer dissemination *in vivo* and enhanced cellular invasion *in vitro.* For example, experiments that involve manipulating the levels of the plasminogen activator system in cells *in vitro* have shown a strong correlation between expression of plasminogen activators and invasion of the ECM (Andreasen et al., 1997). In u-PA-deficient mice, the invasive potentials of carcinogen induced blue naevi and their progression to malignant melanomas were significantly reduced compared to mice carrying the wild-type gene (Shapiro et al., 1996). In human cancers, u-PA expression has been shown to associate with poor prognosis in a variety of cancers, including breast, lung, bladder and many others (Duffy, 1996). Similarly, u-PAR expression also appears to be a prognostic marker in many cancers, such as breast, stomach

and squamous carcinoma of the lung. Interestingly, in colorectal cancer u-PA has been shown to be expressed by stromal cells around the tumours (Pyke et al., 1991), while tumour cells have high concentrations of u-PAR (Pyke et al., 1994), which constitute an independent prognostic marker (Ganesh et al., 1994).

PAI-1 expression is also associated with aggressive cancers (Grondahl-Hansen et al., 1993). This is unexpected due to the inhibitory role of PAI-1 on the plasminogen activators system. However, it could be that PAI-1 has additional functions than merely inhibiting plasminogen activators (Stefansson and Lawrence, 1996). It is also possible that the associated of PAI-1 with aggressive tumours is due to its production by endothelial cell from angiogenic vessels within tumours.

2.4. Cellular motility

Normal epithelial cells form continuous layer of cells that are polarised and non-motile. During malignant transformation, these highly differentiated epithelial cells convert to motile mesenchymal cells. Indeed, It has been known for a long time that these two cell type can interconvert (Hay, 1990). For example, epithelial cells can convert to fibroblast-like cells following disruption of cadherin function by *v-src* oncogene expression (Behrens et al., 1993) or by growth factor treatment (Weidner et al., 1991). Conversely, fibroblasts can be converted to epithelial-like following artificial expression of cadherins (Nagafuchi et al., 1987) or specific growth factors and their receptors (Tsarfaty et al., 1994). This interconversion illustrates the capability of malignant cells to switch on programs involved in cellular motility during embryogenesis, wound healing, morphogenesis and in immune surveillance.

Cellular motility is central to the process of tumour metastasis. Tumour cell invasion, intravasation, and extravasation as well as angiogenesis all require active cellular motility. The ability of tumour cells to migrate in response to ECM proteins has been found to correlate with *in vivo* invasion and metastatic capacity of tumour cell lines (Lester and McCarthy, 1992).

Cellular locomotion is a complex process that requires the co-ordinated interactions of growth factors, ECM and integrins. These co-ordinated interactions result in the formation of membrane ruffling and pseudopodia, formation of attachments to the ECM at the leading edge of the cell, and release of the cell attachment at the rear of the cell and forward motion of the cell body (Stossel, 1993). Tumour cells become motile in response to a variety of agents, including

host-derived motility and growth factors, extracellular matrix components, and tumour-secreted factors (Kantor and Zetter, 1996).

Several forms of cell motility have been defined. Chemotaxis is a directional migration of cells towards a positive gradient of a soluble factor, such as fragments of ECM or growth factors. Chemokinesis is a randomly directed cellular motility, which occurs in the absence of a concentration gradient, while haptotaxis is a directed cellular motion towards a positive concentration gradient of an immobilised attractant. For example, growth factors immobilised within the ECM.

Scatter factor and hepatocyte growth factor are both autocrine and paracrine growth factors that stimulate scattering of epithelial cells and proliferation of hepatocytes respectively. However, they were later found to be the same molecule (Weidner et al., 1991). HGF is member of a family of soluble factors, which includes the macrophage stimulatory protein (MSP). Both molecules share sequence homologies with the serine-proteases of the blood-clotting cascade. These proteins are secreted as single chain precursors (92 kDa) that are subsequently activated in the ECM by serine- proteases to form α and β subunits that are linked by disulfide bonds (Miyazawa et al., 1993). The α subunits of HGF and MSP have a molecular weight of 60 kDa and contain four kringle domains similar to those observed in coagulation factors. The β subunits have a molecular weight of approximately 30 kDa and are closely related to the catalytic domains of serine-proteases but they exhibit no known protease activity. The profound effects of HGF on cell growth, morphology and motility have been well-documented (Bhargava et al., 1993). A strong association between high levels of HGF in breast tumours and shorter relapse free and overall survival has

been documented (Yamashita et al., 1994). However, the role of HGF in colorectal cancer is more controversial (Fukuura et al., 1998; Hiscox et al., 1997).

HGF binds to specific cell membrane receptor termed c-*met*. This proto-oncogene is a 190 kDa protein and belongs to the tyrosine kinase receptor superfamily (Naldini et al., 1991). C-Met is synthesised as a large precursor that is made up of an extra-cellular α chain and a β chain that spans the plasma membrane. Both chains are necessary for HGF activation and are connected by a disulfide bond. The intracellular domain includes a negative regulation juxtamembrane domain followed by a conserved tyrosine kinase catalytic domain and a C-terminal sequence which is responsible for receptor coupling to intracellular transducers (Bardelli et al., 1994). Upon HGF binding, autophosphorylation of the cytoplasmic domains activates a variety of intracellular molecules including the cytoplasmic tyrosine kinase C-Src, Ras (through Grb2/SoS binding), PI-3K, PLCγ and the MAPK (Ponzetto et al., 1994).

C-*met* is overexpressed in a variety of sporadic tumours. In colorectal cancers, c-*met* is overexpressed up to 50-fold in about half of primary tumours, and in 70 % of liver metastases. Moreover, c-*met* overexpression in colorectal cancer has been shown to be associated with gene amplification (chromosome band 7q31) in a significant proportion of metastases (Di Renzo et al., 1995). Recently, the tyrosine kinase domain of c-*met* has been found mutated in hereditary and sporadic papillary renal carcinoma (Schmidt et al., 1997). Thus, establishing a direct role for c-*met* proto-oncogene in human cancers.

2.5. *Interplay between growth factors, adhesion receptors, extracellular matrix degrading enzymes in cellular motility*

It is evident from the above description of the factors involved in cellular movement that this process is highly complicated and requires the interaction of many factors for its regulation. The way growth factors, integrins, MMPs and the plasminogen activators interact together to facilitate cellular movement will be discussed.

Growth factors such as EGF, PDGF, LPA and insulin induce the formation of stress fibers and focal adhesions that are necessary for cell movements (Ridley and Hall, 1992). This focal contact assembly can be ascribed to the action of the Rho family of GTPase. Rac has been shown to be directly responsible for the formation of membrane ruffling and lamellipodia, and Cdc42 can control the formation of filipodia (Hall, 1998). The Rho family can also be activated by clustering and ligation of integrins (Hotchin and Hall, 1995; Renshaw et al., 1996). The convergence of growth factors and integrin-signalling pathways could have important consequences on enhancing cellular motion. For example, in human pancreatic carcinoma cells, integrin $\alpha_v\beta_5$ mediates adhesion but not migration on vitronectin (these cells readily migrate on collagen in an $\alpha_2\beta_1$-dependent manner). However, following stimulation with EGF, these cells migrate on vitronectin through an $\alpha_v\beta_5$ -mediated protein kinase C-dependent pathway without affecting constitutive migration on collagen (Klemke et al., 1994). Similarly, IGF-1 stimulates motility and invasion of pancreatic and breast carcinoma cells on vitronectin by activating and co-operating with $\alpha_v\beta_5$ integrin (Brooks et al., 1997; Doerr and Jones, 1996). Other growth factors, such as TGFβ-

1, are strong stimulators of integrin and u-PAR expression (Lund et al., 1991). In addition, bFGF can induce activation of latent TGFβ by inducing u-PA synthesis in endothelial cells (Flaumenhaft et al., 1992). The plasminogen activators have also been shown to activate latent TGFβ and HGF (ECM-bound) to their active forms (Odekon et al., 1994). Taken together, these two observations illustrate the possible existence of a positive regulatory cycle that could enhance cellular motility, invasion and metastasis.

The presence of certain ECM molecules also induces the expression of MMPs that degrade them. Indeed, it has been shown that integrins bound to specific matrix molecules induce the production of MMPs that specifically degrade that matrix. For example, integrin $\alpha_2\beta_1$ has been shown to induced MMP-1 (interstitial collagenase) production when cells were grown on collagen gels (Riikonen et al., 1995; Sudbeck et al., 1994). Similarly, interaction of $\alpha_5\beta_1$ with the RGD-domain sequence in fibronectin stimulates the expression of MMP-1, stromelysin-1, and MMP-9 (Werb et al., 1989). Treatment of human keratinocytes with anti-α_3 or anti-β_1 monoclonal antibodies induces the expression of MMP-9 (Larjava et al., 1993). Also, in human melanoma cells the expression of MMP-2 has been shown to be regulated by $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins during melanoma invasion (Seftor et al., 1992; Seftor et al., 1993). On the other hand, integrin $\alpha_4\beta_1$ binding to the CS-1-domain in fibronectin suppresses production of MMP-1, stromelysin-1, and MMP-9 (Huhtala et al., 1995). Functional analysis, at least in the MMP-1 gene, has shown that integrin induction of the MMPs is the function of AP-1/PEA3-responsive DNA sequences (Tremble et al., 1995).

Recent significant findings implicating the co-ordinated action of integrins with the plasminogen activators in tumour cells migration and invasion have been reported. For example, integrins have been shown to induce production of urokinase plasminogen activators in a Ras dependent manner (Irigoyen et al., 1997; Lengyel et al., 1996). Also, the induction of u-PA and its receptor u-PAR by TGFα has been shown to require the $\alpha_v\beta_5$ integrin (Brooks et al., 1997; Yebra et al., 1996). A significant step has been recently made illustrating a direct connection between the MMPs, integrins, and cellular invasion. Brooks et al. (1996) demonstrated that MMP-2 co-localises with integrin $\alpha_v\beta_3$ on the surface of melanoma tumour cells *in vivo* and was shown to directly bind $\alpha_v\beta_3$ (but not $\alpha_v\beta_5$ and $\alpha_5\beta_1$) *in vitro*. That $\alpha_v\beta_3$ promotes cell motility, while MMP-2 potentiates matrix degradation, suggests a close co-operative function between these proteins to favour melanoma dissemination. This observation is not unique to tumour cells, but has been also observed on angiogenic blood vessels endothelial cells during active angiogenesis (Brooks, 1996; Brooks et al., 1995).

It is, therefore, clear that close interaction between growth factors, integrins and the matrix degrading enzymes are important in facilitating and enhancing tumour cell invasion and metastasis. However, the remaining challenge is to dissect the precise role of each element in the transmission of biological signals leading to tumour cell migration.

2.6. The role of angiogenesis in metastasis

Tumour angiogenesis is defined as the process by which new blood vessels sprout from the existing vasculature into developing solid tumours (Folkman, 1995). The main purposes of these vessels are to provide nutrients for the expanding tumour and a mean by which tumour cells can disseminate to distant sites.

The characteristics of the newly formed vessels are intriguing. In contrast to normal vessels, these form multiple branching points, they are spiralled and form extensively convoluted loops (Kus et al., 1981). In addition, the vessels are extensively leaky (fenestrated) due to the absence, in some parts, of a complete basement membrane which in turn may facilitate the migration of tumour cells and growth factors (Roberts and Palade, 1997).

Angiogenesis is a multi-step process that involves the dissolution of old basement membranes, migration, proliferation of endothelial cells, formation of vascular loops, and new basement membrane. This process is initiated when tumour cells start to produce diffusible angiogenesis promoting factors. Many factors are known for their ability to promote angiogenesis. The best-characterised factor is vascular endothelial growth factor (VEGF). It was initially identified as a high specific mitogen for vascular endothelial cells (Leung et al., 1989). It was simultaneously isolated by virtue of its blood vessel permeabilising activity, and is therefore also known by the name vascular permeability factor (VPF).

VEGF belongs to the platelet-derived growth factor (PDGF) family of growth factors because it contains the eight conserved cysteines that are the hallmark of this growth factor family.

Five VEGF mRNA species encoding VEGF isoforms of 121,145, 165, 189, and 206 amino-acids are produced by alternative splicing of VEGF mRNA (Tischer et al., 1991). All of these isoforms are synthesised with a hydrophobic leader peptide for secretion, which is then cleaved to yield the mature protein. However, only VEGF 121, 145, and 165 are actually secreted from producing cells (Poltorak et al., 1997). The two larger VEGF isoforms remain cell associated (Houck et al., 1992). The active forms of the various VEGF proteins were found to be homodimers linked via disulfide bonds (Leung et al., 1989). The existence of so many VEGF forms implies that they may differ in their biological properties, tissue processing, and distribution. Indeed, using rat organs, it has been found that VEGF 188 (in rodents all VEGF forms are one amino-acid shorter) is the predominant form in heart and lung tissues. VEGF 164 mRNA levels are relatively low in heart and lung, but predominant in brain and kidney. VEGF 120 is present in equimolar amounts to VEGF 164 in spleen, and relatively large amounts of VEGF 120 are also found in kidneys and lungs, but only low levels of VEGF 120 have been observed in brain and heart tissue.

VEGF interacts with two known endothelial cell receptors. KDR/FLK1 a kinase insert domain containing receptor, which was first cloned from a human umbilical vein endothelial cell cDNA library (Terman et al., 1992), FLK1 (foetal liver kinase 1), which is the mouse homologue of KDR and was originally cloned from a foetal liver stem cell cDNA library and FLT1 a *fms*-like tyrosin kinase initially cloned from a human placenta cDNA library (Shibuya et al., 1990). Other newly identified or as yet unidentified VEGF receptors also exist (Soker et al., 1996). KDR/FLK1 and FLT1 belong to the receptor tyrosine kinase family based upon similarity in structural features (Fantl et al., 1993). VEGF stimulation

of KDR/FLK1 expressing Porcine aortic endothelial cells showed striking changes in cell morphology, actin reorganisation and membrane ruffling, chemotaxic and mitogenicity (Waltenberger et al., 1994). Although the specific signal pathways responsible for these changes are yet to be identified. On the other hand, VEGF binding to FLT1 leads to phosphorylation of PLC and GAP proteins as well as activation of MAPK (Seetharam et al., 1995). In FLT1-expressing PAE cells VEGF induces phosphorylation of Fyn and Yes onco-proteins (Waltenberger et al., 1994). Cunningham et al. (1995) utilised a yeast two-hybrid system to demonstrate that FLT1 tyrosine kinase domain interacts with the p85 subunit of PI-3K.

Heparan sulfate proteoglycans are required for binding of VEGF to its receptors (Tessler et al., 1994). Interestingly, the large VEGF isoforms (VEGF 165, 189, 206), which contain the 44 amino-acids derived from exon 7 contribute to the heparin binding ability of VEGF. For example, addition of heparin to heparinase-treated cells restores VEGF 165 binding but not VEGF 121 binding indicating that changes in the composition of cell-surface heparin-like molecules may differentially affect the interaction of various VEGF isoforms with VEGF receptors (Cohen et al., 1995).

Over-expression of the VEGF gene has been demonstrated in many types of developing solid tumours (Senger et al., 1993; Tokunaga et al., 1998). In addition, inhibiting the interaction of VEGF with endothelial cells slows the growth of tumours *in vivo* (Millauer et al., 1994).

Studies on tumour angiogenesis have shown that the expression of KDR/FLK1 and/or FLT1 in endothelial cells correlates with vascularity, metastasis, and proliferation of human colon cancer (Takahashi et al., 1995).

Recently, melanoma-derived cell lines SK-MEL-2 which express normal, high and low levels of VEGF were injected into the subdermis of nude mice. These experiments revealed that VEGF over-expression dramatically improves tumour angiogenesis and growth. Also, high levels of VEGF expression demonstrated 50 folds increase in lung tumour colonisation over control or antisense transfected SK-MEL-2 cell (Claffey et al., 1996). The requirement of VEGF for growth of metastatic deposits was also demonstrated by a model of liver metastasis from colorectal cancer cells in nude mice. Metastatic deposits can occur but do not grow beyond a minimal size of 1-3 mm when the animals were treated with anti VEGF antibodies (Warren et al., 1995). Taken together, these findings suggest that VGEF production by tumour cells is critical for tumour growth, extravasation, colonising distant tissues, and to overcome growth restraints in the extravascular space to produce metastatic nodules. The possibility of using VEGF and its receptors as prognostic indicators and for cancer irradication is intriguing. For example, the expression of VEGF and its receptor KDR in breast carcinoma was found to statistically correlate with the number of metastatic nodules (Toi et al., 1995).

Little is known about the mechanisms involved in VEGF induction during disease processes generally and cancer in particularly. The VEGF gene contains four potential AP-1 sites that are probably important in the transcriptional induction of VEGF by cytokines and growth factors (Finkenzeller et al., 1995; Kolch et al., 1995). In contrast, hypoxia, which is a strong inducer of VEGF, increases the stability of VEGF proteins through the activation of *c-src* gene (Mukhopadhyay et al., 1995; Stein et al., 1995).

Puzzling clinical phenomena such as why does the removal of certain primary tumours lead to rapid growth of metastases (O'Reilly, 1997; Sckell et al., 1998), have lead to the discovery of endogenous inhibitors of angiogenesis. The mechanisms by which angiostatin is produced by tumour cells is still unclear. However, angiostatin is a 38 KD protein believed to be an internal fragment of a larger protein plasminogen, which is cleaved by urokinase and free sulphhydryl donors produced by tumour cells (Gately et al., 1997). It is a paradigm of tumourigenesis that a tumour produces both angiogenic stimulators and inhibitors, but this discovery has lead to a concept that tumour angiogenesis depends on a net balance of positive and negative regulators of blood vessels growth (Folkman, 1995). This not only helps to explain the clinical puzzles stated above, but also considerably advanced our understanding of why certain tumours are aggressive, while others remain dormant for long-periods.

The role these molecules play in tumour irradication is intriguing. Indeed, angiostatin or endostatin administration to mice caused regression of their primary tumours. In addition, when both drugs were administered together tumours were irradicated to microscopic size lesions with no toxic effects noticed (O'Reilly, 1997; O'Reilly et al., 1997).

CHAPTER 3. RAS GTPase Superfamily

The p21 proteins encoded by N-, Ki- and H-*ras* are small quanine nucleotide-binding proteins that act as switches in several trunsduction pathways. The large superfamily of Ras-like GTPases is divided into several families. In humans, this superfamily consists of the Ras proteins, four Rap proteins; Rap 1a, Rap 1b, Rap 2a, Rap 2b, two R-ras-like proteins; R-ras and TC21, two Ral proteins; Ral A and Ral B, and a recently identified Rheb protein. Less closely related to the Ras family, are other GTPase protein families. For example, the Rho family, which includes Rho-A, Rho-B, Rho-C, Rho-G, Rac-1 and -2, CDC42 and TC 10. An important concept that has emerged regarding the GTPase superfamily is the ability of some members to interact with one another. For example, microinjection experiments have revealed that CDC42 can activate Rac, which in turn activates Rho (Kozma et al., 1995; Nobes and Hall, 1995). Moreover, it has been shown that Ras, possibly acting via CDC42, requires both Rac-1 and Rho-A in order to establish a fully transformed state (Khosravi-Far et al., 1995).

3.1. The ras genes

The *ras* genes family was first identified as the transforming oncogenes of the Harvey and Kirsten strains of rat sarcomas viruses. Subsequently, the *ras* oncogenes have been identified in a wide range of animal species including mammals, birds, insects, molluscs and yeast. Sequence analysis of *ras* genes and proteins in these species revealed a high degree of sequence homology and conservation, which suggests that they may play a fundamental role in cellular processes. Moreover, a remarkable property of the evolutionary conservation of the *ras* genes is their ability to function in heterologous systems. For instance, mammalian *ras* can complement non-viable *ras1*$^{-}$ *ras2*$^{-}$ yeast mutants. Similarly, yeast *RAS* gene can efficiently transform mouse NIH3T3 cells *in vitro* (DeFeo-Jones et al., 1985).

To date, three *ras* genes have been identified in mammalian cells. The genes have been designated H-*ras-1* (Harvey-ras), Ki-*ras-2* (Kirsten-ras) and N-*ras* (Neuroblastoma-ras). Pseudogenes, that is *ras* genes that lack introns, were also found in mammals. In humans and rats these were designated H-*ras-2* and Ki-*ras-1* (McGrath et al., 1983; Miyoshi et al., 1984). Chromosomal localisations of the *ras* genes in humans and rodents have also been determined. In humans, H-*ras-1* has been assigned to chromosome 11 (11p15.1-p15.5), whereas, Ki-*ras-2* and N-*ras* has been assigned to chromosomes 12 (12p12.1-pter) and 1 (1p22-p32), respectively (Popescu et al., 1985). The coding sequences of each of these genes are found in four exons (I-IV). The exception is the Ki-*ras-2* gene, which possesses two alternatively spliced fourth exons (IVA and IVB) that allow the synthesis of two isomorphic p21 proteins of 188 (Ki-Ras-2B) and 189 (Ki-Ras-

2A) residues that differ in their carboxy terminal domains (Capon et al., 1983; McGrath et al., 1983). Although the spliced junctions of all mammalian *ras* genes correspond precisely, their intron sequences vary considerably. This results in *ras* genes ranging from 4.5 kbp size of H-*ras-1* to 50 kbp of Ki-*ras-2*. In addition, mammalian *ras* genes contain an additional 5' noncoding exon downstream from their respective promoters (Jordano and Perucho, 1986). The *ras* genes promoters lack the characteristic TATA and CAT boxes of other eukaryotic genes and are instead rich in G/C boxes (Ishii et al., 1986).

At protein level, the *ras* genes code for highly conserved proteins known as p21 Ras (188 or 189 amino acid residues), which is present in all cells, with the highest levels found in proliferating cells. However, at the mRNA level H-*ras* is transcribed highest in skin and muscle, Ki-*ras* in gut and thymus, and N-*ras* in thymus and testes (Barbacid, 1987).

3.2. The Ras proteins

Comparison of the mammalian H-, Ki-, and N-Ras protein sequences revealed the presence of three domains. The first domain (amino acids 1 to 85) is identical between the three Ras proteins even among different species. In the second domain (amino acids 86 to 165), the sequence and structure diverge slightly to reach up to 85% homology between any pairs of mammalian Ras proteins. The third domain (amino acids 166-185) is highly different in amino acid compositions not only between species but also between the three Ras proteins; hence, this region is termed the 'hypervariable region'. The last four carboxy terminal amino acids containing the sequence Cys^{186}-A-A-X-COOH (A is aliphatic amino acids and X is any amino acid) are present in all members of the Ras protein family (Barbacid, 1987). This conservation of the last four amino acids appears to be important in triggering post-translational modification and cellular localisation of the Ras proteins. The cytoplasmic pro-21 is farnesylated in the cytoplasm at the Cys 186 (Hancock et al., 1989) after which the three C-terminal amino acids are cleaved and a methyl group is transferred onto the exposed α- carboxyl group of Cys 186 (Gutierrez et al., 1989). The last two reactions seem to occur on the intracellular membrane and are important for efficient localisation of Ras to the intracellular membrane (Hancock et al., 1991; Stephenson and Clarke, 1990). However, farnesylation, cleavage and carboxymethylation are not alone sufficient to localise Ras to the intracellular membrane but efficient localisation requires the reversible palmitoylation of residues Cys181 and Cys184 (Hancock et al., 1989). Interestingly, Ki-Ras-2B lacks these cysteine residues but a stretch of extremely basic region comprising

six lysine residues (177-182) seems to be essential for intracellular membrane localisation (Hancock et al., 1990). Thus, it is likely that the hypervariable region of all the Ras polypeptides confers different signals for intracellular membrane localisation that could potentially target different Ras proteins (H-Ras, Ki-Ras A or B and N-Ras) to specific sites in the membrane where they fulfil different requirements and functions for the cell. Indeed, it has only recently been shown that while oncogenic Ki-Ras blocked integrin β_1 chain maturation and up-regulated CEA expression which disrupts basolateral polarity in colon epithelial cells, oncogenic H-Ras lacked such effects (Yan et al., 1997; Yan et al., 1997). Localisation of Ras proteins to the inner surface of the plasma membrane is important for their function since mutating Cys 186 (the farnesylated residue) or Cys 181 and 184 (the palmitoylated residues) or inhibiting farnesylation reduces the cell transformation capabilities of oncogenic Ras (Hancock et al., 1990).

The p21 proteins encoded by N-, Ki- and H-*ras* are small guanine nucleotide-binding proteins that act as switches in several signal transduction pathways (Figure 5). The GTP-bound protein activates the effector pathways while the GDP-bound form does not (Gibbs et al., 1984). The protein's GTPase activity returns it to the inactive state. The GTPase reaction is a hydrolytic one. In the wild-type Ras-GTP crystal structure a water molecule is seen in just the right position to catalyse the reaction (Pai et al., 1990). This water is correctly oriented by hydrogen bonding to the carboxy side chain of Gln-61. The oncogenic position 12 mutants may exert their major effect by the side chain sterically preventing correct access of this water molecule to the active site, or by the mutation causing a rearrangement of key P-loop residues (Maegley et al., 1996). In either case, the oncogenic position 12 mutants have a lowered GTPase

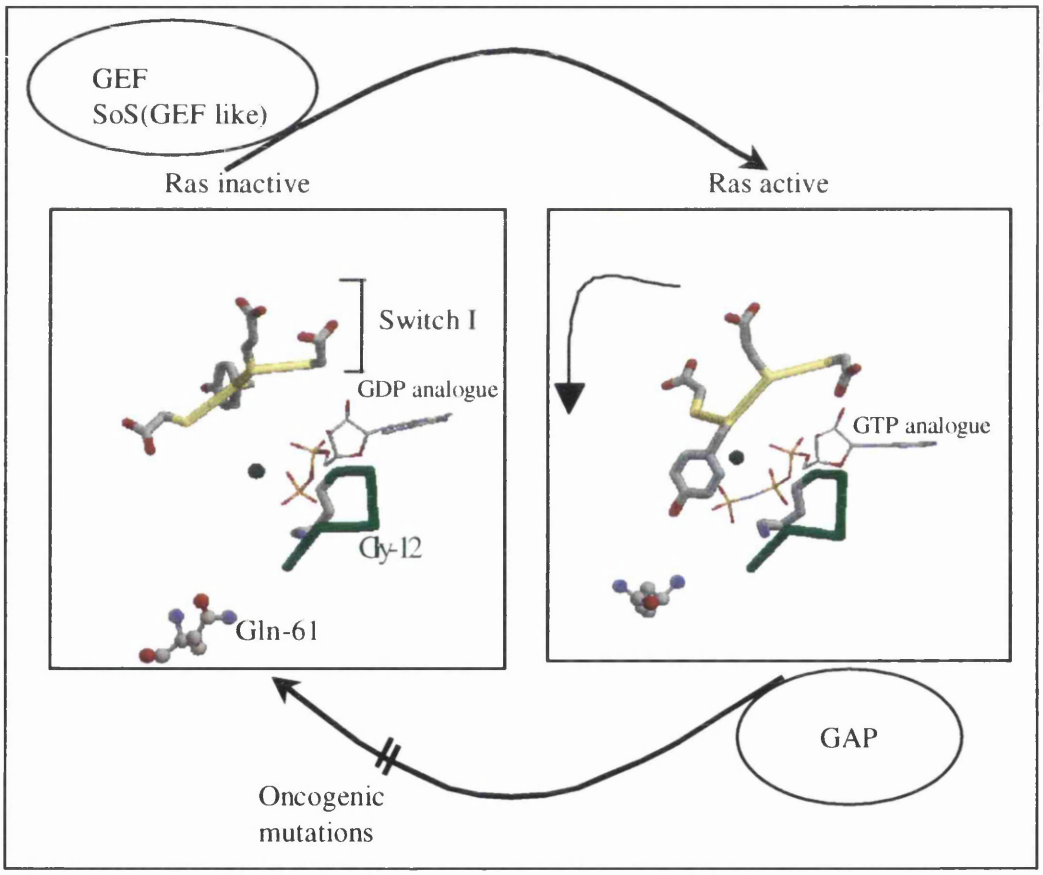
activity, and, perhaps more strikingly, a total absence of ability to be stimulated by GAP.

The three-dimensional structure of two GAP domain- G-protein - nucleoside diphosphate - aluminium fluoride complexes have been determined (Scheffzek et al., 1997). In both, the GAP domain binds directly on top of the nucleotide and also overlaps the effector site. The Switch II region becomes rigid. A conserved arginine protrudes into the nucleotide-binding site, its carbonyl group makes a hydrogen bond with the carboxylate group of glutamine-61 and its guanidino group is near to the γ-phosphoryl group such that it has a key role in GTPase catalysis. The complex also reveals that Gly-12 contacts part of GAP closely so that there is little space for a side chain, and the authors argue this is the reason the GTPase activity of most Gly-12 mutants is insensitive to stimulation by GAP.

Figure 5: *Conformational changes in key residues of the Ras protein on GTP binding*

The switch I region bearing the Tyrosine-32 residue is coloured yellow. Glycine-12 (part of the P-loop) is coloured green and the water molecule is denoted by a green ball. Glutamine-61 (part of switch II) is shown in balls and sticks.

The thin arrow indicates the extent and the direction of the conformational change of the switch I region of Ras after its activation by GTP binding. On binding to GTP exchange factors (GEF) or Sons of Sevens (SoS), GDP is exchanged for GTP and Ras is activated by the subsequent conformational change. The GTPase activating protein (GAP) stimulates the GTPase activity of Ras and the GTP is hydrolysed to GDP. This causes the switch I region to "flip" back to the inactive conformation.



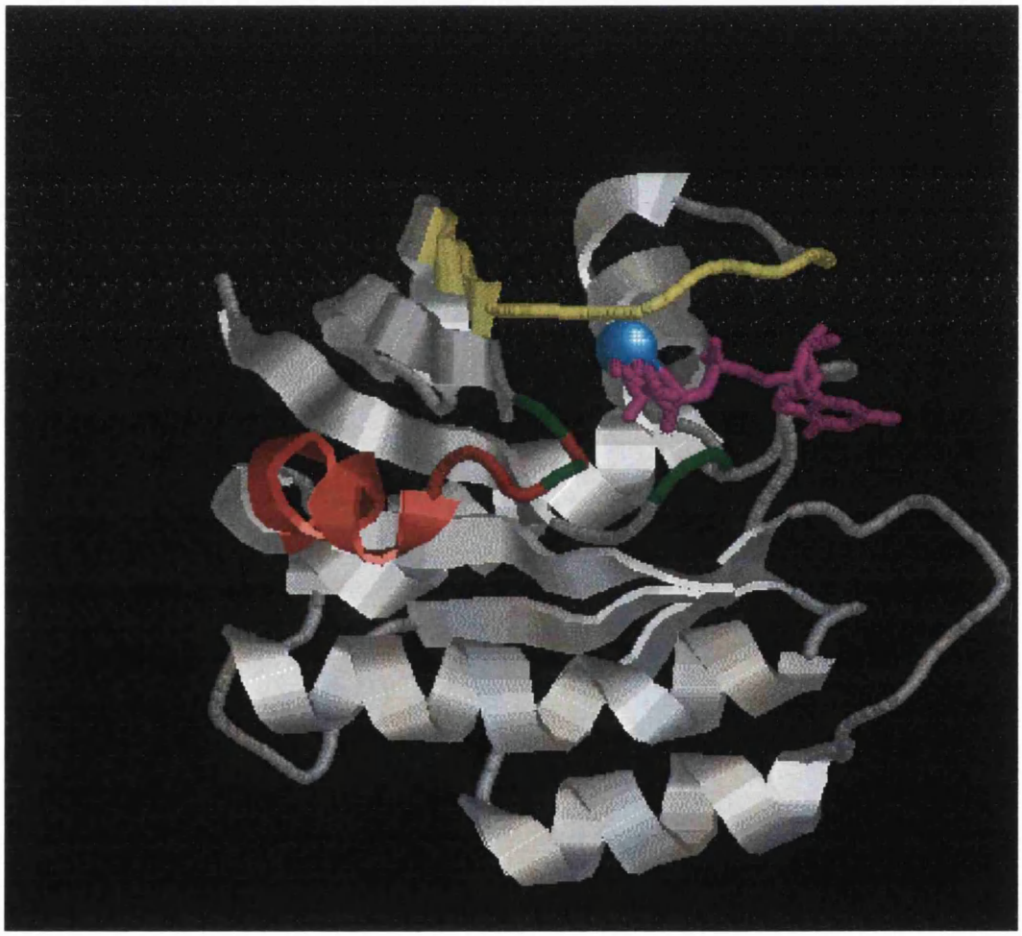
3.3. Structure of Ras protein

Much is known about the tertiary structures of Ras protein molecules (Sprang, 1997). The nucleotide sits in a crevice at the surface and three regions closely surrounding the di- or tri-phosphate part can be distinguished: the P-loop, which binds the triphosphates and Switch I and Switch II, which are the regions that change conformation between the GTP- and GDP-bound forms (Figure 6). The P-loop (residues to 10-17) contains the GxxxxGKS/T sequence motif, which has the same 3D structure in a wide variety of proteins including GTPases, ATPases and kinases. Functionally it can be regarded as a triphosphate-binding site because several amide groups lie together in an anion hole (Dreusicke and Schulz, 1986) or nest (Sayle and Milner-White, 1995) that neatly accommodates the triphosphate by multiple hydrogen bonding to the oxygen atoms. In Ras the sequence is GxGxxGKS and it is the second glycine (residue 12) which is so oncogenic.

The effector region, also called Switch I, comprising residues 30 to 40 which are mostly exposed at the surface, is found by genetic analysis to be the most important in determining the transducing effect of Ras. It shows differences in conformation between the GDP and GTP-bound forms of the protein (Figure 5). The nature of the effector proteins for Ras was controversial, but it is now clear that Raf is a major effector protein (Moodie et al., 1993; Wittinghofer and Nassar, 1996). However, other proteins, including GAP (Trahey and McCormick, 1987; Trahey et al., 1988), neurofibromin (Bollag and McCormick, 1991), RalGDS (White et al., 1996) and PI-3K (Rodriguez-Viciana et al., 1997) bind to this region of Ras and may also act as effectors (Katz

Figure 6: *3-Dimensional-cartoon structure of the Ras protein in the active conformation*

The switch I effector region (residues 30-40) is coloured yellow. Glycine-12 and aspartate-13 (the frequently mutated residues) are coloured green and the switch II region is coloured red (residues 61 and 59 within switch II, which are also hot-spots for mutation) are coloured green. The GTP analogue is coloured magenta and the water molecule is coloured blue.



and McCormick, 1997). Although the area to which they bind overlaps the Raf effector region, their binding sites on Ras are distinct (Winkler et al., 1997).

The switch II region (residues 60 to 70) contains the Gln-61 residue that is of interest because its amide side chain has been found to be necessary for the catalysis of GTP hydrolysis (Pai et al., 1990). The region exhibits relatively large conformational changes but, because its electron density has a high temperature factor, with the exception of that of Asp-12 Ras, it is found to be mobile and there can be little reliability about the apparent conformational changes that can be seen. The switch II region in Asp-12 mutants is different in that it displays a more fixed conformation than those in wild-type or other mutant Ras molecules (Franken et al., 1993). The switch II has also been shown to be important in binding of Raf-1 cysteine-rich domain (residues 139-184). Mutation in switch I (T35A and E37G) and switch II of Ras (G60A and Y64W) diminished binding of Ras to Ras binding domain of Raf-1 (residues 55-131) or the Raf-1 cysteine-rich domain (139-184) respectively (Drugan et al., 1996). Both of these interactions of Ras with the two domains of Raf-1 are necessary for transformation by Ras and for activation of Raf-1 (Drugan et al., 1996; Hu et al., 1995).

*3.4. **Ras the oncogenic protein***

There is compelling evidence that mutations in codons 12, 13 or 61 of the *ras* genes render them oncogenic. These mutations are frequently found in human neoplasms, especially in adenocarcinomas of colon (30-50%), lung (30%) and pancreas (90%), with mutations in codon 12 being by far the most frequent (Bos, 1989). This finding led to the discovery that Ras mediates a wide variety of signal transduction from the cell surface receptor tyrosine kinases. Upon receptor activation by ligand, receptor tyrosine kinases, such as EGFR, PDGFR, IGFR and heterotrimeric G proteins-coupled receptors, SoS (ubiquitous GEF) is recruited to the plasma membrane where it catalyse the exchange of GDP for GTP on Ras. The association of SoS with the receptor tyrosine kinases is mediated by (PH) a membrane attachment domain of SoS and the SH2- and SH3-domain-containing adaptor protein Grb2 (Byrne et al., 1996). In other types of receptors, such as NGFR, SoS and Grb2 are associated with these receptors via tyrosine phosphorylated Shc proteins. Serpentine receptors activate Ras by releasing the βγ-subunit of the heterotrimeric αβγ-G protein complexes (van Biesen et al., 1995). The βγ-subunit activates PI-3 kinase γ, which by ill-defined pathway involving Src or a Src-like tyrosine kinase, induces the tyrosine phosphorylation of Shc and subsequently SoS-mediated activation of Ras (Lopez-Ilasaca et al., 1997).

Ras activation leads to activation of multiple signalling pathways (Figure 7). However, the mechanisms of activation as well as the direct effects of activation of the downstream pathways are not understood in most cases. Nevertheless, Ras activation in immortalised cells results in proliferation (entry

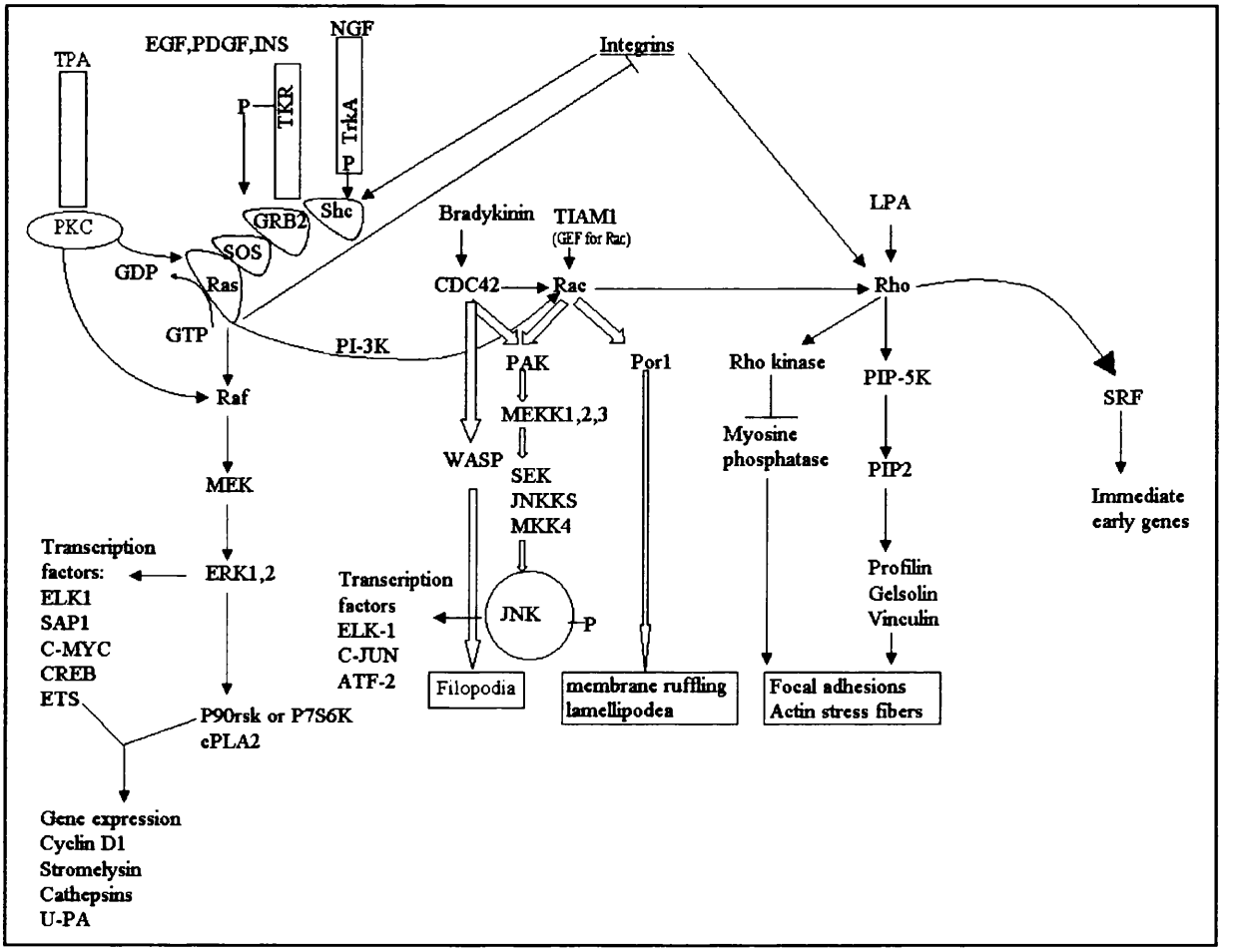
into S phase of the cell cycle) via the activation of cyclin D1 through Ras/Raf/MAPK pathway (Aktas et al., 1997; Mittnacht et al., 1997; Peeper et al., 1997), differentiation and cellular transformation.

3.4.1. Role of Ras in the cell cycle

It has been known for a long time that Ras alone is unable to transform normal primary cells but requires the presence of cooperating oncogenes such as C-*myc*, N-*myc*, *cyclin D1,* adenovirus E1A, polyoma large T or the loss of specific tumour suppressor genes such as *p53*, *p16* and *p21* (Weinberg, 1989). Interestingly, the reasons for requirement of cooperating oncogenes in Ras transformation has only recently been elucidated where it has been shown that in primary cells (cells with normal DNA), transfection of activated *ras* gene resulted in cellular growth arrest and senescence (Ridley et al., 1988; Serrano et al., 1997). Cellular senescence in valine-12 Ras transfected cells has been recently shown to be a consequence of increased intracellular H_2O_2 (Lee et al., 1999). The authors have demonstrated that the ability of Ras proteins to increase H_2O_2 levels was essential for Ras induced senescence because valine-12 Ras-expressing cells grown in the presence of either a peroxidase-scavenging antioxidant or low oxygen were rescued from senescence. On the other hand, inhibition of cell growth has been shown to be a consequence of activation of the Ras/Raf/MAPK pathway that leads to the induction of the universal cell cycle kinase inhibitor (CDKI) $p21^{WAF1/Cip1}$ in a p53-dependent manner (Lloyd et al., 1997). Similarly, in another study, the inhibition of cell growth by Ras was shown to be associated

Figure 7: *A schematic presentation of Ras protein signal transduction pathways*

Ras activation of the MAPK pathway is denoted by red arrows and the activation of PI-3K/Rac/Rho pathway is illustrated by green arrows. See throughout Chapter 3 for details.

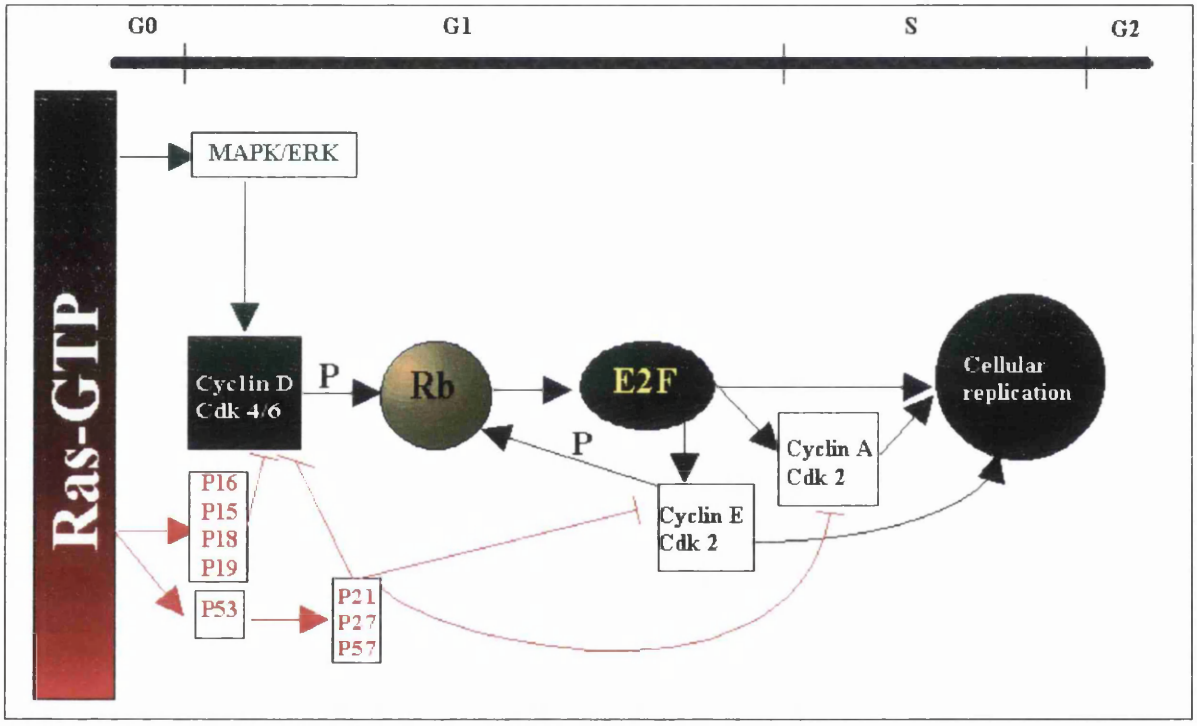


with induction of $p21^{WAF1/Cip1}$ and $p16^{INK4a}$, which binds and inhibits kinases associated with cyclin D1 (Serrano et al., 1997). Recently, oncogenic Ras was shown to induce the expression of p19 ^{ARF} in MEF mouse cells (Palmero et al., 1998). $p19^{ARF}$ (the human homologue is $p14^{ARF}$) can activate p53 in a DNA-damage-independent manner (Kamijo et al., 1998; Kamijo et al., 1997; Pomerantz et al., 1998; Zhang et al., 1998). Taken together, these data indicate that oncogenic Ras elicits an anti-tumour response mediated by the upregulation of $p16^{INK4A}$ and $p19^{ARF}$, which in turn activate the tumour suppressors retinoblastoma and p53 respectively (Figure 8). Homozygous deletion of the *INK4a-ARF,* located on human chromosome 9p (Haber, 1997), is common in human tumours (Hall and Peters, 1996). The loss of these and other tumour suppressor genes could render tumour cells unprotected against the action of oncogenes. Thus, during the process of immortalisation, the presence of cooperating oncogenes or the deletions of suppressor genes render the overall signal from Ras proteins one that is associated with cell growth and proliferation. Indeed, NIH3T3 fibroblasts, a cell line in which many early transformation assays were performed are known to have deleted their *p16* gene (Quelle et al., 1995). Similarly, Rat-1 cells have reduced expression of p21 protein (Perez-Roger et al., 1997). The Myc protein is a classical cooperating partner of Ras. Recently, the collaborative interaction between Myc and Ras has been spiked by the finding that Ras enhances the accumulation of Myc activity via the Ras/Raf/MAPK pathway by preventing its degradation (Sears et al., 1999). In transformation assays, Myc has been shown to be able to overcome a p53-dependent growth arrest by sequestering p21 (Hermeking et al., 1995), likewise, Myc is able to antagonise p16-induced cell cycle arrest (Alevizopoulos et al., 1997; Serrano et al., 1995).

Figure 8: *Consequences of Ras activation on the cell cycle*

Activated Ras is thought to induce the production of cyclin D1 via the MAPK pathway (green arrows).
In normal cells, this induction is seen on release from G0 and more strongly in mid G1.

Oncogenic Ras or Raf may also produce such effects on cells. However, constitutively activated Ras or Raf blocks cell cycle progression via the induction of CDK inhibitors (red pathways). This could represent a safety mechanism, which blocks inapropriately activated Ras. Deletions of retinoblastoma (Rb) and/or the CDK inhibitors would favour a positive effect of Ras on cell cycle progression. P, indicates phosphorylation.



Human papilloma E6, E1A and SV40LT viral oncogenes cooperate with Ras in transformation assays by virtue of their ability to block p53 function, or in the case of human papilloma E7, SV40LT and E1A block p16 by virtue of binding to Rb or block p21 function as seen with E7 viral oncoprotein (Funk et al., 1997; Vousden, 1995). Interestingly, other oncogenes such as Cyclin D1 and cdc25 A and B have been shown to cooperate positively with Ras on the cell cycle and it could be that the overexpression of these proteins may counteract CDKIs (Lovec et al., 1994).

It is now becoming apparent that Ras activates at least two signal transduction pathways. The Ras/Raf/MAPK pathway that regulates gene expression (Thorburn et al., 1994) and the other Ras/PI-3K/Rac/Rho pathway controls actin cytoskeleton organisation (Bar-Sagi and Feramisco, 1986; Ridley, 1994) (Figure 7). Clearly, these two pathway can be dissociated since H-Ras V12C40 mutants could induce membrane ruffling and JNK1 activity but failed to bind Raf-1 or induce MAPK activation, while H-Ras V12S35 mutants were able to bind Raf-1 and induce MAPK activation but failed to stimulate membrane ruffling. However, both mutants were defective in stimulating DNA synthesis but expression of both mutants resulted in stimulation of DNA synthesis that was comparable to Ras valine-12 mutants (Joneson et al., 1996). Similarly, other studies have shown that while Ras V12S35 and V12G37 mutants were defective in transforming fibroblasts in culture, co-transfection of cells with both mutants resulted in an increase in focus formation approximating that obtained using Ras valine-12 mutants (White et al., 1995). Moreover, V12G37 mutant Ras can cooperate with V12C40 Ras in transformation assays. Neither Ras mutant binds or activates Raf-1, indicating that Ras activated Raf-independent signalling pathway

is important in cellular transformation (Khosravi-Far et al., 1996). These results argue strongly that different Ras effector pathways have to interact to produce full transformation.

3.4.2. Role of Ras in apoptosis

Apoptosis is a morphologically distinct process of cell death (Kerr et al., 1972). It is now well recognised that tumour growth is a consequence of disturbed balance between cellular proliferation and cellular death (Kerr et al., 1994).

As with the effects of Ras on the cell cycle, Ras has been shown to have similar double-effects on apoptosis. Several studies have shown that Ras can induce cellular apoptosis under stressful conditions (Kauffmann-Zeh et al., 1997; Vater et al., 1996).

The mechanism by which Ras promotes cellular apoptosis is not well defined. However, it has been found that the Raf/MAPK pathway is involved in this pro-apoptotic effect of Ras (Kauffmann-Zeh et al., 1997). As discussed in section 3.4.1, the ability of Ras to induce apoptosis is tied closely to its ability to induce the production of $p16^{INK}$ and $p21^{WAF1}$ via p53 that are important in cellular senescence. Loss of p53 has been shown by several reports to abrogate the induction of apoptosis in activated Ras-expressing fibroblasts (Fukasawa et al., 1997; Nikiforov et al., 1996). On the other hand, Ras has also been shown to protect cells from apoptosis. In colorectal cancer, activated Ki-*ras* is associated with tumours that have lower apoptotic counts (Ward et al., 1997). It has recently been shown, by using the effector specific mutants of Ras, that activation of the PI-3K effector arm of Ras significantly reduces the apoptosis of Rat-1 cells (Kauffmann-Zeh et al., 1997). PI-3K is known to activate PKB/Akt and this

pathway has been found to be activated by Ras in a variety of cell lines (Marte and Downward, 1997; Marte et al., 1997; Yao and Cooper, 1995). The way PKB/Akt inhibits apoptosis is not well understood but could involve the phosphorylation and the inhibition of BAD (a known inhibitor of Bcl-2) (Datta et al., 1997; Zha et al., 1996).

Ras has also been shown to activate the transcription factor NF-κB, which has anti apoptotic properties. Inhibition of NF-κB (by using a super-repressor form of the inhibitor of NF-κB (IκB), which cannot be phosphorylated or degraded) has been shown to induce significant apoptosis in Ras transformed fibroblasts in a p53-independent manner (Mayo et al., 1997). Thus, activated Ras can have both apoptotic and anti-apoptotic effects on cells. In general, the anti-apoptotic Ras/MAP kinase is dominant over the pro-apoptotic pathway (Kauffmann-Zeh et al., 1997). However, the final effects on cells depend on a fine balance between the two effectors of Ras (Raf and PI-3K). In tumour cells, activating Ras could shift the balance towards anti-apoptosis since p53 and p16 are frequently altered. Similarly, different cell types will respond differently to activation of Ras depending on which arm of the Ras effector pathways is altered.

3.4.3. Role of Ras in genomic instability

An emerging theme arising from studies comparing DNA content between adenomas and carcinomas is that carcinomas are frequently more aneuploid than adenomas. Although small adenomas are more likely to be deploid, large displastic adenomas and adenomas containing foci of carcinomas are frequently found to be polyploid (Suzuki et al., 1995). Since mutated Ki-*ras* gene has been associated with the transition of early adenomas to displastic adenomas

(Vogelstein et al., 1988) and that activated *ras* genes are frequently found in aggressive and heteroploid tumours, it is tempting to speculate whether activated *ras* could be associated with genetic instability. Recent literature has indeed shown such an association to be possible. For example, expression of activated human H-*ras* oncogene in NIH3T3 cells induced rapid production of chromosomal aberrations ranging from multicentric, acentric, double minute chromosomes and gene amplifications to induction of heteroploidy. These effects have been shown to be induced by activated *ras* since they were repressed by a dominant negative effector of Ras, Raf 301 (Denko et al., 1995). Similarly, other studies, using a variety of cell lines, have shown a direct association between activated *ras* and genomic instability (de Vries et al., 1993; Giaretti, 1997; Ichikawa et al., 1990). Notably, the induction of chromosomal aberrations by activated *ras* has been shown to be rapid and occurred during a single cell cycle (Denko et al., 1994). It should be noted, however, that the cell line used to study the effects of activated *ras* on genomic instability contained other genetic defects such as mutated p53 that are important in permitting such *ras*-effects on the genome. Interestingly, it has been shown, although in a small number of adenomas, that codon-12 G→ C and G → T tranversion mutations of Ki-*ras* oncogene were more significantly associated with DNA aneuploidy than the more common G → A transition mutations (Giaretti et al., 1995).

Since metastasis is a multi-step process requiring the functions or de-functions of many genes, it has been a scientific dogma that a single gene, such as activated *ras*, could activate such a complex process. Thus, the ability of activated *ras* to induce metastatic behaviour on cells could be ascribed partly to its ability to destabilise the genome in addition to its ability to activate many down-

stream signals discussed below. However, it should be noted that fibroblasts transformed with activated H-*ras* oncogene, although were induced to metastasise, they remained diploid (Muschel et al., 1986). How do activated Ras proteins induce genomic instability is not well understood. However, the ability of Ras to induce reactive oxygen species production in cells lacking a functional senescence program or possessing a defective DNA repair machinery could result in oxidative DNA damage and genomic instability.

3.5. The role of Ras in the metastatic process

It has been known for a long time that introducing activated *ras* genes in immortalised cells render them more invasive *in vitro* and metastatic in nude mice (Chambers and Tuck, 1993). In this section, I will discuss some of the ways by which activated Ras proteins could induce cellular motility, invasion and metastasis.

3.5.1. Ras and cell adhesion molecules

As discussed in section 2.2, alterations of cellular adhesion can have profound effects on cellular behaviour. Transformation of cells with the small GTPase Ras results in increased tyrosine phosphorylation of β-catenin and decreased amount of β-catenin in the E-cadherin-β-catenin complex (Kinch et al., 1995) that could explain the disruption of cadherin mediated cell adhesions in v-*ras* transfected cells. Also, Ras and APC proteins seem to act synergistically in transforming cultured murine colon epithelial cells (D'Abaco et al., 1996). Recently, phosphorylation of Akt by the Ras/PI-3K pathway has been shown to phosphorylate and inactivate glycogen synthase kinase 3, which may disrupt the degradation pathway of free β-catenin in cells (Sutherland et al., 1993). This is significant because excess β-catenin has been shown to play a role in tumorigenesis due to its activity in transcription of genes (Rubinfeld et al., 1997; Tetsu and McCormick 1999).

Activated Ras has been shown to disrupt the interaction of AF-6 with ZO-1 by binding to AF-6 (Han and Colicelli, 1995; Yamamoto et al., 1997). AF-6 and

ZO-1 proteins co-localize and accumulate at tight junctions in epithelial cells and at cell-cell junctions in non-epithelial cells and are important in cell-cell adhesions (Itoh et al., 1993; Yamamoto et al., 1997). In addition, activated Ki-Ras protein has been shown to increase CEA expression on epithelial cells *in vitro* resulting in disruption of epithelial cell polarity (Yan et al., 1997). Such disruption of cell-cell adhesions is necessary during mitosis. However, the disruption of epithelial cell-cell junction by constitutively activated Ras could represent an important step in epithelial to mesenchymal cellular transformation.

Ras has been shown to affect integrins in at least two ways. Plantefarber and Hynes (1989) have shown that rat cells transformed with *ras* or Rous sarcoma virus expressed decreased amount of β_1 integrin. In addition, Yan et al. (1997) have shown that activated Ki-Ras reduced the maturation of β_1 integrin in colon epithelial cells. Recently, H-Ras has been shown to inactivate integrin binding to the extracellular matrix (Hughes et al., 1997). The discovery that oncogenic H-Ras can reduce integrin affinity suggests a possible connection between reduced affinity and a more motile and invasive phenotype in cancer cells. Indeed, DeMilla et al. (1993) have shown using smooth muscle cell migration on fibronectin matrix that the optimal cellular migration occurred at an intermediate attachment strength. Both low and high levels of cell adhesion on fibronectin inhibited cell migration.

3.5.2. Ras in cellular motility and invasion

It has been known for some time that *ras*-transformed cells undergo significant morphological changes such as loss of stress fibers, decreased matrix adhesions, increased membrane ruffling and motility (Bar-Sagi and Feramisco,

1986). Although the precise mechanisms of these changes are not well understood, they appear to involve the Rho family members of proteins. For example, activated valine-12 mutant of H-Ras induction of membrane ruffling was blocked by dominant negative Rac1 and bacterial C3-transferase, which inactivates Rho (Ridley and Hall, 1992). In addition, Rac1, RhoA and Cdc42 have been shown to promote cellular motility and invasion. Notably, Rac has been shown to be directly responsible for forming membrane ruffling and lamellipodia and Cdc42 can control the formation of filipodia (Hall, 1998; Hall, 1994). These observations established that the ability of activated Ras to cause membrane ruffling and motility could be ascribed to its ability to stimulate the Rac-Rho cascade (Figure 7).

Genes of matrix degrading enzymes are induced by Ras. Several such genes contain regulatory sequences that are activated by Ras through binding of FOS/JUN (AP-1) family and the PEA3/Ets family of transcription factors. For example, Ras induce the expression of stromelysin-1 and collagenase via the AP-1 elements (Matrisian et al., 1991; Schonthal et al., 1988), MMP-9 through NF-κB, SP-1, AP-1 and ETS (Gum et al., 1996; Himelstein et al., 1997), uPa via PEA3/AP-1 (ERK and JNK) and Cathepsin L through the ERK pathway activation (Silberman et al., 1997). The increased invasive and metastatic abilities of tumour cells harbouring activated Ras proteins could be in part be due to the increased expression of these matrix degrading enzymes, which have been shown to be expressed in aggressive tumours *in vivo* and *in vitro*.

Ras is also responsible for transmitting signals from growth and motility factors that induce invasion and metastasis. For example, recently, using signalling mutants of c-Met, it has been shown that coupling of the receptor to the

Ras pathway is both essential and sufficient for proliferation, whereas, activation of PI-3K alone is sufficient to induce motility (Ponzetto et al., 1996). Notably, the activation of Ras and PI-3K by the receptor is both necessary and sufficient to induce invasion and metastasis (Giordano et al., 1997). In addition, migration of keratinocytes stimulated by HGF is inhibited by treatment of bacterial C3-transferase, which inactivates Rho (Takaishi et al., 1994). These results argue for an essential role of the GTPase super family in mediating the motility signals of HGF-Met to the cytoskeleton of cells (Hartmann et al., 1994). Also, murine NIH 3T3 and C127 cells transformed by oncogenic Ras have been shown to over express the c-Met receptor and display increased invasive properties in response to HGF (Webb et al., 1998). Taken together, these results show that Ras can activates and induces many signals necessary for cellular invasion and metastasis.

3.5.3. Ras and angiogenesis

Angiogenesis, as discussed in section (2.6), is a critical biological process for tumour growth. It is now well established that activated Ras induces or upregulates the production of various pro-angiogenic factors such as VEGF (Bouck et al., 1996). Indeed, cells containing the activated Ras has been shown to induce significantly larger number and sized angiogenic vessels when grafted in mice compared to cells infected with other retroviral constructs (Thompson et al., 1989). Moreover, Grunstein et al. (1999), by using VEGF null fibroblasts (in which VEGF can be conditionally deleted), have shown VEGF expression to be critical for ras-mediated tumourigenesis. Similarly, knocking-out *ras* in human colorectal cancer cell lines by homologous recombination (these cells contain a single mutated Ki-*ras* allele) suppressed VEGF production and reduced the ability

of these cells to form tumours in nude mice (Rak et al., 1995). The authors argued that reduced VEGF production in these cells was partly to blame for their reduced tumuorigenicity since suppression of VEGF by antisense in the parental cells (containing activated Ras) reduced their tumourigenicity and also because the growth of *ras* knock out cells *in vitro* was unaffected (Okada et al., 1998). Although, subsequent work, by the same authors demonstrated a weak but detectable restoration of tumourigenicity when *ras* knock-out cells were transfected with one of the VEGF isoforms (VEGF 121). The inability to fully restore tumourigenicity by VEGF 121 made the authors conclude that VEGF up-regulation by activated Ras was necessary but not sufficient for tumourigenicity (Okada et al., 1998). This is not surprising because activated Ras is required for induction of other molecules necessary for tumourigenesis. Although it can also be argued that restoration of only one of the VEGF isoforms and not the others is a major flaw in this work since the other isoforms could be necessary in fully inducing angiogenesis by activated Ras.

The mechanisms by which activated Ras induces VEGF production are not fully understood. Although the *VEGF* gene is known to contain AP-1 sites for binding of FOS/JUN dimers (Tischer et al., 1991), Ras has been recently shown to induce VEGF production via the PI-3K pathway. For example, the inhibition of PI-3K by Wortmannin reduced VEGF production, angiogenesis and tumour volumes of SVR cells in nude mice (Arbiser et al., 1997). The authors also demonstrated that Ras induced angiogenesis by up-regulating the matrix metalloproteases and down regulating their inhibitors. As discussed in section (2.5), matrix metalloproteases are important in the process of angiogenesis and the ability of activated Ras to up-regulate MMP-2 and MMP-9 and down regulate

TIMP could be an additional mechanism by which Ras induces angiogenesis (Arbiser et al., 1997).

CHAPTER 4. Tumour heterogeneity

4.1. Genetic diversity in solid tumours

Tumours arise from single cells. This statement infers that tumours are monoclonal in origin. Numerous cancer biology researchers using a variety of experimental procedures demonstrated that tumour growth represents clonal expansion from a single aberrant cell (Fialkow, 1976; Nowell, 1976; Nowell, 1986). One approach, utilised X-linked RFLP to examine the pattern of X chromosome inactivation in colorectal tumours of females and showed that in carcinomas and adenomas the same member of the X chromosome pair is functional in all cells of a given tumour thus demonstrating the monoclonal patterns of X chromosome inactivation (Fearon et al., 1987). It is of course possible that tumours may develop independently from a number of transformed cells but that only one of these will ultimately survive or overgrow to give rise to a clinically detectable tumour. Another obvious problem in correlating the pattern of X chromosome inactivation with tumour clonality is the fact that methylation pattern of DNA can be abnormal in malignancy. A fact that is frequently ignored in this field of study. Thus, the use of X chromosome inactivation by itself may not be a valid indicator of tumour clonality. For example, using tissue from a Y chromosome mosaic individual (XO/XY) and Y-chromosome probes in a *in situ* hybridisation experiment, Novelli et al. (1996) have shown that in this patient, while the crypts of the small and large intestine were clonal, at least 76 percent of the microadenomas were polyclonal in origin. Although this study is based on a single and a rare individual, it raises considerable doubt on the clonal origin of tumours. With this notion aside, it is now generally accepted that single-cell origin

does not rule out subsequent variation in cellular composition as tumours progress (Dexter and Calabresi, 1982; Mitelman, 1971). Tumour heterogeneity is now a well-accepted phenomenon in cancer biology. Although it has been reported as far as the 1950's.

The term heterogeneity requires a careful definition. Tumours are architecturally complex, differing regionally in differentiation status, growth potential, cell cycle phase, vasculature, immune-cells infiltrate, connective tissue compositions and other characteristics that can influence identical tumour cells to a different extent. However, the types of variability usually meant by the term heterogeneity are differences in the genetic composition of cells within a single neoplasm. This definition is intended to discriminate between variability that arises from genetic, as distinguished from non-genetic processes because this usage allows a more focused experimental analysis of at least one type of neoplastic variability.

The development of cellular heterogeneity is a prime manifestation of tumour progression. This in turn is a consequence of the stepwise emergence of subclones, a few of which possess selective growth advantage. This dynamic and Darwinian evolutionary process is driven by random or induced genetic or epigenetic factors and host selection pressures.

Another prediction of the tumour progression hypothesis is that metastatic cells arise stochastically during primary tumour growth (Nowell, 1976). Fidler et al. (1973) have shown earlier that re-injecting cells established from lung metastases enhanced their metastatic potential (Fidler, 1973). Also, by deriving single-cell clones from a parent culture of murine malignant melanoma cells Fidler and Kripke (1977) have shown that these clones varied in their ability to

produce metastatic colonies in the lungs upon i.v. inoculation into syngeneic mice. Fidler's work, therefore, suggests that a malignant tumour is heterogenous and that highly metastatic cell populations preexist in parental tumours. However, it should be noted that other investigators were unable to confirm the pre-existence of subpopulations of metastatic cells in primary tumours (Milas et al., 1983; Weiss, 1980).

There is numerous evidence in the literature supporting the existence of heterogeneity within tumours (Dexter and Calabresi, 1982; Dexter et al., 1978; Shapiro et al., 1981). However, most of the evidence is circumstantial and based on isolating different subclones from the same tumour in culture and thus could be artificially induced. If tumour heterogeneity exists, it should be directly evident in solid tumour *in situ*. Recently, microdissection techniques have shed more light on the extent of intratumour heterogeneity. For example, Nagel et al. (1995) studied 20 gastrointestinal cancers obtained from female patients and found that while these tumours were clonal in origin based in the patterns of X chromosome inactivation, a significant number of tumours had LOH in mini and microsatellite in restricted areas of single tumours and not others. Other researchers have shown similar evidence of intratumour heterogeneity using a variety of techniques (Dracopoli et al., 1985; Giaretti et al., 1996; Heim et al., 1997; Hiddemann et al., 1986; Koha et al., 1992; Kuwabara et al., 1998; Simpson et al., 1996). On the other hand, recent work using LOH on 5q, 18q and 17p from colorectal tumours demonstrated pronounced allelic heterogeneity in high-grade dysplasia and in carcinomas within the high-grade dysplastic adenomas, while allelic losses in carcinomas were uniform (Boland et al., 1995). Similarly, in breast cancer and its precursor, ductal carcinoma *in situ* (DCIS), multiple microsatellite markers for

allelic loss in multiple separate microdissected regions of more than 40 cases were evaluated (Fujii et al., 1996). Different microdissected foci of intraductal carcinoma in the same sample often differed from one another in their LOH pattern, while individual carcinomas exhibited much less intratumour diversity than their preinvasive stages. This clonal convergence seems in line with earlier reports form Kerbel et al. where it has been shown using a variety of genetic tagging methodologies that spontaneous metastases developed in a non-random fashion from genotypically distinct cell clones and that the progeny of a single metastatic clone could eventually overgrow the primary tumour (Bell et al., 1991; Kerbel, 1990; Korczak et al., 1988). Although many other researchers confirmed these findings (De Both et al., 1997; Price et al., 1990; Waghorne et al., 1988), work by Moffett et al. (1992) using identical techniques have shown that when a genetically tagged heterogenous cell population is injected in mice the resulting primary tumours and their metastases remained polyclonal and only in few cases there was evidence of clonal dominance of the primary tumours by a single metastatic clone. Nevertheless, comparing primary tumours and their metastases could shed more light on the clonal dominance theory put forwards by Kerbel et al. The work of Boland et al. (1995) and Fujii at al. (1996) give further support for the prediction made from the tumour progression hypothesis in that tumours may be subclonally highly heterogenous during early stages of tumour growth but relatively homogenous during advanced stages of growth as the progeny of one or few aggressive subclones dominate a tumour.

The presence of heterogeneity in tumours seems to be in conflict with the clonal dominance hypothesis. However, the concept of clonal dominance is not incompatible with the concept of cellular heterogeneity of tumours, since a

dominant clone can be homogenous for one marker and heterogenous for other markers. For example, while a specific clonally dominant subclone of the mouse mammary carcinoma SP1/B5 was found to be homogenous for the plasmid-based genetic marker used in tagging the parent cell line, the subclone was in fact karyotipically heterogenous (Bell et al., 1991).

An important concept that emerges from the clonal dominance theory is that results of any comparison between the primary tumours and their metastases could be profoundly affected by the stage at which the primary tumour is removed and analysed. Thus, genetic analysis of early primary tumours (containing a very small proportion of metastatic cells) with their metachronous metastases (removed years after the primary tumour) could yield erroneous results on the clonal relationship between the two tumours. Indeed, recent work comparing the genetic progression of primary breast cancer with their metachronous metastases using CGH showed that primary tumours, in some significant number of cases, to share no genetic relationship to their metastases (Kuukasjarvi et al., 1997). On the other hand, genetic comparisons between advanced primary tumours and their synchronous metastases would, as expected, show closely related genetic changes between the two lesions. Similarly, orthotopic implantation of advanced colon cancer (Dukes' D) was shown to be capable of metastasis if injected subcutaneously in nude mice, whereas earlier staged (Dukes' B) cancer was comparatively deficient (Giavazzi et al., 1986; Morikawa et al., 1988; Morikawa et al., 1988; Naito et al., 1986). Taken together, the data suggest that the genetic composition of tumours could be different at different stages of their progression and that metastasis depends on the intrinsic metastatic capability of primary tumours. Therefore, results that compare primary tumours with their earlier or late

metastatic lesions or with other seemingly similar staged tumours or even within each tumour should be interpreted with caution.

Thus, the need to develop comparable staging systems is crucial if we are to understand the role and extent of cellular heterogeneity in tumour progression. Indeed, recently a report from the Kananaskis, Alberta, Canada working group on quantitative methods in tumour heterogeneity, emphasised the need for quantifying heterogeneity and standardising methodologies used in assessing tumour heterogeneity (Chapman et al., 1998).

4.2. Mechanisms for the generation of genetic diversity

Colorectal cancer studies have brought to light the existence of at least two separate mechanisms for generating genetic instability (Ionov et al., 1993; Perucho et al., 1994). The first is found in about 15 % of colorectal cancers and involves mismatch repair deficiencies resulting in point mutations, microdeletions, and microinsertions (Marra and Boland, 1995). Tumours with microsatellites instability usually involves the proximal colon (Thibodeau et al., 1993), have a distinctive undifferentiated mucinous appearance in which necrosis is a prominent feature (Kim et al., 1994) and usually remain diploid or near diploid even in advanced stages (Remvikos et al., 1995; Schlegel et al., 1995). Patients with this form of colorectal cancer usually have a better prognosis than the second form (Bubb et al., 1996).

One of the distinctive features of microsatellite instability is that it develops early in the course of the disease. It first becomes apparent in early adenomas and progresses with sufficient regularity that some have suggested its use as a molecular genetic evolutionary clock (Shibata et al., 1996). This form of colorectal cancer is clinically similar to HNPCC that commonly exhibit inherited defects in enzymatic repair of DNA. However, patients with sporadic colon cancer whose tumours exhibit microsatellite instability, the DNA mismatch repair enzymes are frequently normal (Konishi et al., 1996; Liu et al., 1995).

The second more common form is found in mismatch repair proficient cells and involves gains and losses of whole chromosomes (Lengauer et al., 1997). Tumours with this form of instability are usually aneuploid, involve the distal colon (Delattre et al., 1989; Ionov et al., 1993; Lothe et al., 1993) and

frequently exhibit loss and mutations of wild type p53 alleles (Boland et al., 1995; Carder et al., 1993). Indeed, microdissection studies have indicated that p53 mutations precedes the development of aneuploidy in colorectal cancer (Carder et al., 1995). Interestingly, introduction of specific mutant p53, termed class II mutants, that affects residues which contribute to the maintenance of the tertiary structure or conformation of the protein (Cho et al., 1994; Prives, 1994), rather than DNA binding capabilities, in human cells with null p53 or wild type p53, induced altered spindle checkpoint control and cells underwent repeated rounds of DNA synthesis without chromosomal segregation, generating polyploid cells. These specific p53 mutations were shown to act in a dominant fashion and represent gain rather than loss of p53 function (Gualberto et al., 1998).

By comparing colorectal cancer mismatch repair deficient cell lines with chromosomally unstable cell lines, it has been demonstrated that colorectal tumours with chromosomal instability exhibited a striking defect in chromosome segregation, resulting in gains or losses in excess of 10^{-2} per chromosome per generation. In contrast, tumours with microsatellite instability maintained a diploid or near diploid genotype. Also, while mismatch repair deficiency was a recessive trait, chromosomal instability appeared to be a dominant one. The authors also demonstrated that chromosomal instability is the cause of the observed aneuploidy and not *vice versa*. Interestingly, only one of the eight cell lines studied had both genetic defects (MIN and CIN), suggesting that either type of instability may be sufficient for driving the neoplastic process, and that MIN does not preclude CIN and *vice versa*. Equally interesting, is the fact that p53 mutation did not necessarily correlate with chromosomal instability. For example, p53 was found to be mutated in one of the chromosomally stable cell lines, but

was wild type in one of the chromosomally unstable lines. Thus, gene(s) other than p53 could be responsible for generating chromosomal instability in these cell lines (Lengauer et al., 1997).

Recently, a striking difference in methylation ability between MMR-proficient (CIN) and MMR-deficient (MIN) cells has been suggested to play a role in chromosome segregation processes (Ahuja et al., 1997; Lengauer et al., 1997). While mismatch repair deficient cells could methylate exogenously introduced retroviral genes, mismatch repair proficient cells were unable to do so. The authors hypothesised that methylation abnormalities are intrinsically and directly involved in the generation of chromosomal instability. This is supported by the observation that demethylation is associated with chromosomal aberrations (Schmid et al., 1983; Schmid et al., 1984) and is consistent with the hypothesis relating methylation and aneuploidy (Thomas, 1995). Moreover, hypomethylating agent, 5-azacytidine, has been shown to induce cell variants in several tumour cell lines (Kerbel et al., 1984).

Another mechanism by which significant genetic diversity is generated involves the generation of hydrogen peroxide species by tumours. This radical attack on primary tumour DNA is likely to result in additional mutagenic damage and genetic instability and further increase the number of structurally diverse DNA populations from which highly malignant clones could be selected (O'Donnell-Tormey et al., 1985; Malins et al., 1996).

4.3. Aims of the Ph.D. project

It is clear from the foregoing introduction that, although much is known about the metastatic process, much is still to be learned. A great emphasis has been previously placed on the progression of adenomas to carcinomas in colorectal cancer and much is still to be learned about the genetic aberrations associated with its progression to a metastatic phenotype, and about which ones are causative and which are the results of metastasis. So, the aims of this study were to find genetic aberrations associated with advanced colorectal cancer and relate them to cancer progression. Colorectal cancer was chosen for the following reasons:

(i) It has very well characterised stages of progression.

(ii) It progresses relatively rapidly.

(iii) Materials from primary and their synchronous metastases are readily available.

(iv) Metastases to either or both lymph nodes and liver are available, so there is an apportunity to compare two metastatic pathways.

Ultimately, if genetic aberrations are characteristic of tumour aggressiveness, then their prognostic value could be of immense importance clinically.

MATERIALS AND METHODS

CHAPTER 5. MATERIALS

5.1. ANTISERUM

Supplier: *Affinity, Mamhead, Exeter, UK*

-anti-β_1 integrin monoclonal antibody

-anti-E-cadherin monoclonal antibody

Supplier: *Boehringer Mannheim UK, Lewes, East Sussex, England*

-anti-digoxigenin-alkaline phosphatase conjugated antibodies

-sheep anti-digoxigenin

Supplier: *Chemicon, Cardinal way, Harrow, UK*

-anti-stromelysin-1 polyclonal antibody

Supplier: *Gibco Europe Life Technologies Ltd., Paisley, Scotland*

-streptavidine-alkaline phosphatase conjugated antibodies

Supplier: *Santa Cruz, Autogen Bioclear U.K Ltd, Wiltshire, England*

-anti-Ras monoclonal antibody

-anti-VEGF polyclonal antibody

Supplier: *Serotec Ltd., Oxford, England*

-anti-α_6 monoclonal antibody (Clone 4F10)

Supplier: *Stratech, Luton, Beds., UK*

-donkey anti-sheep Texas-red antibody

Supplier: *Vector Labs., Peterborough, UK*

-FITC-avidin DCS

-biotinylated anti-avidin D

5.2. CELL LINES, MEDIA AND TISSUE CULTURE

All cell lines used in this thesis were from The Beatson Institute Laboratory stocks.

Rat-1 cells were a kind gift from Professor B. Ozanne (Hennigan et al., 1994).

Supplier: *Beatson Institute Central Services*

-sterile water

-sterile PBS

-sterile glassware and glass pipettes

Supplier: *Becon Dickinson Labware, Plymouth, England*

-60 mm and 100 mm diameter tissue culture dishes

Supplier: *Becon Dickinson,Collaborative Research,Franklin Lakes, New Jersey, USA*

-matrigel

Supplier: *Costar Corporation, High Wycombe, Bucks, England*

-24 well cluster tissue culture dishes

-sterile disposable cell scrapers

-transwell chambers

Supplier: *Fisons Scientific Equipment, Loughborough, Leics., England*

-DMSO

Supplier: *Gibco Europe Life Technologies Ltd., Paisley, Scotland*

-BCIP

-chromosome medium

-colcemid

-10x DMEM concentrate

-EGF

-foetal calf serum

-200mM glutamine

-NBT

-100mM sodium pyruvate

-7.5% (w/v) sodium bicarbonate

-1M HEPES

-2.5% (w/v) trypsin

-100x non-essential amino acids

*5.3. **CHEMICALS***

Supplier: *Alpha laboratories Ltd., Eastleigh, Hampshire*

-streck tissue fixative

Supplier: *Amersham International plc., Amersham, Bucks., England*

-[α-^{32}P] dCTP ~ 3000Ci/mmol

-[γ-^{32}P] ATP ~5000Ci/mmol

Supplier: *BDH Chemicals Ltd., Poole, Dorset, England*

Unless otherwise stated, all chemicals were obtained from BDH and were of AnalaR grade or better.

Supplier: *Bethesda Research Laboratories, Life Technologies, Inc., USA*

-agarose, ultrapure electrophoresis grade

Supplier: *Boehringer Mannheim UK,Lewes, East Sussex, England*

-BrdU

-10% block

-DAPI

Supplier: *James Burrough Ltd., Witham, Essex, England*

-ethanol

Supplier: *Fluka Chemicals-Biochemica AG, Buchs, Switzerland*

-formamide

Supplier: *Pharmacia Ltd., Milton Keynes, Bucks., England*

-dextran sulphate

Supplier: *Rathburn Chemicals Ltd., Walkerburn, Scotland*

-phenol (water-saturated)

Supplier: *Sigma Chemicals Co.Ltd., Poole, Dorset, England*

-Bicinchoninic acid
-bromophenol blue
-BSA (fraction V)
-Copper (II) sulphate pentahydrate
-dithiotheitol
-ethidium bromide
-NP40
-pepsin
-Polyvinylpyrrolidone
-RNase
-salmon sperm DNA
-TEMED
-tetramethyl ammonium chloride (TMAC)
-thymidine
-tRNA

Supplier: *Victor Labs. Peterborough, UK*

-antifade

*5.4. **COLUMNS***

Supplier: *Quiagen Ltd., Dorking, Surrey, England*

-QIAquick PCR purification columns

5.5. ENZYMES AND INHIBITORS

Supplier: *Bethesda Laboratories Research, Gibco Ltd., Paisley, Scotland*

All DNA modifying enzymes were obtained from BRL and supplied with the appropriate buffers concentrates, with the following exceptions:-

Supplier: *Boehringer Corporation (London) Ltd., Lewes, East Sussex, England*

-Proteinase K

Supplier: *Northumbria Biologicals Ltd., Cramlington, Northumbrerland, England*

-RNase A

Supplier: *Pharmacia Ltd., Milton Keynes, Bucks., England*

-T4 polynucleotide kinase

5.6. KITS

Supplier: *Boehringer Mannheim UK,Lewes, East Sussex, England*

-Biotin and digoxigenin DNA random priming labelling kit
-Biotin and digoxidenin DNA nick-translation labelling kit

Supplier: *Hybaid, Teddington, UK*

-PCR purification kit

Supplier: *Perkin Elmer Cetus, Norwalk, CT 06859, USA*

-GeneAmp PCR reagent kit

-AmpliTaq DNA polymerase sequencing ready mix (part 402079)

*5.7. **MARKERS and DNA***

Supplier: *Amersham International plc., Amersham, Bucks., England*

-Prestained protein standards: Rainbow markers,
range 14 300-200 000 Da

Supplier: *Bethesda Laboratories Research, Gibco Ltd., Paisley, Scotland*

-Cot-1 DNA
-ϕX174 RF DNA/Hae III fragments
-100bp ladder check

*5.8. **MEMBRANES, PAPER AND X-RAY FILMS***

Supplier: *Amersham International plc., Amersham, Bucks., England*

-Hybond N+

Supplier: *Eastman Kodak Co., Rochester, New York, USA*

-X-ray film (XAR)

Supplier: *Millipore, Watford herts, England*

-immobilan P membrane

Supplier: *Whatman International Ltd., Maidstone, Kent, England*

-3MM paper

5.9. ***OLIGONUCLEOTIDES***

Supplier: *Beatson institute Technology Services, Bearsden, Glasgow, Scotland*

-PCR primers

Supplier: *Chruachem LTD. West of Scotland Science Park, Glasgow, Scotland*

-Allele specific oligonucleotides

5.10. ***SOFTWARE PROGRAMMES***

Supplier: *Digital Scientific, Cambridge, UK*

-smart capture CGH software

Supplier: *Micrografx, Arapaho Richardson, Texas, USA*

-photomagic

-micrografx draw

Supplier: *SPSS Science, Chicago, Illinois 60611, USA*

-SPSS software for survival analysis

5.11. ***WATER***

Distilled water for the preparation of buffer stocks was obtained from Millipore MilliRO 15 system, and for protein, RNA or DNA procedures was further purified on Millipore MilliQ system. Sterile distilled water for use in tissue culture media was supplied by the Beatson Institute for Cancer Research Technical Services.

CHAPTER 6. METHODS

*6.1. **In vivo Ki-ras study***

6.1.1. Case selection

Two groups of colorectal carcinoma patients for whom archival paraffin-embedded tissue was available in Pathology Department files at Glasgow Royal Infirmary (Glasgow, Scotland) were studied. One had biopsy-proven hepatic metastasis of adenocarcinoma at presentation or subsequently while the other was free from hepatic or other distant metastases at presentation and remained so. Follow-up data were obtained from case sheets and augmented by cancer registry, primary care, and audit data as appropriate.

6.1.2. Tissue preparation and microdissection

6μm sections of formalin-fixed paraffin-embedded tissue blocks of primary and secondary carcinomas and normal tissues were dried on plain glass slides, dewaxed, dehydrated and stained in 0.1% toluidine blue. They were dissected with microneedles (tip radius about 3μm) using a Leitz model M micromanipulater (Going and Lamb, 1996). The proportion of carcinoma cells in each neoplastic tissue sample was estimated and recorded. Tissue samples (0.1 to 1mm^2) were digested with proteinase K at 37 °C for 18h. After inactivation of the

proteinase K at 90 °C for 10 min the material was used directly for amplification of Ki-*ras* sequence by PCR.

The median estimated percentage of carcinoma cells in 254 microdissected carcinoma samples from the complete series of 78 patients was 75%. Of these samples, 244 (96%) contained 60% or more of carcinoma cells, and all more than 50%.

6.1.3. PCR

Part of Ki-*ras* first exon was amplified with the outer 5' and 3' primers (Table 3) using a commercial kit (Geneamp; Perkin Elmer/Cetus). PCR reactions consisted of 34 cycles of 92 °C for 30 s, 60 °C for 90 s, and 72 °C for 150 s followed by a final extension at 72 °C for 5 min.

To obtain consistent amplification 1μl of the first PCR reaction was reamplified with the inner 5' and 3' primers (Table 3) in the following PCR reaction: 92 °C for 30 s, 60 °C for 1 min, 72 °C for 30 s for 34 cycles, with final extension for 5 min at 72 °C. In all reactions positive and negative controls were included. For the positive controls, an adenocarcinoma-derived cell line with GTT mutation in codon 12 of Ki-*ras* was used (Williams et al., 1993). For the other codon 12 and 13 mutations, patient specimens known to have these mutations in Ki-*ras* by direct sequencing were used as positive controls.

For negative controls, samples without DNA were included in the PCR reaction mixture and samples representing mock microdissections were also amplified to evaluate the possibility of DNA carry-over during microdissection.

The PCR products were examined for amplification of the expected 121 bp fragment on 2% agarose gels (Figure 9).

Table 3: PCR primers and ASO probes

PCR primers used to amplify DNA from microdissected tissues for (a), allele-specific oligonucleotide hybridisation and (b), sequencing. Anti-sense probes used to detect various codon-12 and 13 mutations of the Ki-*ras* gene using allele-specific oligonucleotide hybridisation are listed in (c)

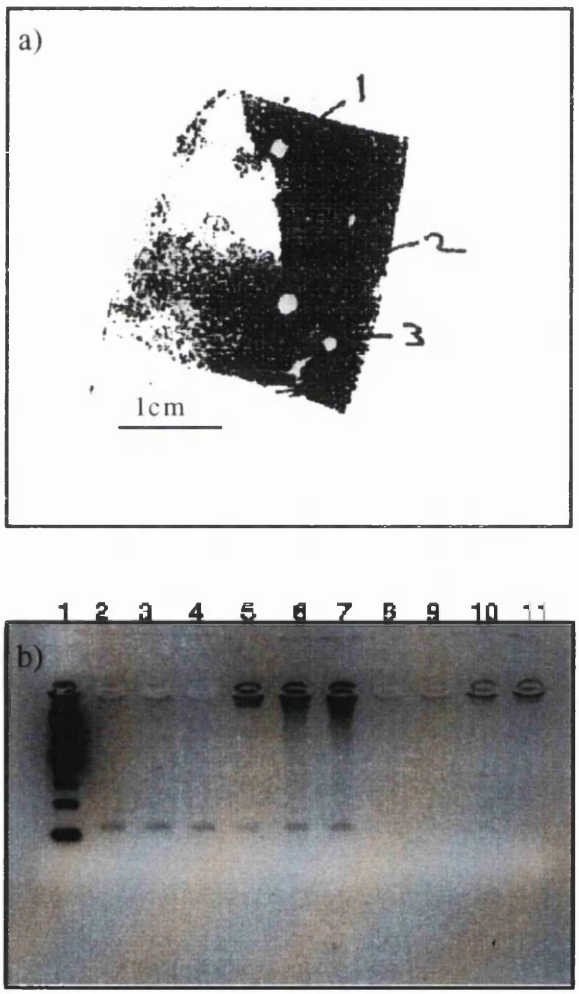
(a)	5' outer	5'-AGGCCTGCTGAAAATGACTGAATA-3'
	3' outer	5'-CTGTATCAAAGAATGGTCCTGCAC-3'
	5' inner	5'-CTCTATTGTTGGATCATATTCGTC-3'
	3' inner	5'-GCCTGCTGAAAATGACTGAATATAA-3'
(b)	5' outer	5'-TAGTGTATTAACCTTATGTGTGAC-3'
	3' outer	5'-CTGTATCAAAGAATGGTCCTGCAC-3'
	5' inner	5'-AGGCCTGCTGAAAATGACTGAATA-3'
	3' inner	5'-CTCTATTGTTGGATCATATTCGTC-3'
(c)	GGT-12 probe	5'-CCTACGCCACCAGCTCCAA-3'
	GAT-12 probe	5'-CCTACGCCATCAGCTCCAA-3'
	GTT-12 probe	5'-CCTACGCCAACAGCTCCAA-3'
	GCT-12 probe	5'-CCTACGCCAGCAGCTCCAA-3'
	TGT-12 probe	5'-CCTACGCCACAAGCTCCAA-3'
	AGT-12 probe	5'-CCTACGCCACTAGCTCCAA-3'
	GAC-13 probe	5'-CCTACGTCACCAGCTCCAA-3'
	Mock control probe	5'-CCTACGCGACCAGCTCCAA-3'

Figure 9: *Microdissection of liver metastasis*

a) An illustrative example of microdissection of normal liver (1) and adjacent areas of adenocarcinoma (2 and 3).

b) 2% agarose gel of nested PCR products generated from microdissected tissue.

Lane 1: 100 bp ladder
Lane 2-7: 121 bp Ki-*ras* products
Lane 8 and 9: Mock microdissection
Lane 10 and 11: PCR negative controls



6.1.4. Allele-specific oligonucleotide hybridisation

This was done as described previously (Verlaan-de Vries et al., 1986). Hybond Nylon N+ membranes (Amersham International) were soaked in distilled water then 10× SSC (20× SSC: 3M sodium chloride, 0.3M sodium citrate, pH 7.0) and air dried. Equal volumes of 20× SSC were added to the PCR reactions and the mixture boiled for 5 min and quenched on ice. 5µl of the denatured PCR product was then dotted on the membranes. The membranes were placed carefully in denaturing buffer (1.5M NaCl, 0.5M NaOH) for 5 min and then in neutralising buffer (1.5M NaCl, 0.5M Tris-HCl pH 7.2, 0.1mM EDTA) for 1 min. The filters were allowed to air dry and the DNA fixed to the membranes by U.V. light crosslinking. The membranes were blocked in pre-hybridisation solution (3M TMAC, 50mM Tris pH 8, 2mM EDTA, 100µg/ml sonicated salmon sperm ssDNA boiled just before use, 0.1 % SDS, 5× Dehardt's solution) at 56 °C for 16 h in a Hybaid rotating oven. 500 ng of codon-12 specific oligonucleotides (Table 3) were end labelled in a reaction volume of 10µl containing 1× T4 polynucleotide kinase buffer (10× PNK buffer: 0.5M tris-HCl pH 7.6, 0.1M $MgCl_2$, 50mM dithiothreitol DTT, 1mM spermidine, 1mM EDTA), 10 µCi [γ-^{32}P] ATP and 20 units of T4 polynucleotide kinase enzyme. The mixture was incubated at 37 °C for 1 h. The reaction was terminated with 2.5µl of 0.25M EDTA. Unincorporated nucleotides were removed from the oligonucleotide by addition of 10 μg t-RNA, 7.5M ammonium acetate (v/v), 10 volumes 100 % ethanol, the mixture incubated at -70 °C for 1 h and centrifuged in a 4 °C bench top microfuge for 20 min at 14K rev/min. The supernatant was then removed and the pellet resuspended in distilled

water. ^{32}P incorporation was estimated using the scintillation counter. 2×10^7 counts of each oligonucleotides were then added to each membrane in the pre-hybridisation mix and left for 2 h at 56 °C in the hybaid rotating oven. After hybridisation, the membranes were washed at room temperature in 2× SSPE containing 0.1 % SDS for 5 min (20× SSPE: 3.6M sodium chloride, 0.2M sodium phosphate, 0.02M EDTA, pH 8.3). The filters were washed again for 5 min in 5× SSPE at 63 °C and for a further 1 h at 59 °C in hybridisation buffer without Denhardt's and ssDNA. Finally the membranes were dried and exposed to Kodak XAR films at -70 °C using intensifying screens for various times.

6.1.5. Sequencing

The DNA was amplified as before, using the primers in Table 3. PCR products were isolated using QIAquick system (QUIAGEN GmbH) according to the maufacturer instructions, and were then cycle-sequenced using AmpliTaq DNA polymerase ready mix (part 402079) and 3' primer 5'-CTCTATTGTTGGATCATATTCGTC-3'. The products were analyzed using the ABI 373 system.

6.1.6. Statistical analysis

Categorical variables were analyzed by Chi-square and Fisher's exact tests. Disease-free and overall survival were analyzed by constructing Kaplan-Meier survival curves, and the log rank test was used to test for difference in survival between groups.

6.2. *In vitro Ki-ras study*

6.2.1. *Ki-ras plasmid construct*

The pCEXV-3 expression vectors containing wild-type or GTT mutated codon-12 of Ki-*ras* cDNA insert were a kind gift from professor Chris Marchall (Miller et al., 1993). The vectors and inserts were confirmed by sequencing. The GAT mutated codon-12 of Ki-*ras* cDNA was generated by PCR (Perkin Elmer) using the wild-type Ki-*ras* pCEXV-3 vector as template and 5' oligonucleotide 5'-TCAGTGAGCTCCACCATGACTGAATATAAACTTGTGGTAGTTGGAGCTGATGGCGTAG -3' and the 3' oligonucleotide 5'-TCAGTTCTAGATTCACAGGCATTGCTAGT-3' using the following PCR conditions: 95 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min for 30 cycles and a final elongation step in 72 °C for 15 min. The PCR product was then purified from agarose using Hybaid recovery DNA purification kit (Hybaid). The purified product was then digested with sst/xba1 and ligated into sst/xba1 digested and phosphatased pCEXV vector. E.Coli (Novablue) was then transformed with the ligated vector and the recombinant plasmids were isolated and checked by sequencing using the forward primer 5'-TGTACGGAAGTGTTACT-3' and the reverse primer 5'-CTTATCATGTCTGGATCG-3'.

6.2.2. *Cell culture and transfection*

Rat-1 cells were grown in DMEM containing 10% FCS. The cells were transfected by electroporation with either the plasmid (pUM-1) conferring

resistance to G418 (neo) alone, or co-transfected with neomysin resistance plasmid together with pCEXV containing Ki-*ras* cDNA inserts (with different codon-12 i.e. GGT, GAT, GTT). The plasmids were mixed in a ratio of 20 (pCEXV) : 1 (neo) (i.e. 10μg pCEXV: 0.5μg neo) in a total volume of 50μl T.E. (Tris-EDTA).

After two days, G418 (600 μg/ml) was added to the cell culture medium and G418-resistant clones were picked (approximately after 10 days). Clones expressing the correct Ki-*ras* gene product were characterised by Western analysis and RT-PCR. The cDNA generated was subjected to allele specific oligonucleotide hybridisation using the procedure in section 6.1.4 and the probes specific for GAT-12, GTT-12 and GGT-12 mutations listed in Table 3.

The preparative work described in sections 6.2.1 and 6.2.2 was contributed by Mrs Elaine MacKenzie.

6.2.3. Western analysis

Cells were grown to sub-confluence in 10cm tissue culture dishes, washed twice with cold phosphate-buffered saline (PBS) and the excess PBS aspirated off. Cells were immediately transferred onto ice and 0.2 ml of stock lysis buffer (0.5% NP40, 250mM NaCl, 50mM HEPES pH 7, 5mM EDTA) containing fresh protease inhibitors (50mM NaF, 200μM sodium orthovanadate, 50mM β-glycerophosphate, 1mM phenylmethylsulphonyl fluoride (PMSF), 10μg/ml leupeptin, 10μg/ml aprotinin) was added to cells, scraped immediately using a disposable cell scraper and transferred to pre-chilled screw cap eppendorf tubes.

Samples were then centrifuged at 14K rev/min for 5 min at 4 °C. Supernatants were transferred in aliquots to fresh eppendorf tubes and stored at -70 °C until required. The protein concentrations were measured with a commercial reagent (Bio-Rad). 50μg aliquots of proteins were resolved on SDS-polyacrylamide gels by electrophoresis according to the apparent molecular weights. Typically, the resolving gels were 7.5% or 10% with respect to the acrylamide concentration and stacking gels ranged between 4-5% acrylamide. Acrylamide solutions of the required concentration were mixed with the appropriate gel buffer to a final concentration of 1× with dH_2O. ([4× resolving gel buffer: 1.5M tris pH 8.9, 0.4% (w/v) SDS][4× stacking gel buffer: 0.5M tris pH 6.7, 0.4% (w/v) SDS]). Prior to pouring of either resolving or stacking gels, 100μl of a 10% (w/v) ammonium persulphate solution and 10μl of TEMED were added per 10mls of gel solution, mixed by gentle swirling and poured in ATTO mini-gel apparatus. Resolving gels were overlaid with butan-2-ol and allowed to polymerize at room temperature for 2 h. Excess butan-2-ol was then removed with a pipette, washed with dH_2O and then 1× running buffer. The stacking gel was subsequently poured, appropriate comb inserted and the gel allowed to polymerize for 1 h. Both reservoirs were filled with 1× SDS-PAGE running buffer (10× SDS-PAGE running buffer: 522mM tris, 4% (w/v) glycine, 1% (w/v) SDS), the bubbles and comb carefully removed and the wells rinsed with running buffer before samples were loaded. 50μg of proteins were denatured prior to loading by heating to 100 °C for 5 min in 2× SDS sample (62.5mM Tris pH 6.8, 2% SDS, 10% Glycerol, 0.1% Bromophenol blue, 4% β-mercaptoethanol) and quenched in ice. Equal amounts of proteins were added in each lane as calculated using the BCA/$CuSO_4$ protein

assay (BCA: Bicinchoninic acid solution [Sigma] 5mls, $CuSO_4$: Copper (II) sulphate pentahydrate 100µl of 4% (w/v) solution [Sigma]). Protein extracts were resolved together with the appropriate molecular weight markers (Rainbow markers, Amersham UK).

Following electrophoresis, the gels were transferred to nitrocellulose membranes (Immobilon P; Millipore) using the dry blotting method. For this procedure, twelve pieces of Whatman 3MM paper and a single piece of nitrocellulose were cut to the size of the gel. The nitrocellulose membrane was wetted in methanol briefly then soaked in dH_2O for 5 min before left in 1× blotting buffer (10× blotting buffer: 0.6M tris, 0.5M glycine, 16mM SDS) containing 20% (v/v) methanol with the Whatman papers. The gels were carefully rinsed in 1× blotting buffer and placed on top of six pieces of pre-soaked Whatman papers in a Transblot wet blotting apparatus (Bio-Rad), the nitrocellulose membrane was then laid on top of the gel and finally the remaining six pieces of Whatman papers were placed on top of the nitrocellulose membrane. Air bubbles were carefully removed and transfer effected towards the anode at 20 volts and 200 mA for a maximum of 1 h. after transfer the membrane could be either used immediately for blotting or alternatively, air-dried and stored at 4 °C until required.

Immunoblots were blocked in PBS containing 0.1% Tween 20 and 5% skim milk (Marvel) for 1 h at room temperature and probed with anti-Ras MAbs (Santa Cruz), anti-β_1 Integrin MAbs (Affinity), anti-E-cadherin MAbs (Affinity), anti-Stromelysin-1 polyclonal antibody (Chemicon), and anti-VEGF polyclonal antibody (Santa Cruz) according to the manufacture's instructions. Membranes were subsequently washed 3 times with PBS containing 0.1% Tween 20 and

incubated for 1 h with the appropriate peroxidase-conjugated secondary antibodies (1:5000). After 3 washes with PBS-0.1% Tween 20, band were detected with Enhanced Chemiluminescence Western Blotting system (Amersham) according to the manufacturer's instructions and visualized by exposure to Kodak X-Omat film for various times.

To check for efficiency of transfer and equal loading of proteins, nitrocellulose membranes were stripped with strip solution (0.2M glycine, 1% (w/v) SDS, pH 2.5) for 1 h with vigorous shaking at room temperature. Membranes were then washed 3 times for 10 min in PBS containing 0.1% Tween 20 at room temperature and blocked for 1 h in PBS containing 0.1% Tween 20 and 5% skim milk (Marvel) at room temperature and probed with anti-vinculin MAbs (Sigma) according to manufacturer instructions.

6.2.4. In vitro invasion assay

Inverse invasion assay in serum-free conditions was performed and quantitated as previously described (Hennigan et al., 1994). 2×10^5 cells were allowed to adhere to 8-μm-pore-size filters in Transwell chambers (Costar) containing 100 μl of growth factor-depleted Matrigel (Collaborative Research) for 4 to 6 h in a humidified 5% CO_2 37 °C incubator. Filters were then washed in serum free DMEM to remove unattached cells and placed back in chambers filled with serum free DMEM. The top of the matrigels were filled with serum free DMEM with or without growth factor and the trays placed in a humidified 5% CO_2 37 °C incubator for 3 days. In this assay, cells are first chemoattracted across the 8-μm-pore-size filters, those that then migrate into Matrigel are visualized, and cell numbers are quantitated in 10-μm confocal optical sections. In assays

containing Growth factor, human recombinant EGF (Life Technologies) at a concentration of 40 ng/ml was added above the Matrigel layer in serum-free DMEM as previously described (Hennigan et al., 1994).

For inhibition of invasion by oligonucleotides, GTT-12 specific Ki-*ras* sense, 5'-TTGGAGCTGTTGGCGTAGG-3' or antisense, 5'-AACCTCGACAACCGCATCC-3' were used as described (Kawada et al., 1997). 20 μM/day of sense or antisense Ki-*ras* oligonucleotides were added above the Matrigel each day for 3 days. For anti-α_6 inhibition of invasion, the anti-α_6 antibody (Serotec) (a kind gift from Dr. Brunton; CRC Beatson laboratories) was applied above the Matrigel in serum-free DMEM at a final concentration of 20 μg/ml after the cells were allowed to attach to the filter. After 3 days, For assays of invasion, the Transwells were washed twice in PBS then fixed with ice-cold methanol. Cell nuclei were stained with Propidium-iodide and visualised by a Bio-Rad MRC 600 confocal illumination unit attached to Nikon Diaphot inverted microscope with various magnification. Cells at 20-μm and above in the Matrigel are considered to be invasive. To account for variations in cell numbers plated on the Transwell filters, results were quantitated by counting cell nuclei at 20-μm and above as a percentage of cells in all optical sections. Assays were performed three-times in duplicates.

6.3. Comparative genomic hybridisation study

6.3.1. Patients

Paraffin-embedded, formalin-fixed tissue blocks were retrieved for 12 patients who had been operated on for metastatic colorectal cancer at the department of surgery, Glasgow Royal Infirmary between 1984-1997. Patients had not been given chemotherapy or radiotherapy before surgery. Primary tumours were excised and their synchronous metastases were removed or biopsied at the time of initial surgery. Dukes' staging was assessed and sections were confirmed histologically to contain areas of adenocarcinoma by an experienced pathologist.

For the isolation of DNA, Four 7μm thick tissue sections from each sample were dewaxed and rehydrated and areas of high tumour content (more than 60%) were dissected and pooled into sterile Eppendorf tubes. The samples were then dried and resuspended in lysis buffer (0.1M EDTA, 0.01M Tris-HCL, 0.02M NaCl, 0.4% Sarkosyl) containing proteinase K to a final concentration of 1mg/ml and incubated at 37 °C for 3 days with gentle tumbeling. After extraction with phenol-chloroform and ethanol precipitation, the DNA was dissolved in 10mM Tris-HCl, 1mM EDTA (pH 7.4) with RNAase at a final concentration of 0.4mg/ml, and incubated at 37 °C for 1 hour. The DNA was purified by a second phenol-chloroform extraction.

6.3.2. Comparative genomic hybridisation

Normal DNA was prepared from the peripheral blood of healthy female and male volunteers. Tumour DNA (500ng) was labelled with biotin-16-dUTP and normal DNA (500ng) was labelled with digoxigenin-dUTP using a nick-translation (1500ng of DNA) for 75 min at 16 °C or random primed labelling kit (less or equal to 500ng of DNA) (Boehringer Mannheim) at 37 °C for 10 h depending on the amounts of DNA extracted from samples. To ensure labelling of the DNA, 1μl of the labelling mixture and serial 1 in 10 dilutions were dotted onto nylon membranes (separate membranes for Biotin and Digoxigenein labelled DNAs) and crosslinked with UV Stratalinker. The membranes were washed on a rocking table with buffer A (0.1M Tris pH 7.5, 1× SSC) for 5 min, the solution was poured out and the membranes were washed in buffer B (0.1M Tris pH 7.5, 1× SSC, 3% BSA) at 4 °C on a rocking table for 30 min. 5 ml of buffer B containing either 5μl Streptavidin Alkaline Phosphatase conjucated antibody for biotin labelled DNAs (Gibco-BRL) or Anti-Digoxigenin Alkaline Phosphatase for Digoxigenin labelled DNAs (Boehringer-Mannheim) (according to the manufacturers instructions) was added to the membranes in a plastic sealable bags and left for 30 min in the dark. The membranes were then washed 3 times, 5 min for each wash, in buffer A and a further 5 min in buffer C (0.1M Tris pH 7.5, 1× SSC, 50mM $MgCl_2$). To detect successful labelling, 5 ml of buffer C containing developing solution (22μl NBT and 17μl BCIP, Gibco-BRL) according to manufacturer instructions, and the membranes were left in the dark overnight. The size of the biotin and digoxigenin labelled DNAs was in the range of 500 bp to 2 Kb. To check for this, 1μl of the labelled mixtures were run on 1% agarose gel.

The labelling reaction was stopped by addition of 0.5M EDTA and heating at 100 °C for 10 min. The labelled DNAs were ethanol precipitated in the presence of 50μg of Cot-1 human DNA (Gibco BRL, Gaithersburg, MD) and 3mM sodium acetate, dried and resuspended in 16μl of hybridization solution (50% formamide, 500 μg/ml salmon sperm DNA, 10% dextran sulphate in 2× SSC) and left to dissolve at 4 °C for 20 min. The DNA was then denatured at 75 °C for 9 min and allowed to reanneal at 37 °C for 1 hour.

Target metaphase chromosomes were prepared from peripheral blood lymphocytes obtained from a healthy male using a standard protocol. 200μl of whole venous blood was added to 5ml of chromosome medium (Gibco-BRL) and incubated at 37 °C in 5% CO_2 for 72 h with gentle mixing every day. 294μl of 6mg/ml Thymidine (Sigma) was added to the medium and incubated for a further 15-17 h in 5% CO_2 at 37 °C. Samples were then washed three times in Hanks balanced salt solution, HBSS, after centrifuging at 1.5K rev/min for 7 min for each wash. The pellet was then resuspended in 5ml fresh chromosome medium containing 50μl of 3mg/ml Bromodeoxyuridine, BrdU (Boehringer) and incubated in the dark for a further 7-8 h at 37 °C in 5% CO_2. 50μl of 10μg/ml Colcemid (Gibco-BRL) was then added to each 5mls samples and incubated at 37 °C for a further 1-3 h. The samples were then centrifuged at 1.5K rev/min for 5 min and the supernatant carefully removed. The pellets were resuspended in 10ml of hypotonic solution (0.075M KCl) and incubated at 37 °C for 10 min. the cells were then fixed by the addition of 2-3ml freshly made methanol/acetic acid (3:1v/v). Samples were centrifuged at 1.5K rev/min for 5 min, resuspended in 10ml of methanol/acetic acid and incubated at 37 °C for 10-15 min. This step was

repeated at least 3 times. Finally, the samples were centrifuges at 1.5K rev/min for 5 min, resuspended in a small volume of methanol/acetic acid, and stored at -20 °C until used (maximum of 2 weeks).

To prepare the metaphase slides, a small volume of cells was dropped onto clean slides from a height and areas with usable metaphase spreads were marked with a diamond pen under phase contrast microscopy. The slides were fixed in methanol/acetic acid (3:1v/v) at room temperature for 1 h and left to air dry. After rinsing the slides in 2× SSC they were incubated in 2× SCC containing 100μg/ml RNase (Sigma) at 37 °C. The slides were rinsed in 2× SSC twice and incubated in 10mM HCl solution containing 0.01% pepsin (Sigma). Slides were rinsed in 2× SSC, fixed for 10 min in streck tissue fixative STF (Alpha labs.) and dehydrated for 2 min in 70 % ethanol then 2 min in 100 % ethanol and air dried. The metaphase slides were denatured in 70% formamide in a water bath at 73 °C for 3 min and dehydrated in ice-cold 70%, 85% and 100% ethanol. The DNA solution (15 μl), containing equal amounts of tumour and normal DNA, was applied to each denatured metaphase slides under a coverslip, sealed with rubber cement and left to hybridise in a humidified chamber at 37 °C for 72 hours.

After the slides were washed in denaturing solution (50% formamide in 2× SSC) for 20 min at 42 °C, then 2× SSC for a further 20 min, the slides were blocked with 4× SSCTB (4× SSC containing 0.4% Tween 20 (v/v) and 10% block (v/v)) (Boehringer Mannheim, UK) for 10 min under parafilm coverslips. 100μl of 1:200 dilution of FITC-Avidin DCS (Vector Labs. Peterborough, UK) in 4× SSCTB was added to each slide for 45 min at room temperature. Slides were then washed in 4× SSCT (4× SSC containing 0.4% Tween 20 (v/v)) for 10 min at room

temperature. 100μl of the second layer of antibodies containing 1:200 dilution of biotinylated anti-avidin D (Vector Labs. Peterborough, UK) and 1:200 dilution of sheep anti-digoxigenin (Boehringer Mannheim, UK) in 4× SSCTB was applied onto slides for 45 min at room temperature. Slides were then washed in 4× SSCT for 10 min at room temperature. Finally, 100μl of the third layer of antibodies containing 1:200 dilution of FITC-Avidin DCS and 1:300 dilution of donkey anti-sheep Texas-red in 4× SSCTB was added to slides for 45 min at room temperature. Slides were then washed in 4× SSCT for 20 min at room temperature, dehydrated in 70% then 100% ethanol, air dried and mounted in antifade (Vector Labs. Peterborough, UK) containing 0.1μg/ml DAPI (Boehringer Mannheim, UK) to identify the chromosomes.

In all experiments green-labelled normal *versus* red-labelled normal DNA hybridizations were included as controls. Hybridisation of green-labelled normal male DNA *versus* red-labelled normal female DNA (Karhu et al., 1997) and of DNA from a characterised colorectal cancer cell line (Colo-320) against normal female DNA was also done for quality control. Chromosome 19 was excluded from the study because of the reduced hybridization of normal DNA to chromosome 19, a problem which has been reported previously (Kallioniemi et al., 1994). The sex chromosomes were excluded because hybridisation of DNAs from different genders (e.g. female normal DNA and male tumour DNA) could give false signals reflecting the differences in the number of the X chromosomes in the samples (Karhu et al., 1997).

6.3.3. Image collection and analysis

Images were collected using a cooled charge-coupled-device (CCD) camera mounted on an epifluorescence microscope (Zeiss) equipped with filters to detect DAPI, fluorescein, and Texas Red. Uniformly labelled metaphases with minimal overlaps were chosen in each case. Five metaphases were collected from each sample and analysed using CGH computer program (SmartCapture, Digital Scientific, Cambridge, UK).

Stringent criteria were used to identify gains and losses. Amplifications were scored if the median ratio was above or equal to the 1.25 threshold and deletions were below or equal to the 0.75 threshold. Secondly, the genetic aberration was scored if the standard errors representing 99% confidence intervals of the tumour *versus* normal profiles were outwith the 99% confidence intervals of the normal *versus* normal control profiles. Three-colour images were processed using image processing software (Photomagic, Micrografx, Arapaho Richardson, Texas, USA), annotated in Micrografx Draw (Micrografx) and printed using a dye sublimation printer (Colour Ease, Kodak, Harrow, UK) to be used as a visual support and to reinforce fluorescence analysis. All original unedited images were stored on optical disks and have been retained.

6.3.4. Statistical analysis

Statistical differences in the prevalence of the most commen gains and deletions between the primary tumours and their metastases and between Dukes' stages C and D were performed using Fisher's exact test.

To calculate the probability of clonal relationship between primary tumours and their metastases a model developed previously was used (Kuukasjarvi et al., 1997). This model evaluates the probability that a set of gains and losses shared by a pair of tumours might occur by chance alone. If a_1, a_2, a_3,.....*etc* are specific gains or losses, the probability of a particular gain or loss in an individual tumour is estimated from its frequency in the group, as

$$p(a_n)= n(a_n)/N$$

where $n(a_n)$ is the number of occurrences of a_n in the N tumours analysed. If P = the set of aberrations present in a particular primary tumour, and M = the set of aberrations in an associated metastasis, then events in common are $P \cap M = c_1,c_2,c_3,\ldots c_n$, the probability that shared events occurred independently in the two tumours being compared is

$$p(c_1) \times p(c_2) \times \ldots \times p(c_n) = X$$

If X is small, we reject chance as a plausible explanation of the shared events, and accept the non-random explanation, ie that the shared abnormalities are a consequence of a clonal relationship. The model assumes that the paired specimens must be both losses or both gains and that the breakpoint along the chromosome arm is the same.

RESULTS AND DISCUSSION

SECTION 1

CHAPTER 7. The Role of Mutant codons-12 and 13 of Ki-*ras* gene in *in vivo* Colorectal Cancer Progression

Ras proteins play a fundamental role in signal transduction pathways in cells. Mutations at codons 12 and 13 of the Ki-*ras* gene appear to be a relatively frequent and early event in colorectal cancer. During the last decade, a large number of publications have tried to address the role and dissect the precise signal transduction pathways activated by Ras proteins in cells. These studies have immensely clarified our understanding of the role activated Ras proteins play in human tumours in general and colorectal cancer in particular. However, the importance of activated Ras proteins in tumour progression *in vivo* is still largely controversial. Moreover, the role different codon 12 mutations play in colorectal cancer progression and aggressiveness is even more controversial. For example, as will be discussed later, some authors have suggested that certain codon 12 mutations are associated with more aggressive colorectal cancer than others. If this is correct, could specific codon 12 mutations in the Ki-*ras* gene be used clinically as prognostic indicators or as markers of aggressive cancers? In addition, since Ki-*ras* mutations appear early in the adenoma-carcinoma sequence, are these mutations also present in metastases? Thus, are these mutations important in colorectal progression into a metastatic phenotype or are they a consequence of tumour progression?

This study was designed to shed some light on the role of Ki-ras codons 12 and 13 mutations in colorectal cancer and to attempt to address these questions.

*7.1. **Patients and sample characteristics***

DNAs from 86 patients with colorectal adenocarcinoma were analyzed for codon 12 and 13 mutations in the first exon of Ki-*ras* by ASO hybridisation and direct sequencing (see for example, Figures 10 and 11). Data from 8 patients were not interpretable, and they were excluded from the study. Six patients were classed as Dukes' A, 25 as Dukes' B, 21 as Dukes' C, and 26 as Dukes' D. The patients were 40-96 years old (mean $\pm$ SD, 68.7 $\pm$ 11.3). A total of 145 specimens from primary carcinomas were analysed (duplicate samples of the same primary were analysed where available), 57 from 32 lymph node metastases and 52 from 26 liver metastases from Dukes' D patients. Twenty-three adenoma specimens from 11 of these patients were analysed. As controls, 158 specimens of morphologically normal mucosa from the 78 patients were microdissected from the same primary carcinoma histological block or an adjacent block, and analysed for Ki-*ras* mutation. A total of 435 tissue samples were analysed.

Figure 10

Figure 10: *Detection of Ki-ras mutations by allele-specific oligonucleotide hybridisation*

Detection of Ki-*ras* mutations by allele-specific oligonucleotide hybridisation to PCR-amplified DNA from multiple microdissected liver metastases and adjacent normal liver (nlv) from 29 Dukes' D patients. Hybridisations were with ^{32}p-labelled probes specific for codon-12 and 13 mutations as indicated beside each membrane.

The coloured panel illustrates the nature of the dotted DNAs. Multiple samples from each patient are given identical colour shade. Control DNAs with known sequences and mock dissections are illustrated in rows H and F respectively. A mock codon 13 probe (CAC) was also included to exclude non-specific hybridisation.

The GGT (wild-type) probe shows DNA present in dots since the mutations occur in one allele only. In certain samples the GGT probe shows no hybridisation. In these cases, either the DNAs were not amplified or, in the presence of a mutation, the mutation is homozygous or one allele is mutated and the other is deleted.

Normal liver sample B4 has a codon 12 AGT mutation. All Samples were subjected to at least three separate ASO hybridisations.

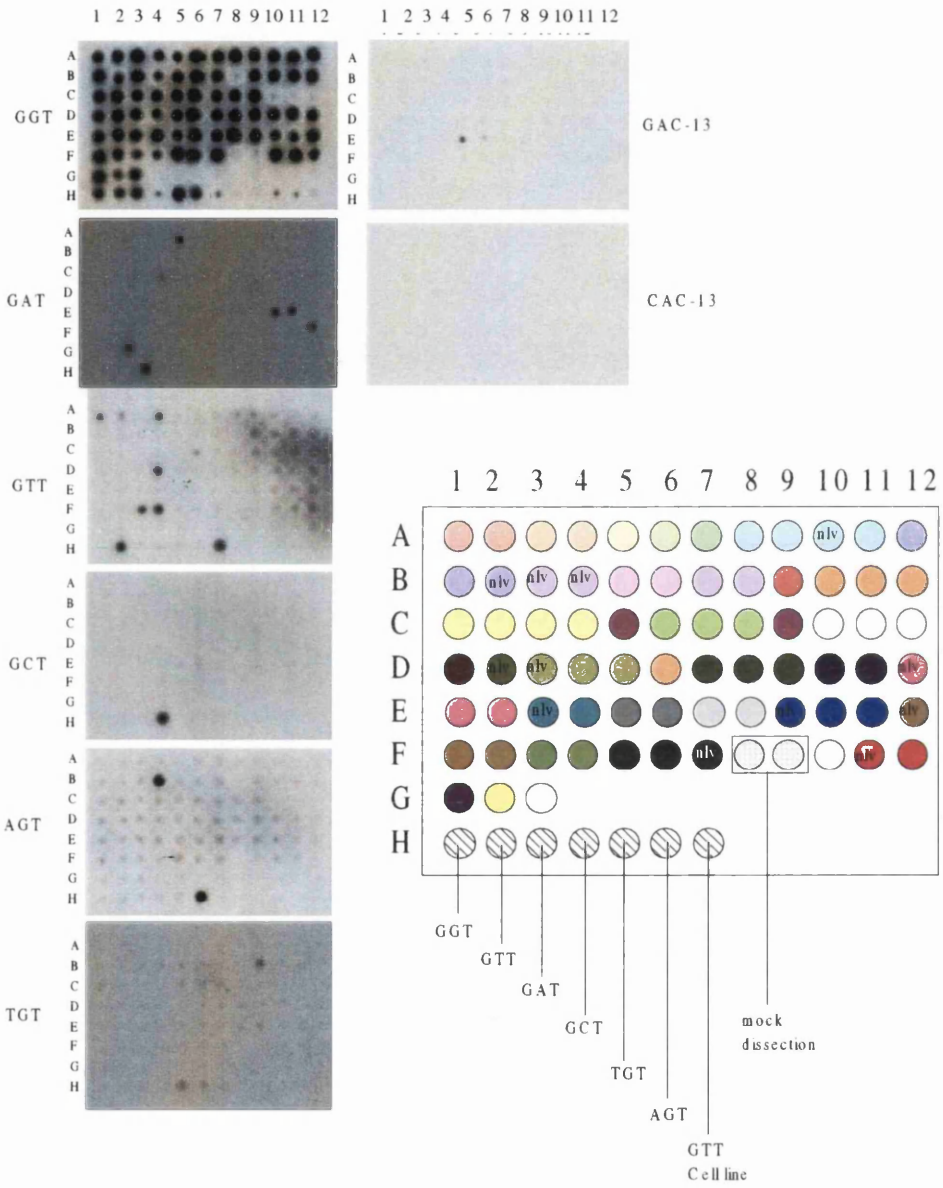

1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
GGT
GAT
GTT
GCT
AGT
TGT
GAC-13
CAC-13
nlv
GGT
GTT
GAT
GCT
TGT
AGT
GTT
Cell line
mock
dissection

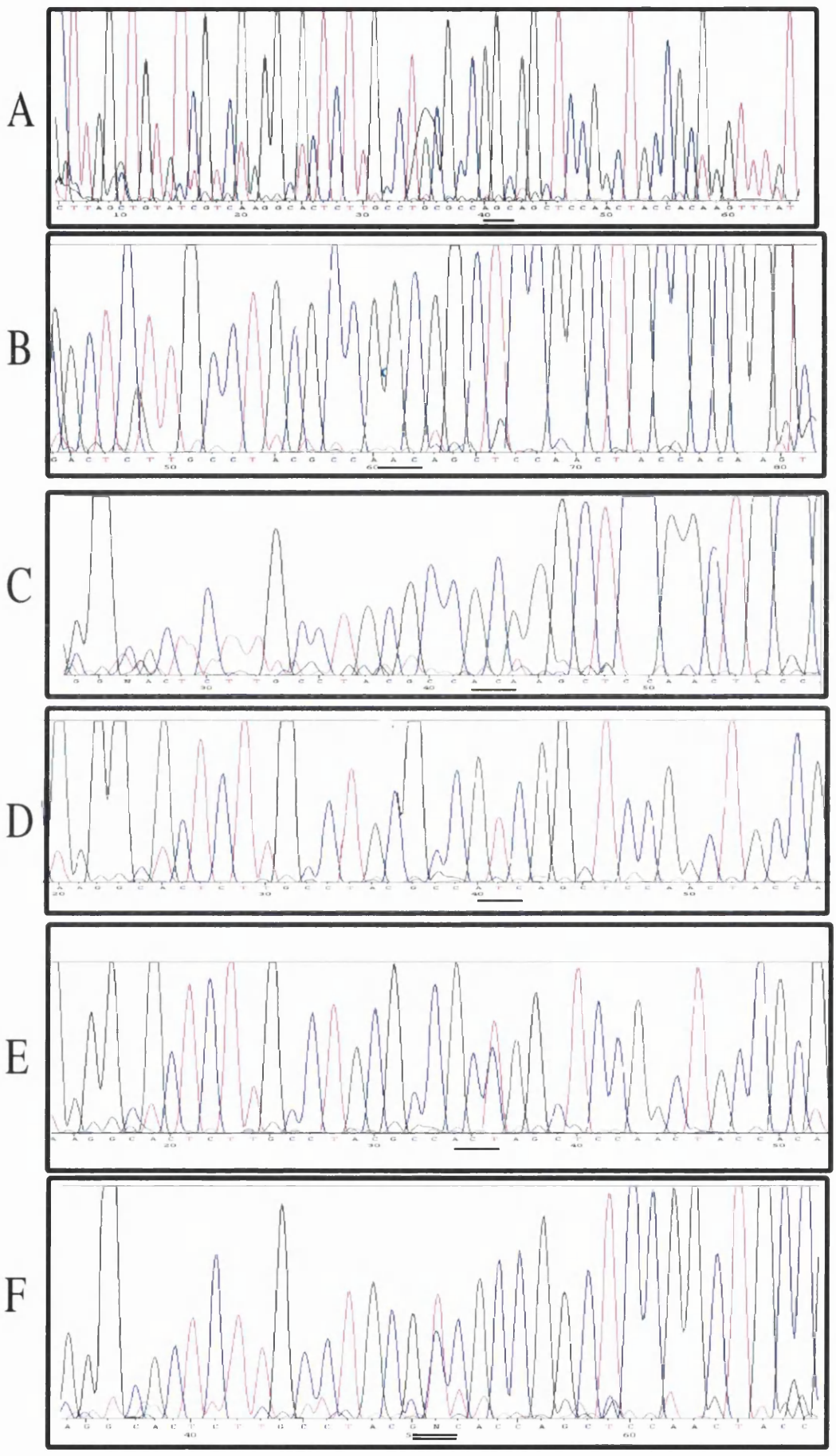
Figure 11

Figure 11: *Selected examples of sequence data around codons-12 and 13 of the Ki-ras exon-1*

Part of the Ki-*ras* exon-1 was amplified by PCR as described in section 6.1.5 and sequenced using the antisense primer 5'-CTCTATTGTTGGATCATATTCGTC-3'. The products were analysed using the ABI 373 system.

The figure shows sequences obtained from tumours of five different patients and a cell line with a homozygous GTT-12 mutation. A) GGT-12 to GCT mutation, B) GGT-12 to GTT mutation, C) GGT-12 to GAT mutation, D) GGT-12 to TGT mutation, E) GGT-12 to AGT mutation and F) GGC-13 to GAC mutation. Codon-12 is underlined and codon-13 is double underlined.

Samples A, C and D have either a homozygous mutation in codon-12 or the wild-type allele is deleted. Sample B has a homozygous codon-12 mutation. Samples E and F are heterozygous for codon-12.


A
B
C
D
E
F

7.2. Frequencies of Ki-ras mutations

The overall incidence of mutations (34-38%) was the same in primary carcinomas, lymph node metastases, liver metastases and adenomas (Table 4). Of the 158 histologically normal mucosa specimens analysed, one was found to have a GGT to GTT transversion in codon 12. Also, 1 of 23 apparently normal microdissected areas of lymph nodes was found to have a GAT mutation in codon 12 of Ki-*ras*. In all but two of the DNAs analysed, the normal allele of Ki-*ras* had

Table 4: Frequency of Ki-*ras* codon 12 and 13 mutations in adenomas, primary carcinomas, lymph node and liver metastases from 78 patients with colorectal adenocarcinoma

Tissue	No. with mutation (%)	No. without mutation (%)	Total
Primary adenocarcinoma	26 (34)	52 (66)	78
Lymph node metastasis	10 (32)	21 (68)	31
Liver metastasis	10 (38)	16 (61)	26
Peritoneal metastasis	1 (100)	0 (0)	1
Colon adenoma	4 (36)	7 (64)	11

been retained. The data were analysed with respect to specific type of Ki-*ras* mutation (Table 5). Little difference in the incidence of the various mutations between this and other similar studies (Bos et al., 1987; Boughdady et al., 1992; Finkelstein et al., 1993; Moerkerk et al., 1994; Span et al., 1996) were noted except that transversions to GTT tended to be less prevalent than in Yugoslavia (Urosevic et al., 1993). Mutation at codon 13 was limited to the GAC

transversion. Also, no codon 12 GGT to CGT transversion was found though this is a common mutation of Ki-*ras* in pancreatic adenocarcinoma (Bos, 1989).

Table 5: Type and frequency of specific Ki-*ras* codon 12 and 13 mutations in primary and secondary carcinomas (lymph node and liver metastases) in patients with colorectal adenocarcinoma

Mutation	Primary carcinoma (%)	Secondary carcinoma (%)
Codon-12		
AGT	2 (8)	0
GAT	10 (38)	8 (38)
CGT	0	0
GCT	3 (11)	1 (5)
GTT	4 (15)	7 (33)
TGT	1 (4)	2 (10)
Total	20 (77)	18 (86)
Codon 13		
GAC	6 (23)	3 (14)
Codons 12 + 13	26 (100)	21 (100)

Metastatic deposits showed a similar trend. There were no statistically significant differences in the incidence of particular mutations between primary carcinomas and metastatic lesions. However, when the data were viewed with respect to Dukes' stage (Table 6), the codon 12 GGT to GTT transversion was exclusively found in primary carcinomas and metastatic deposits of Dukes' C and D patients. The incidence of all other types of mutation was not significantly different in terms of Dukes' classification.

Of possible significance, five of the Dukes' D patients, of whom two had been staged as Dukes' A, and three as Dukes' B on presentation, had later relapsed with liver metastases; in three of these patients (one Dukes' A and two Dukes' B) the primary carcinoma harboured a GTT mutation also found in the

subsequent metastatic deposits. In another, the primary carcinoma had a GAC mutation in codon 13, while in the remaining patient the primary and the metastatic carcinoma harboured wild-type Ki-*ras*.

Table 6: Type and frequency of specific Ki-*ras* codon 12 and 13 mutations in relation to Dukes' classification

Mutation	Dukes' A and B (n = 31)	Dukes' C (n = 21)	Dukes' D (n = 26)
Codon 12			
AGT	2	0	0
GAT	6	1	4
CGT	0	0	0
GCT	2	1	0
GTT	0	2*	5*
TGT	0	0	1
Total (%)	10 (32)	4 (19)	10 (38)
Codon 13 GAC	3 (10)	1 (5)	4 (15)
Codons 12 + 13 (%)	13 (42)	5 (24)	14 (54)

***Frequency of codon 12 GGT to GTT transversions in primary and secondary carcinomas of Dukes' C/D patients is highly significant (P = 0.01) as measured by Fisher's exact test. No statistical differences were observed between the other types of Ki-*ras* mutations in relation to Dukes' classification (P>0.05).**

*7.3. **Ki-ras mutations and survival***

The relationship between Ki-*ras* mutations and aggressiveness was investigated further by examining disease-free and overall survival using Kaplan-Meier survival curves. No significant difference in disease-free and overall survival was observed between carcinomas with Ki-*ras* codon 12 and 13 mutations and carcinomas carrying wild type Ki-*ras* (Figure 12). However, overall survival was significantly (P=0.001) decreased in patients with GGT to GTT transversions compared to patients with no Ki-*ras* mutation (Figure 13). Moreover, a comparison between Dukes' D patients with wild type Ki-*ras* and Dukes' D patients with GGT to GTT transversions in codon 12 of Ki-*ras* showed that overall survival of the latter was significantly shorter (P=0.012) (Figure 14). No other statistically significant effects of Ki-*ras* mutations on overall or disease-free survival were found.

Figure 12: *Survival curves of patients with colorectal cancer*

A) Disease-free survival curves from 43 patients with wild type Ki-*ras* (black line) compared to 28 patients with mutated codons-12 and 13 Ki-*ras* (red line). No statistical differences in survival were observed between the two groups (P>0.05).

B) Overall survival curves from 43 patients with wild type Ki-*ras* (black line) compared to 28 patients with mutated codons-12 and 13 Ki-*ras* (red line). No statistical differences in survival were observed between the two groups (P>0.05).

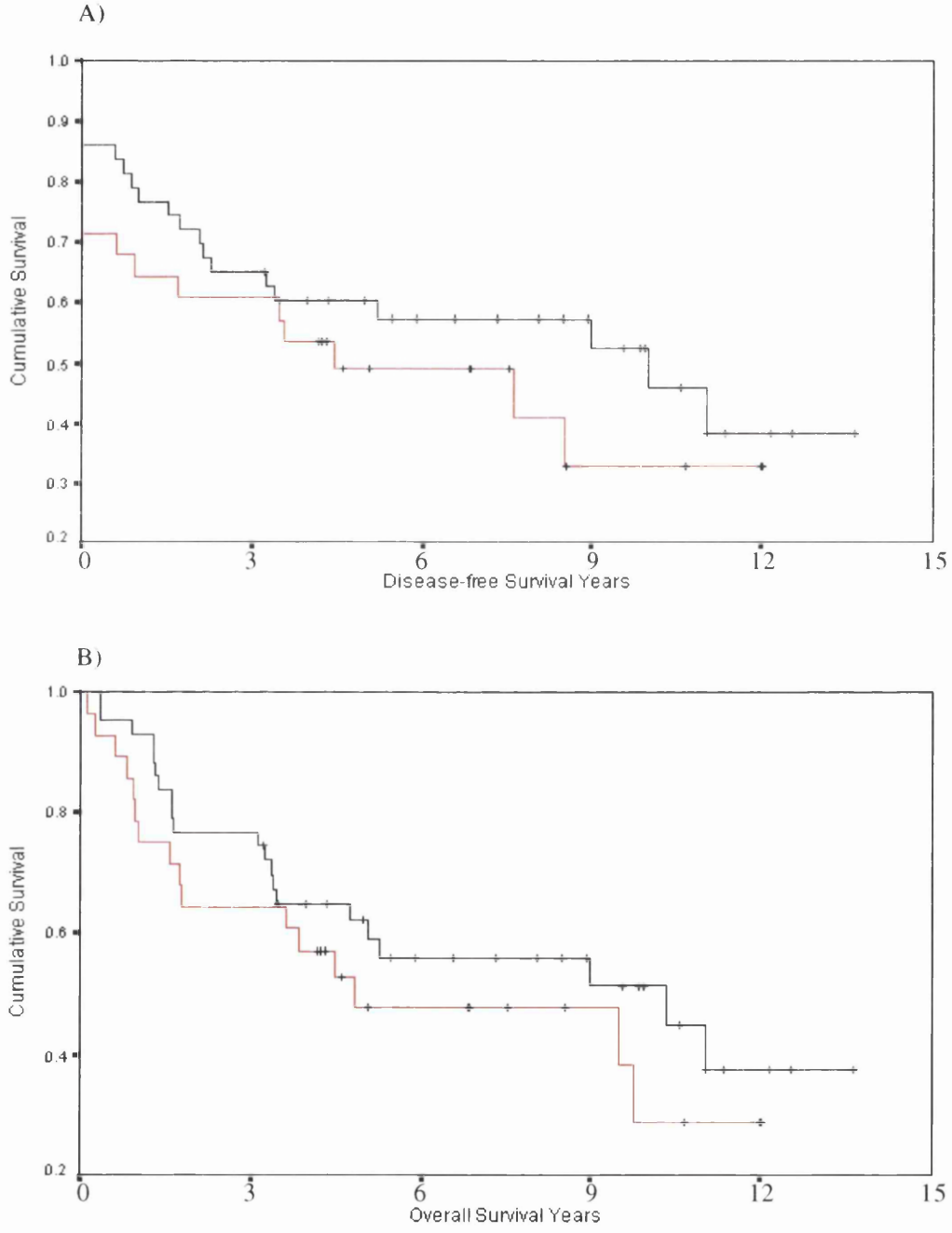


Figure 13: *Kaplan-Meier survival analysis of patients with colorectal cancer*

Survival curves from 43 patients with wild type Ki-*ras* (black line) compared to 8 patients (A) with codon-13 mutated Ki-*ras* (blue line) and 11 patients (B) with GAT mutated codon-12 (green line).
No statistical differences in survival were observed between the three groups (P>0.05).

C) Seven patients with GGT to GTT transversion in codon 12 of Ki-*ras* (red line) and 43 patients with wild type Ki-*ras* (black line). Overall survival was significantly reduced in patients with the GTT mutation (P=0.001; log-rank test).

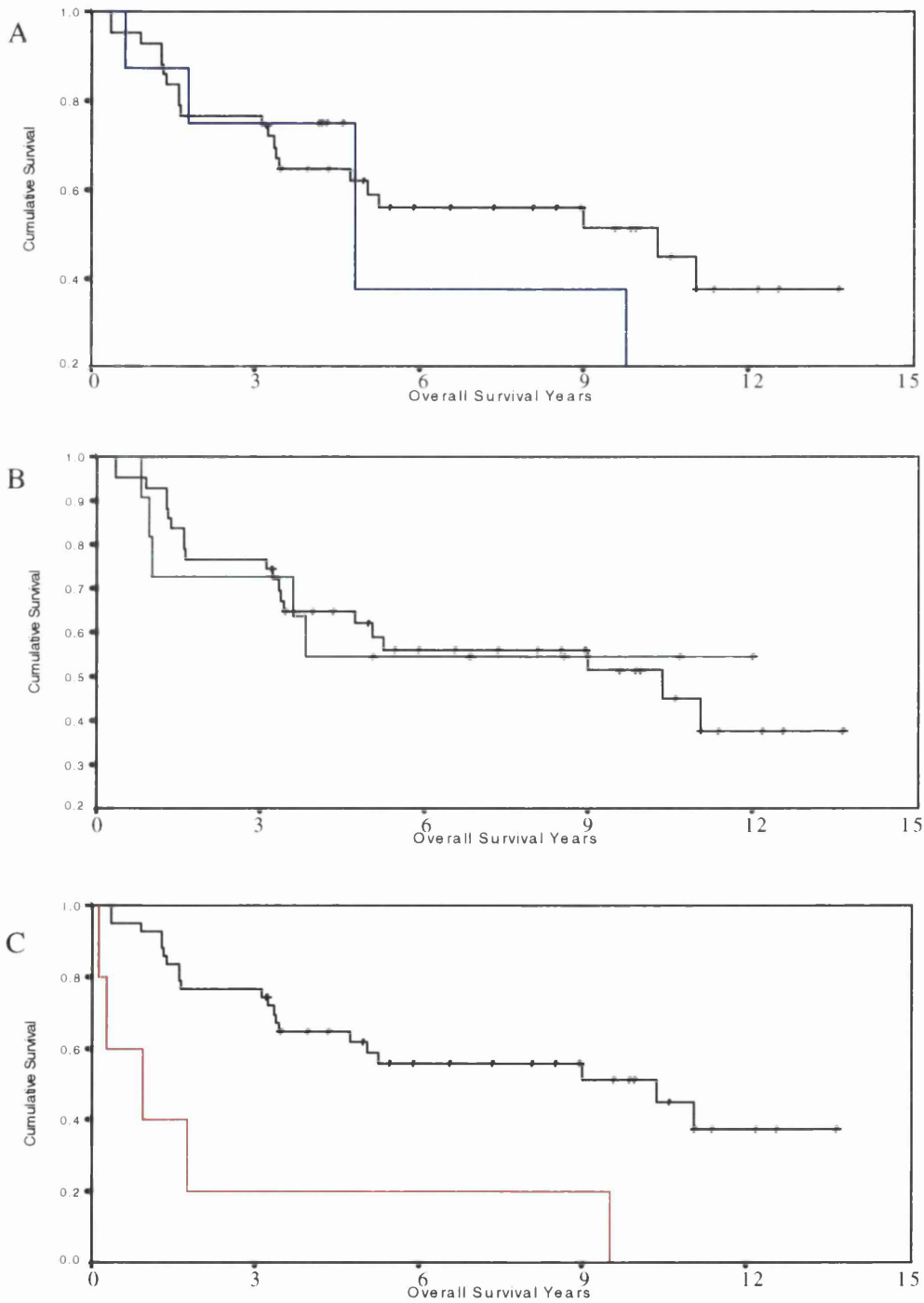
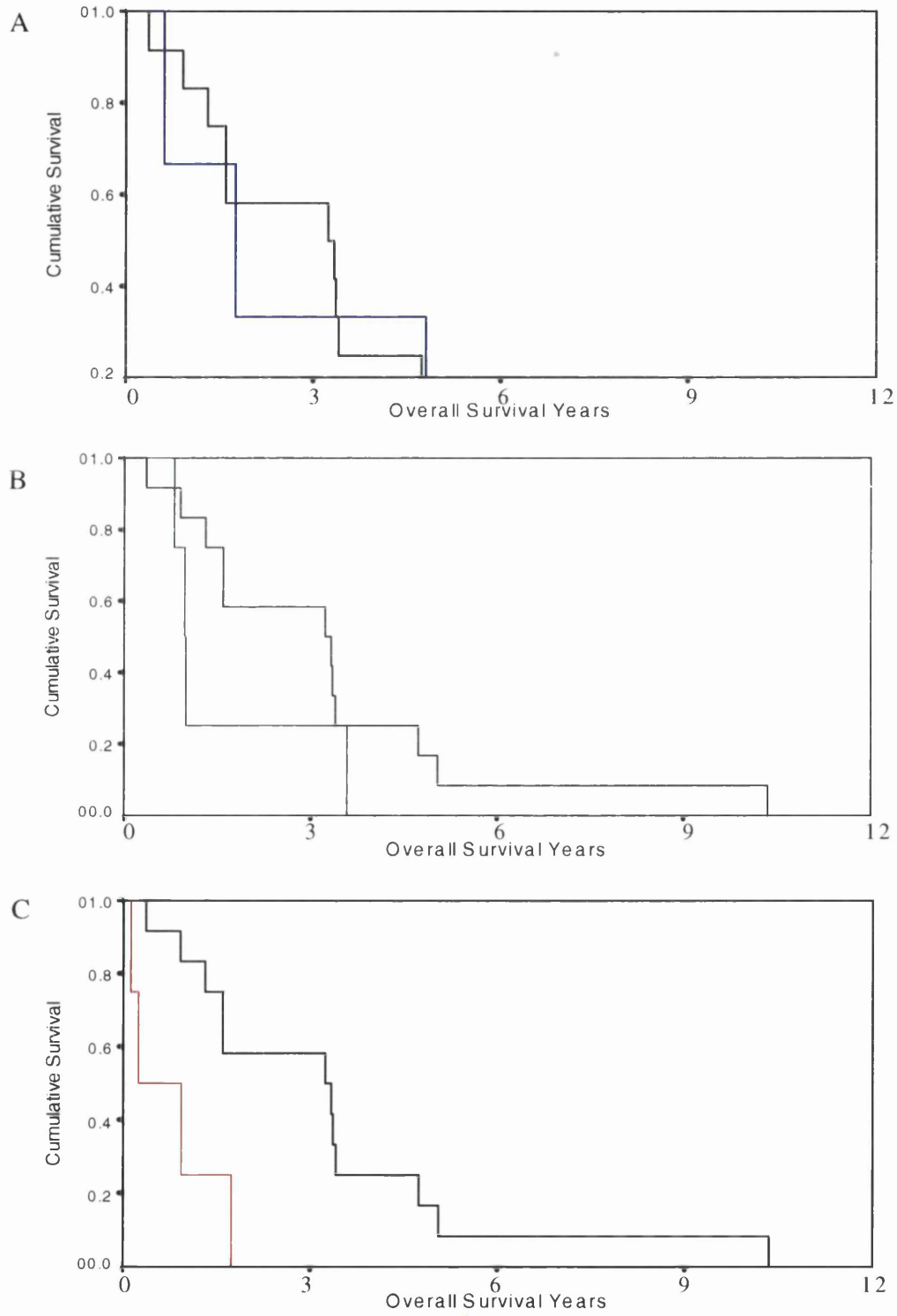


Figure 14: *Kaplan-Meier survival analysis of Dukes' D patients*

Kaplan-Meier survival analysis of (A) 3 Dukes' D patients with GGC to GAC mutation in codon 13 of Ki-*ras* (blue line), (B) 4 Dukes' D patients with GGT to GAT transition in codon-12 of Ki-*ras* (green line), and (C) 5 Dukes' D patients with GGT to GTT transversion in codon 12 of Ki-*ras* (red line) compared to 12 Dukes' D patients with wild type Ki-*ras* (black line). Patients with only a GTT mutation have a significantly shorter survival than patients with the same Dukes' stage but harbouring the wild type Ki-*ras* (P=0.012; log-rank test).



7.4. Heterogeneity of tumours

In the majority of patients with Ki-*ras* mutations the same mutation was found in adenomas, primary carcinomas and their metastases, but there were exceptions. For example (Figure 15), both specimens of primary carcinoma from one patient had a GAT mutation (A9, A10), as had the corresponding liver metastasis (B1), but three adenomas from the same patient had three different mutations, one to GAT (A5), one to GTT (A8) and one to TGT (not shown). As these were probably independent neoplasms, this result is not surprising. In a second patient, three specimens of primary carcinoma (B4-6) and two of a lymph node metastasis (B7, B8) had a codon 12 GTT mutation, but only one of two liver metastasis specimens (C1) carried this mutation (Figure 15).

No instances of multiple mutations in a single carcinoma, as described by Span et al., (1996), were found even when multiple sections of carcinomas were analysed. However, heterogeneity in the presence of a Ki-*ras* mutation within and between primary and secondary carcinomas was frequent, both between different sections of the same carcinoma, and between closely adjacent regions of carcinoma cells from a single section (Table 7). Moreover, in 2 of 26 Dukes' D patients (numbers 3 and 10) a mutation was found in the primary carcinoma but none in liver metastases and, conversely, in 6 of 47 Dukes' C and D patients (numbers 4, 5, 6, 7, 11 and 15) there was a Ki-*ras* mutation in a liver or lymph node metastasis, but none was found in the primary carcinomas (Table 7).

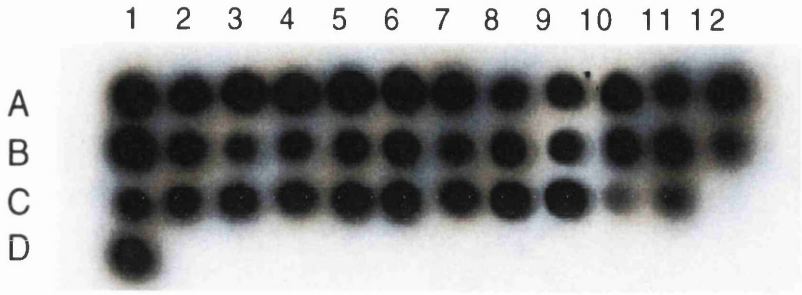
Figure 15

Figure 15: *Detection of Ki-ras heterogeneity by allele-specific oligonucleotide hybridisation*

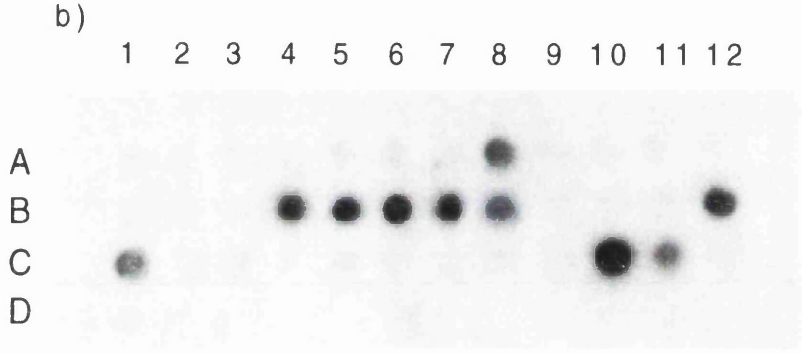
Detection of Ki-*ras* mutations by allele-specific oligonucleotide hybridisation to PCR-amplified DNA from microdissected normal mucosa, adenoma, primary and secondary tumours from three patients, two showing heterogeneities.
Hybridisations were with ^{32}P-labelled oligomers specific for (a), GGT (gly); (b), GTT (val); (c) GAT (asp) at codon 12.

A5 to B3-DNAs from one patient: A5, A8, A12, from 3 adenomas; A9, A10, adenocarcinoma from two parts of the same section of primary tumour; A6, A11, adjacent normal mucosa; B1, B2, liver metastasis; B3, adjacent normal liver.
B4 to C1 - DNAs from a second patient: B4, B5, B6, adenocarcinoma from three parts of the same section of primary tumour; B7, B8, B12, lymph node metastases; B9, C1, two parts of the same section of a liver metastasis; B10, B11, adjacent normal liver and mucosa, respectively.
Controls: C12, negative control (no DNA); C10, GTT-positive mutant DNA; D1, GGT-positive wild-type DNA.

a)


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D

b)


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D

c)

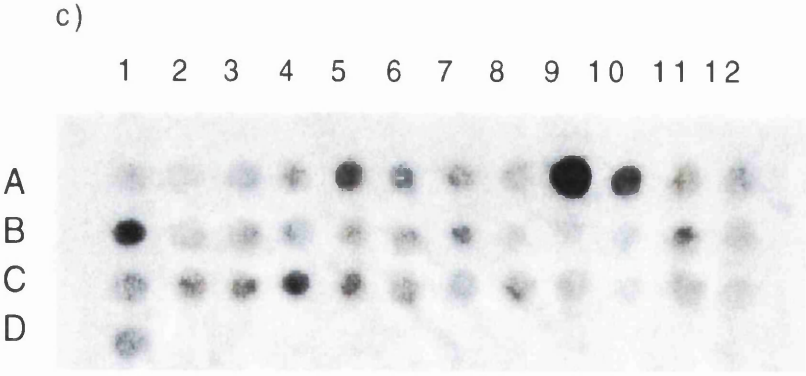

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D

Table 7: Heterogeneity of Ki-*ras* mutations within and between primary and secondary carcinomas

Patient	Adenoma	Primary carcinoma	Lymph node metastasis	Liver metastasis
1	GCC/GGV/N[1]	GN		GN
2		GGNN		
3	GG	GGGN		GG
4		GG	N	NN
5		GG		GV
6		GG	VV	
7		G	GV	
8		GCC	CCC	C
9		GV	GG	
10		GN		GGGG
11	GGG	G	N	N
12		GS		
13		GN		
14		GN		
15		GGG	GN	

Results for replicate samples from the same section shown by individual symbols; G, wild-type codon 12 and 13; codon 12 mutations: N, GAT; V, GTT; C, TGT, S, AGT; codon 13 mutation: N, GAC. Each sample was assayed at least 3 times by ASO hybridization by two independent observers; most were also confirmed by direct sequencing.

[1]Sections from three separate adenomas.

CHAPTER 8. Characterisation of two different Ki-*ras* codon-12 mutations *in vitro*

An important concept that emerged from the analysis of Ki-*ras* mutation *in vivo* is that transversion mutation GGT-12 to GTT appears to be associated with aggressive colorectal tumours compared to the more common GAT-12 transition mutation. The reasons behind such difference in the biological behaviour of the two mutants can be ascribed to many factors including differences in tumourigenicity, invasion, growth and VEGF production to mention a few. It was decided to compare some of the characteristics of the two mutants *in vitro* to see whether the difference in the behaviour found *in vivo* could also be reflected *in vitro.*

8.1. ***Ki-ras expression***

As described in section 6.2.1, Rat-1 cells were transfected with wild-type or GTT-12 (valine) or GAT-12 (aspartate) mutant human Ki-*ras* expression constructs along with the plasmid (Pum-1) conferring resistance to G418. Of six Rat-1 clones transfected with each type of Ki-*ras*, two clones of each Ki-*ras* type expressing the appropriately transfected Ki-*ras* were selected for subsequent analysis. Allele-specific oligonucleotide hybridisation of RT-PCR products and Western blotting were used to demonstrate the expression of the appropriate type of Ki-ras in these cells (Figure 16). Of the two Rat-1 clones expressing mutant GAT-12 mutant Ki-Ras, GAT clone 6 expressed lower levels of Ras as assessed by Westen blotting. However, Ras mRNA expression of the transfected GAT-12 Ki-*ras* could be detected by RT-PCR from this clone (Figure 16). Due to this inconsistency, it is advisable to treat the results obtained from this clone cautiously.

*8.2. **Invasion assay***

Invasion by tumour cells of the extracellular matrix and the basement membrane is a crucial event in tumour metastasis. Therefore, the ability of the various codon-12 Ki-Ras clones to invade matrigel *in vitro* was tested. All cells, including controls were able to invade matrigel in the presence of EGF (Figure 18a). Consistently, valine-12 (GTT-12) mutants Ki-Ras clones demonstrated 3 to 5 folds increased invasion *in vitro* compared to the aspartate-12 mutant clones in the absence of growth factors (Figure 17 and 18b). The invasive capability of valine-12 clone 6 in the absence of growth factor was particularly as efficient as in the presence of growth factor. Remarkably, clone 4, which expressed high levels of aspartate-12 mutant Ki-Ras demonstrated significantly lower invasive capability compared to valine-12 mutant clones (Figure 18b and c). To demonstrate that mutant valine-12 Ras (GTT-12) and not random genetic events was directly responsible for the enhanced *in vitro* invasion, antisense oligonucleotide against valine-12 was used. Antisense oligonucleotides centred along the GTT codon-12 mutation reduced invasion back to control levels. On the other hand, sense oligonucleotides had no effect on invasion (Figure 18d).

Figure 16: *Western analysis and allele-specific oligonucleotide hybridisation showing relative abundance of Ras proteins and mRNA expressed by control and Ki-ras transfected Rat-1 cells*

A) 10 % polyacrylamide immunoblot detecting the relative abundance of Ki-Ras protein. B) Detection of the appropriate Ki-*ras* mutations by allele-specific oligonucleotide hybridisation to amplified cDNA as described in section 6.2.2. Membrane probes with a) GGT-12 specific probe (Wild type), b) GTT-12 specific probe and c) GAT-12 specific probe as described in section 6.1.4.

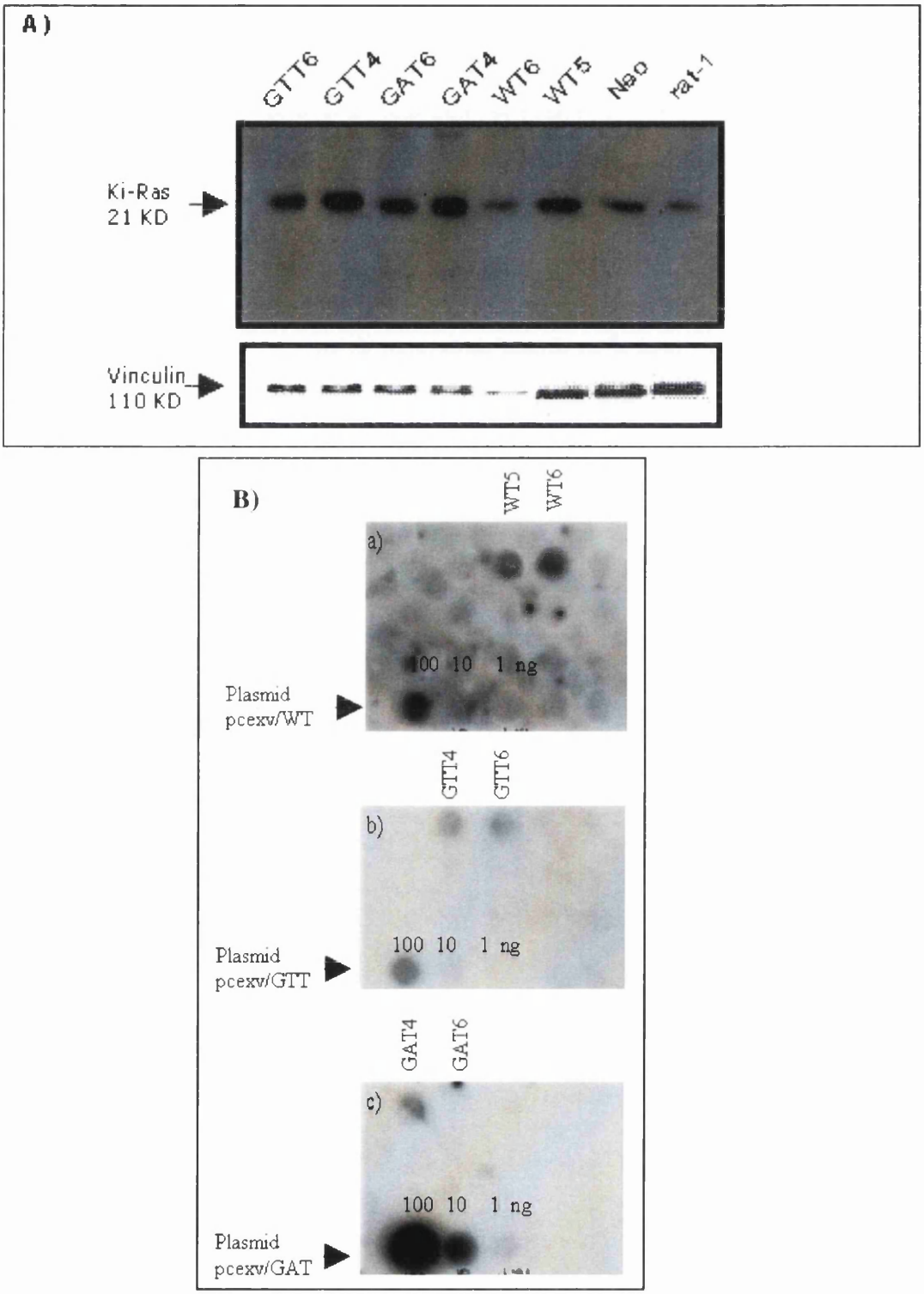
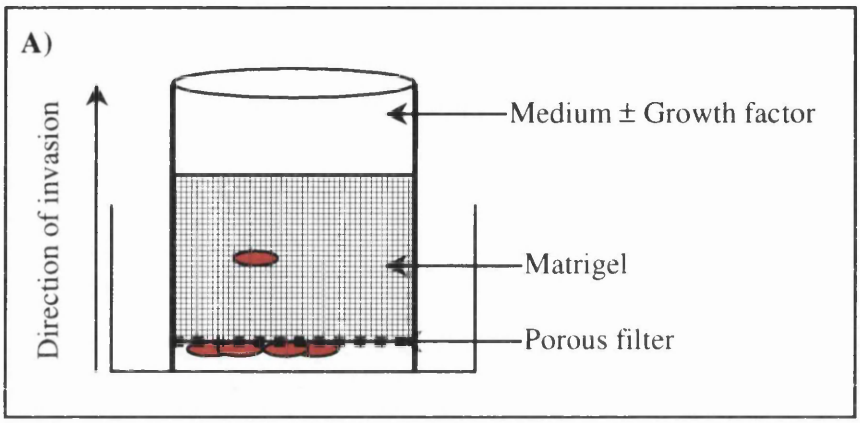


Figure 17: *In vitro invasion assay*

Confocal microscopy images of propedium iodide-stained cell nuclei (red dots) at 0, 10, 20, 30 µm in Matrigel of Rat-1 cells transfected with valine-12 (GTT clone 6), aspartate-12 (GAT clone 4), wild-type Ki-Ras (WT clone 5) and control neo transfected cells. A) represents a cartoon of the invasion well.



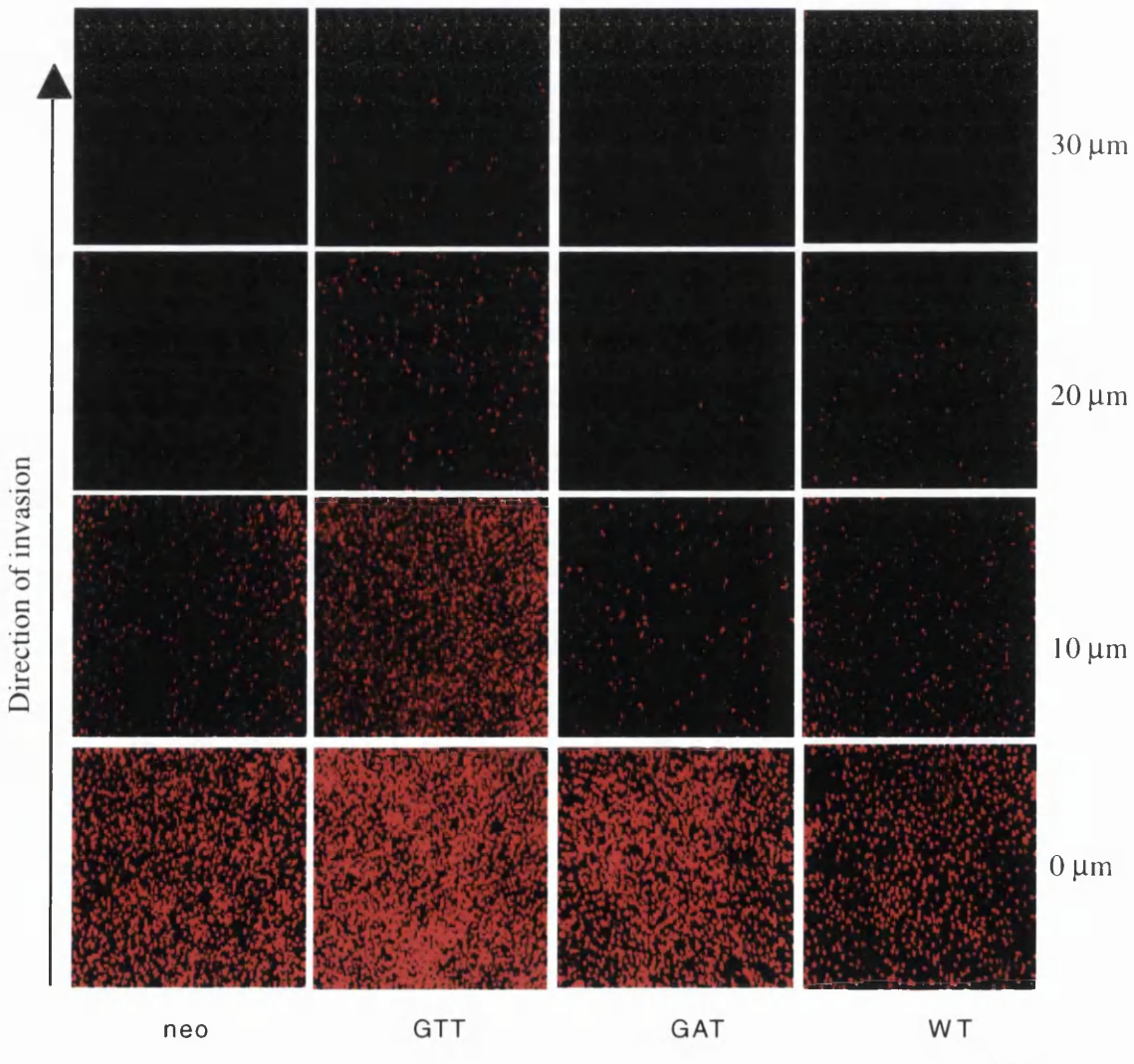
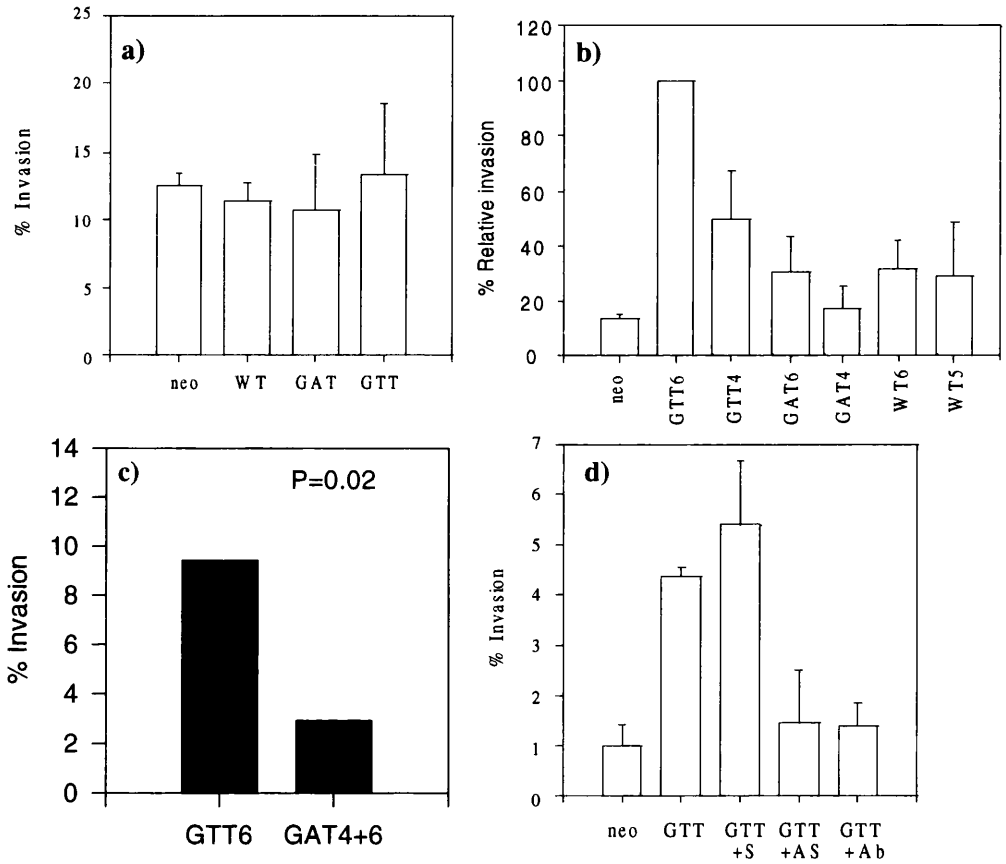


Figure 18: *Quantitative analysis of invasion.*

Invasion assay results were quantitated as described in the material and methods with a Bio-Rad program (Comos) and represent the average of two separate assays from three experiments. a) Invasions of GAT, WT, GTT or neo clones in the presence of epidermal growth factor. b) Invasions of GAT, WT, or neo clones are expressed as percentages of GTT clone 6 (100 %). c) The differences in invasion between GTT clone 6 and GAT clones 4 and 6 is statistically significant p =0.02 using student's *t*-test. (If GTT clone 4 is included in the analysis, the statistical difference in invasion becomes insignificant). d) Invasion of Rat-1 cells transfected with valine-12 mutant Ki-Ras (GTT 6 clone) with no treatment (GTT), Ki-ras antisense (GTT+AS), sense (GTT+S) or anti-α_6 integrin antibody (GTT+Ab).

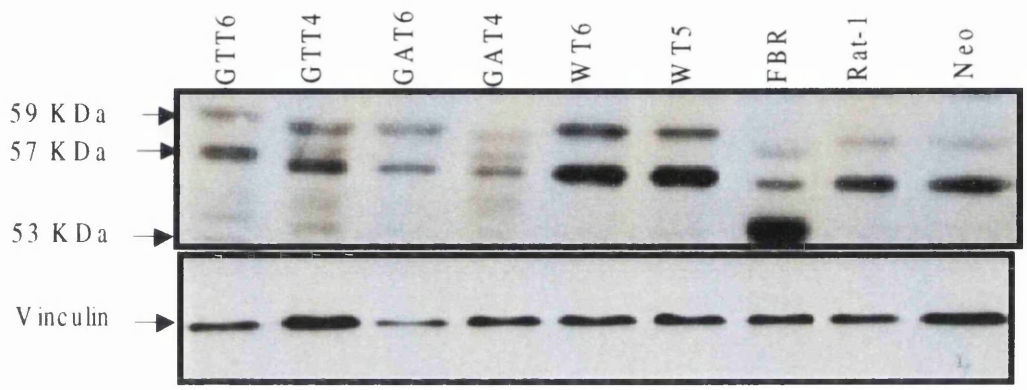


The difference in Matrigel invasion capabilities of the two mutants could partly be due to differences in matrix metalloproteases production. Stromelysin-1 was chosen for three reasons. Firstly, Ras has been previously shown to increase stromelysin-1 production (Matrisian et al., 1991). Secondly, Matrigel is primarily made up of laminin, collagen VI, and proteoglycans, which are prime substrates of stromelysin-1 (it is devoid of fibronectin and vitronectin) (Coussens and Werb, 1996; Kleinman et al., 1986) and thirdly, Matrigel invasion has been shown to depend primarily on the expression of stromelysin-1, rather than on other protease families (Lochter et al., 1997).

Stromelysin-1 (57 and 59 KDa) was surprisingly upregulated in cells expressing wild type Ki-*ras*. Notably, cells containing valine-12 (clones 4 and 6) activated Ras expressed 2-fold more stromelysin-1 than clone 4 expressing GAT-12 Ras. Although clone 6 containing GAT-12 Ras still expresses high levels of stromelysin-1. However, it should be noted that results from GAT-12 clone 6 should be interpreted cautiously. The reduced expression of stromelysin-1 demonstrated by GAT-12 clone 4 cells is consistent with their decreased ability to invade Matrigel (Figure 19).

Figure 19: *Western analysis showing relative abundance of stromelysin-1 expressed by control and various Ki-ras transfected Rat-1 cells*

7.5 % immunoblot for Stromelysin-1 (Transin), arrows showing pro-stromelysin (59 KD) and stromelysin-1 (57 KD). The blot was re-probed with antivinculin MAb to control for loading variations and transfer. The Table shows relative abundance of the 59 and 57 KDa stromelysin-1 normalised against the loading control. This was repeated twice using different protein preparations with consistent findings.



	GTT6	GTT4	GAT6	GAT4	WT6	WT5	FBR	Rat-1	Neo
59 KDa	0.66	0.7	0.9	0.35	1	0.82	0.42	0.5	0.28
57 KDa	0.71	0.6	0.68	0.5	1	1	0.5	0.86	0.64

8.3. Transformation of Rat-1 cells in vitro

The increased *in vitro* invasiveness of Rat-1 cells containing the mutant GTT-12 Ki-*ras* could be ascribed to many factors including increased tumourigenicity, transformation and changes in adhesion molecules. Although, neither tumourigenicity nor anchorage independent growth were studied, the ability of mutant Ras to induce cellular transformation *in vitro* was assessed. Both valine-12 and aspartate-12 mutant Ki-Ras were able to equally disrupt the quiescent monolayer (this was more pronounced in GAT-12 clone 4 than GAT-12 clone 6) and form disordered network of spindled refractile Rat-1 cells compared to the flat morphology of Rat-1, neo and wild type Ki-Ras transfected cells. Wild type Ki-Ras transfected cells demonstrate close cell to cell contacts, whereas, both mutant Ki-Ras transfected cells demonstrated significant loss of cell-cell adhesions (Figure 20).

The effects of mutant and wild type Ki-Ras on adhesion molecules were also investigated, since the dramatic cell shape, cell to cell contacts and cellular invasion could be due to perturbation of adhesion molecules involved in cell to cell and cell to matrix adhesions. Both mutant Ki-Ras transfected cells demonstrated complete loss of E-cadherin, whereas cells containing wild-type Ki-Ras maintained E-cadherin expression (Figure 21). Similarly, both mutants Ki-Ras containing cells showed a dramatic loss of β_1 integrin (Figure 21). The effects of mutant Ki-Ras on these adhesion molecules could explain the dramatic cell shape changes seen in mutant Ki-Ras transformed cells. It can also explain the reduced ability of wild-type Ki-*ras* expressing cells to invade the Matrigel compared to cells expressing the mutant GTT-12 Ki-*ras*. Although the former,

expressed higher levels of stromelysin-1. Thus, the maintained expression of E-cadherin and β_1 integrin could have played a major part in reducing *in vitro* invasiveness of Rat-1 cells expressing the wild-type Ki-*ras*. However, the loss of E-cadherin and β_1 integrin expression was comparable between the two mutant Ki-*ras* expressing cells, which could indicate that the reduced expression of stromelysin-1 by clone 4 GAT-12 expressing cells was probably partly responsible for their reduced invasiveness *in vitro*. Notably, the loss of E-cadherin and β_1 was not as pronounced in GAT-12 clone 6 as GAT-12 clone 4 or GTT-12 clone 6.

Integrin α_6 has been shown to be associated with tumour aggressiveness *in vivo* and to stimulate chemotactic migration of cells *in vitro* (O'Connor et al., 1998). Therefore, anti-integrin α_6 antibody was used to test its effect on invasion. The antibody was able to reduce *in vitro* invasion of the valine-12 mutant clones demonstrating the importance of this integrin monomer in the invasive process (Figure 18d). Integrin α_6 monomer can dimerise with β_1 and β_4. Since β_1 is significantly reduced in these cells, it was of interest to investigate the levels of β_4 integrin in the cells. Unfortunately, during the time of analysis, there were no suitable antibodies against β_4 monomer available for Westen analysis.

Figure 20: *Phase-contrast microscopy images of Rat-1 cells growing in DMEM containing 10% FCS*

A) Rat-1 cells expressing valine-12 mutated Ki-Ras protein (GTT clone 6). B) Rat-1 cells expressing Aspartate-12 mutated Ki-Ras protein (GAT clone 4). C) Rat-1 cells expressing wild-type Ki-Ras protein (WT clone 5). D) Control Rat-1 cells transfected with plasmids carrying neomycin resistance only (neo). Magnification 100 ×

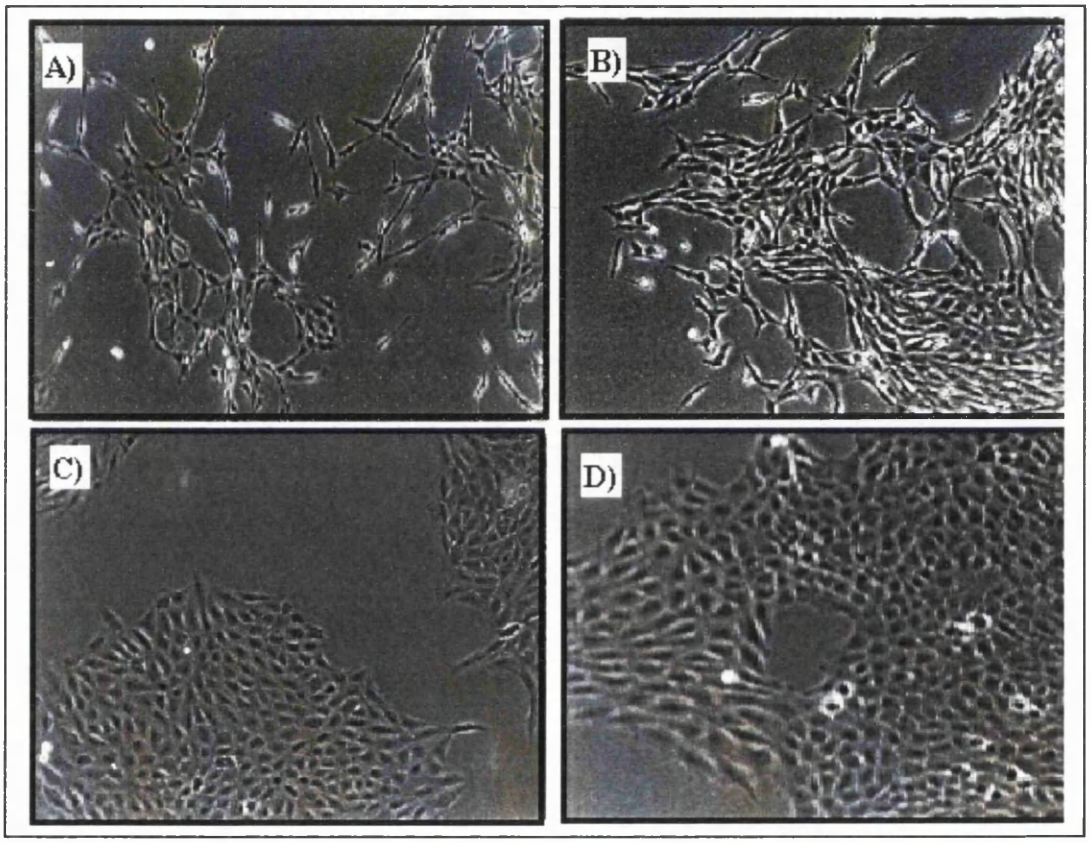
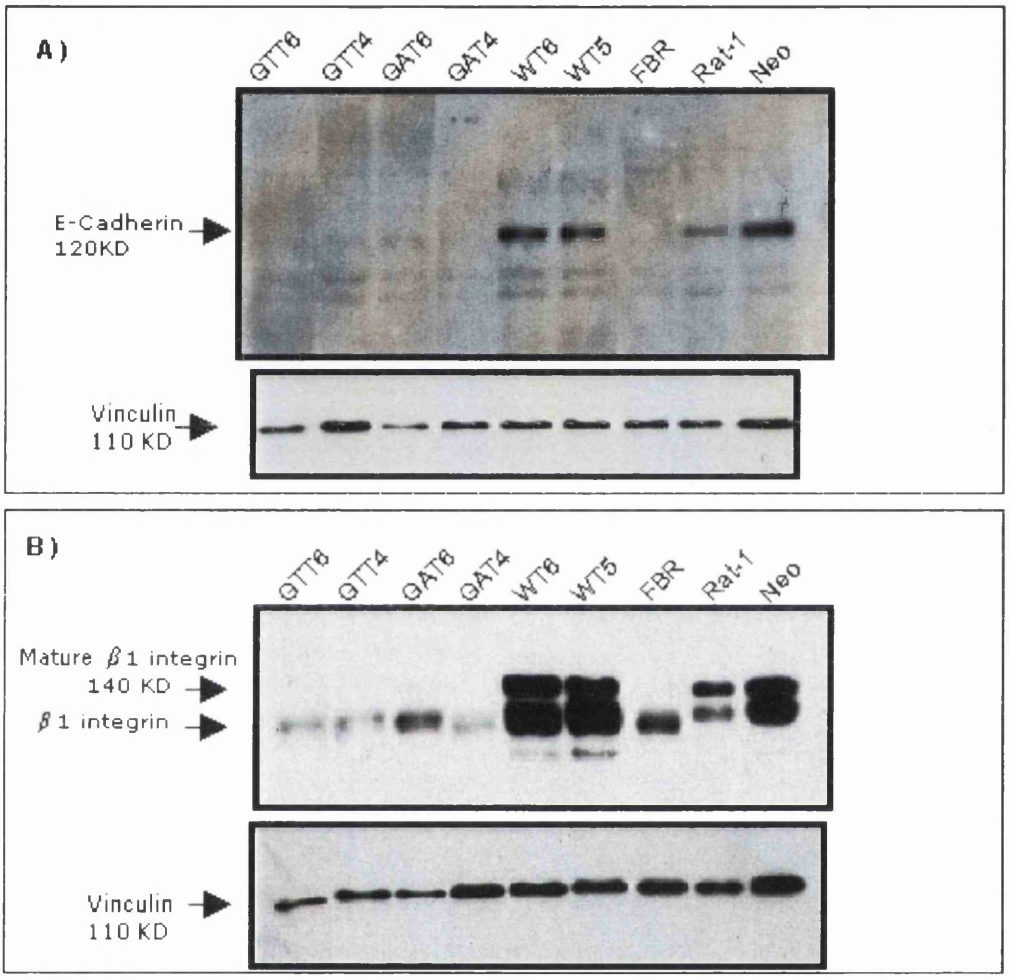


Figure 21: *Western analysis showing relative abundance of E-cadherin and β_1 integrin proteins expressed by control and Ki-ras transfected Rat-1 cells*

A) 7.5 % immunoblot for E-Cadherin. B) 7.5 % immunoblot shows β_1 integrin. Blots were probed with anti-vinculin MAb to control for loading variations and transfer.



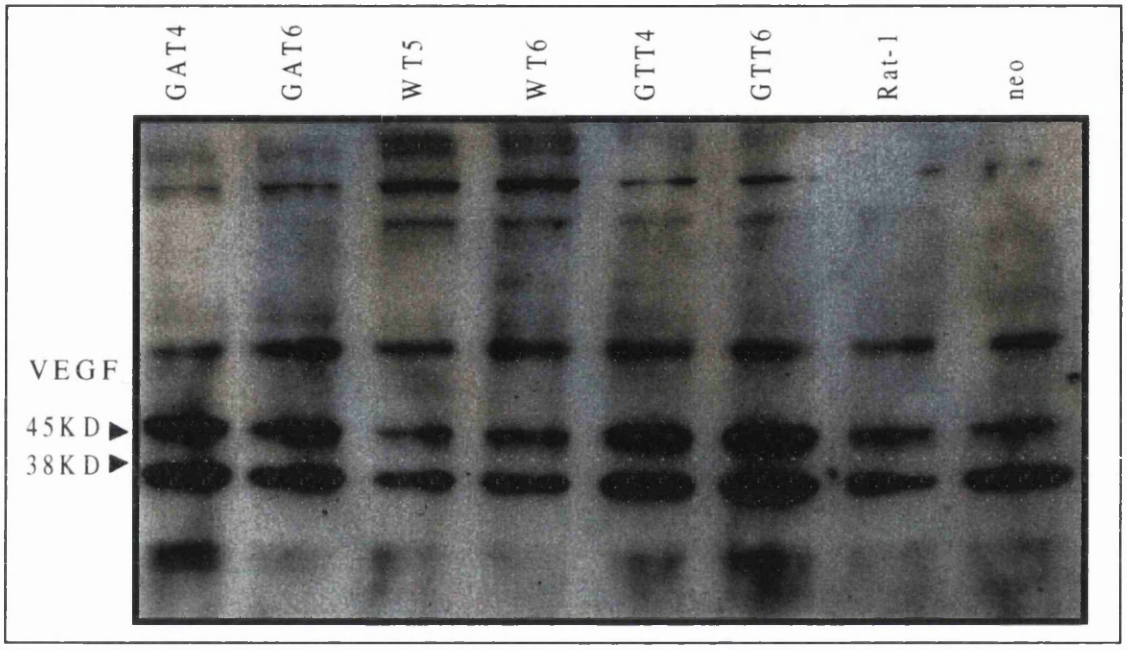
8.4. VEGF production

It is now well established that VEGF production by tumour cells improves not only their growth but also their subsequent dissemination. Thus, genetic or epigenetic events that induce VEGF production could play an important role in inducing tumour growth and metastasis.

As discussed in section 3.5.3, activated Ras has been shown to induce VEGF production. However, most, if not all, work done in this area has used valine-12 activated Ras to establish such association. It was, therefore, of interest to examine the extent to which the two mutants differ in stimulating VEGF production. Therefore, valine-12 (GTT), aspartate-12 (GAT) and wild-type (GGT) Ki-*ras* transfected cells were analysed with respect to their ability to induce VEGF production. Western analysis against VEGF demonstrated the ability of the two mutant Ki-Ras bearing cells to increase production of various isoforms of VEGF. On the other hand, Rat-1 cells transfected with wild type Ki-*ras* or control plasmids had no effect on VEGF production (Figure 22).

Figure 22: *Western analysis showing relative abundance of VEGF protein expressed by control and Ki-ras transfected Rat-1 cells*

10 % immunoblot for VEGF. The arrowheads indicate the major isoforms. Equal loading was confirmed by staining the membrane with Giemsa. Mrs Elaine MacKenzie kindly contributed this result.



CHAPTER 9. Structural characterisation of Valine-12 and Aspartate-12 Ki-*ras* mutants

The data described above linking valine-12, but not aspartate-12, mutations with more aggressive disease *in vivo* and more invasive phenotype *in vitro* raise the important question of how differences in the amino acid residue substituted for glycine at codon 12 cause differences in the biological activity of Ras. One idea is that it is due simply to differential GTPase inhibition. However, the difference in this respect between G12D and G12V is relatively small (Table 8) and it is necessary to consider other possible ways in which these mutants could affect downstream events to different extents. Although the structures of various Ras mutants have been analysed in detail, there has as yet been no attempt to correlate conformational differences between Ras mutants with their differences in signal transduction activity and tumour aggressiveness. To address this question, comparison of the molecular structures and other biochemical characteristics of the more aggressive G12V mutant Ras with the more common, but less aggressive, G12D mutant, was made.

9.1. Review of published biochemical properties of mutant Ras protein

The known biochemical differences between glycine-12 (wild type), valine-12 and aspartate-12 mutant Ras are summarised and compared to other codon-12 mutant Ras proteins in Table 8.

The GTPase activity of the valine-12 mutant, which is one-fourth of that of the aspartate-12 mutant (Goody et al., 1992) and one-tenth of that of the wild-type Ras (John et al., 1988; John et al., 1989). The importance of this is outweighed by the fact that GAP stimulates the GTPase activity of wild type Ras whereas both the valine-12 and aspartate-12 mutants are unaffected by GAP. These mutants do, however, still bind GAP. The G12D mutant binds as well as the wild type while G12V has a three-fold lower affinity for GAP.

Table 8: Biological and biochemical characteristics of codon 12 mutant Ras proteins relative to wild type

Codon 12	Transformation	GTPase	GTP dissociation	GAP affinity	GAP activation	Raf-1 binding
WT	-[1]	1	1	1	+++	1[2]
G12 V	++++[1]	0.1[3,4]	1[3,4]	0.3[5]	-[5]	2.25[2]
G12 D	++[1]	0.2[7] -0.4[6]	8[7]	1[8]	-[7]	ND
G12 P	-[1]	2.7[7]	1[7]	0.3[7]	+[7]	1[2]
G12 R	++++[1]	0.05[3,4]	1[3,4]	0.2[5]	-[5]	1[2]

The GTPase, GTP dissociation, GAP affinity, and Raf binding in wild-type Ras (WT) were arbitrary chosen as 1. ND is not determined. Numbers in brackets are references.

1. (Seeburg et al., 1984) 2. (Herrmann et al., 1995) 3. (John et al., 1988) 4. (John et al., 1989) 5. (Krengel et al., 1990) 6. (Goody et al., 1992) 7. (Franken et al., 1993) 8. (Bollag and McCormick, 1991).

One notable difference in Table 8 is that the rate at which the GTP analogue GppNp dissociates from its complex with Ras is 8-fold greater with the

Asp-12 mutant Ras (Franken et al., 1993) than with either wild type or Val-12 mutant Ras (John et al., 1988; John et al., 1989).

The X-ray structure of the complex between GppNp and G12D shows that the Asp-12 side chain is hydrogen bonded to the side chains of both Gln-61 and Tyr-32. The Asp-12 side chain oxygen hydrogen-bonded to Tyr-12 is also very close to the γ-phosphate of the GppNp (the O...O distance is 2.65Å, 1Å=10^{-1} nm). Under physiological conditions, both are negatively charged so it is likely one or other is protonated, in which case there is a hydrogen bond between them. ^{31}P NMR evidence with the Ras-GppNp complex indicates that it is the γ-phosphate that is protonated (Franken et al., 1993).

The γ-phosphate of GTP is less able to become protonated as its pKa is about 2.0 pH units (100-fold) lower than that of GppNp (Yount, 1975), so binding of GTP by Asp12 Ras is likely to be even weaker than that of its analogue. Taken together, with the observation that the GppNp dissociation rate from G12D is 8-fold faster than that from Ras (Table 8) the binding of GTP to the aspartate mutant could be as much as 800-fold weaker than for the wild type. However, dissociation is not the same as hydrolysis to GTP, since after dissociation of GTP, a likely next stage is reassociation of GTP (Feuerstein et al., 1987). It also has been observed that Ras in the absence of the nucleotide is unstable, so the weaker GTP binding may make the G12D Ras more susceptible to breakdown by proteolysis (John et al., 1990). Nevertheless, together the kinetics of the Ras-GTP dissociation and the GTPase measurements indicate that Val-12 Ras forms a more stable complex with GTP than Asp-12 Ras does. Thus, probably providing a more constant positive signal to the pathways it stimulates.

9.2. Structural properties of the valine-12 and aspartate-12 mutants

The comparisons of the three-dimensional structures of GppNp-bound G12V and G12D show there to be significant differences between the mutants that may provide reasons for the biochemical and biological differences between them (Figure 23). The hydrogen bond network of the carboxylate side-chain of the G12D mutant involving Asp-12, Gln-61 and Tyr-32 has already been mentioned (Figure 23 b). In contrast, in Gly-12 and Val-12 Ras (Figure 23 a and c), Tyr-32 takes up a distinctive orientation in which it is pointing more away from the surface. (At this point, it should be noted that in the crystal lattice the tyrosine is hydrogen bonded to the γ-phosphoryl group of the nucleotide of an adjacent Ras molecule; however in the molecule in the living cell it is probably mobile.) The hydrogen bonding between, and subsequent orientations of, Asp-12, Tyr-32 and Gln-61 cause the Asp-12 mutant to differ from Gly-12 and Val-12 Ras in at least three ways that could affect the recognition site for an effector protein approaching Ras from the exterior. First, in the Asp-12 mutant, Tyr-32 is pulled towards the interior of the molecule to occupy the b and g crevice for the phosphates of GTP. Second, Gln-61 is pulled towards the γ-phosphate covering Gly-60 which is an important residue for the binding the Cysteine rich domain of Raf-1 (Quilliam et al., 1996). Third, Pro-34 is fully exposed at the surface, presumably because Tyr-32 is hydrogen bonded to Asp-12 (Figure 23 c). In G12V Ras, Tyr-32 is not displaced and sits above Pro-34 (Figure 23 d).

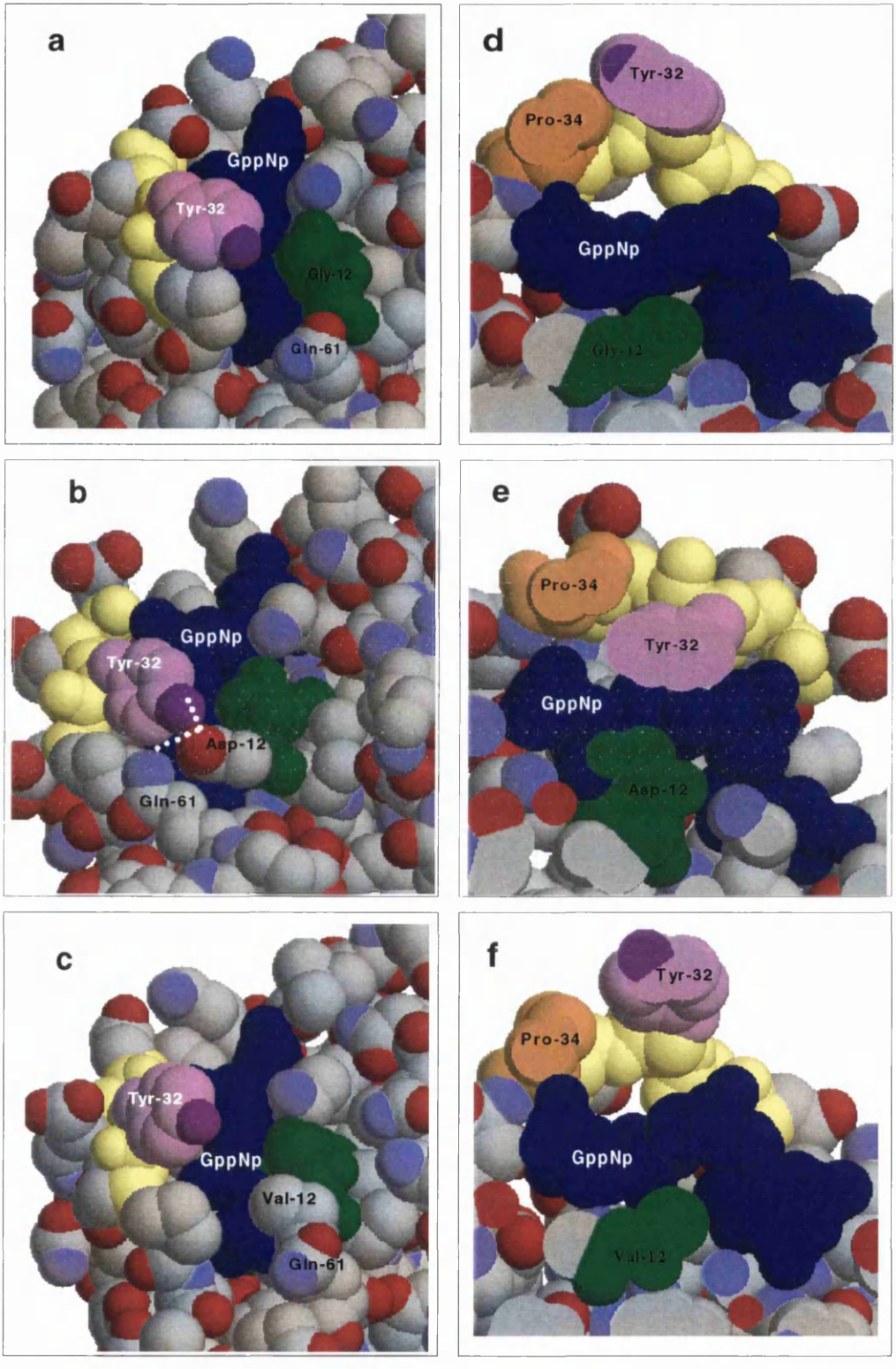
It is, thus, deduced that the arrangements of Gln-61, Tyr-32 and Pro-34 differ markedly between the G12D mutant and both wild type and G12V Ras. A protein such as Raf-1 recognising the surface including Tyr-32 and Pro-34 (switch

Figure 23

Figure 23: *Crystal structures of H-Ras protein*

The wild type and codon-12 mutant Ras proteins were extracted from the Brookhaven Protein Data Bank (Bernstein et al., 1977). The Ras PDB files give the coordinates of the crystal structures of the truncated versions (residues 1-166), expressed in *E. coli*, of the human protein Harvey-P21ras complexed to either MgGppNp or MgGDP. (GppNp is an analogue of GTP in which the oxygen atom linking the b,g phosphates is replaced by an NH group.) The molecular graphics package Rasmol (Sayle & Milner-White, 1995) was used to manipulate the Ras PDB images.

Wild type (a,d), G12D (b,e) and G12V (c,f) Ras proteins: a, b and c show views of the surface at the ligand of p21 Ras; all protein and ligand atoms are shown in spacefill. The GppNp atoms are dark blue. The side chain atoms of Tyrosine-32 are coloured violet and its phenolic oxygen is purple. The main-chain atoms of residues 30-33 (part of the effector site) are coloured yellow. Residue 12 is coloured green. In (b) the carboxylate side chain of aspartate-12 is hydrogen bonded (as an acceptor) both to phenol of Tyr-32 and the amide of Gln-61, which are both acting as hydrogen bond donors. The dotted lines represents these hydrogen bonds. Slice sections through the surface of wild-type and mutant Ras molecules, using the Rasmol slab command, are shown in d, e and f. They are designed to highlight the arrangement of Tyrosine-32 and Proline-34 at the surface. Atoms that are sliced appear flat.


a
GppNp
Tyr-32
Gly-12
Gln-61
d
Tyr-32
Pro-34
GppNp
Gly-12
b
GppNp
Tyr-32
Asp-12
Gln-61
e
Pro-34
Tyr-32
GppNp
Asp-12
c
Tyr-32
GppNp
Val-12
Gln-61
f
Tyr-32
Pro-34
GppNp
Val-12

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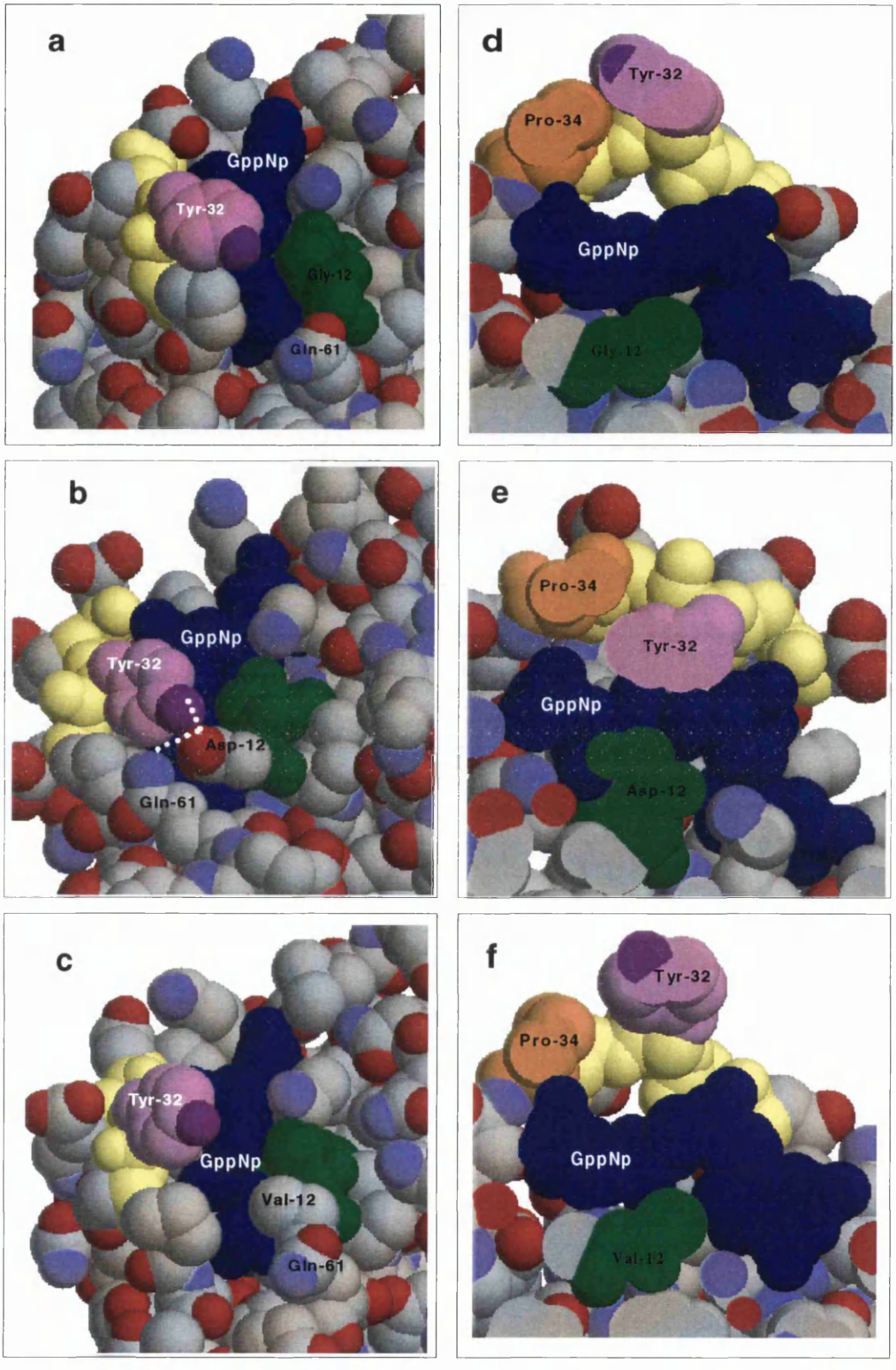

a
GppNp
Tyr-32
Gly-12
Gln-61
d
Tyr-32
Pro-34
GppNp
Gly-12
b
GppNp
Tyr-32
Asp-12
Gln-61
e
Pro-34
Tyr-32
GppNp
Asp-12
c
Tyr-32
GppNp
Val-12
Gln-61
f
Tyr-32
Pro-34
GppNp
Val-12

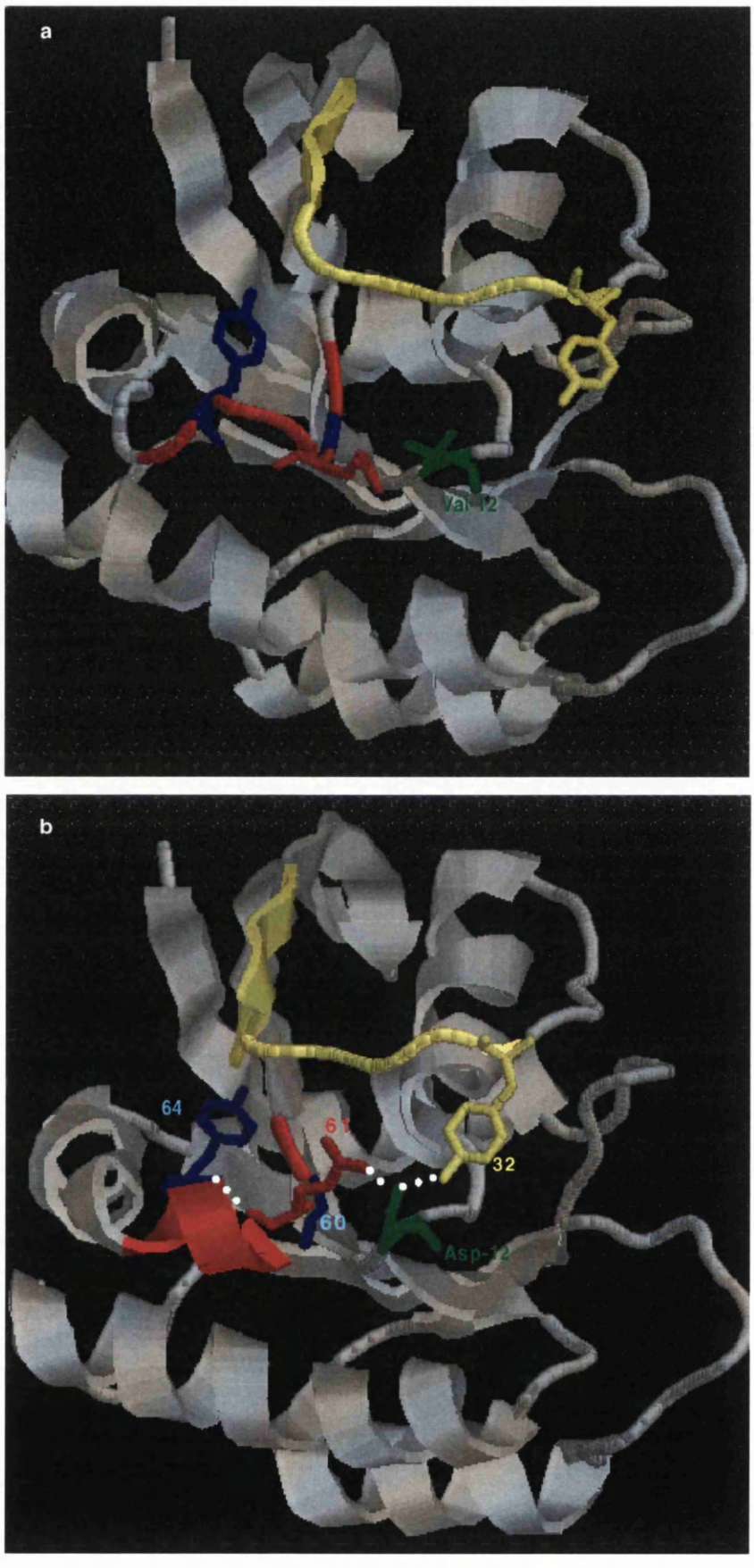
I) and Gly-60 (switch II) would find the G12D mutant presenting a different shape and partial charge distibution from both wild type and G12V proteins (Figure 24). In particular the β, γ-phosphates of the nucleotide sit at the bottom of a trough in the wild type and G12V, whereas in G12D protein the trough is filled by the aspartate and Tyr-32, to which it is hydrogen bonded. Since Tyr-32 is not securely fixed on the surface of Gly-12 and Val-12 Ras it is to be expected that residues from a protein recognising GTP-Ras could protrude into the b, g-phosphate crevice of these proteins, but are less likely to do so for G12D.

Three-dimensional structures of GAP-G12D and GAP-G12V have not been determined but when they are it may be possible to test these expectations. The same principles would also apply to other proteins recognising the nucleotide phosphates and the residues surrounding its trough at the surface of Ras. Another possibility that ought to be considered is that the three-dimensional structure adopted by GppNp-Ras, with the trough filled in by Asp-12 and Tyr-32, is not favoured when the true substrate GTP binds, at least in its complex with GAP, because of its lower affinity for protons. In addition, since Raf is one of the down stream effector of Ras and that GAP and Raf use overlapping binding domains on Ras (residues 30-38 for GAP (Bollag and McCormick, 1991; Gideon et al., 1992) and 32-40 for Raf (Stang et al., 1997) then it can be postulated that increased affinity of GAP to aspartate-12 compared to valine-12 might interfere with Raf binding in aspartate-12 but not with valine-12 mutant, which has low affinity for GAP. This interference in binding between Raf and GAP has been documented recently. The authors show that binding of Raf protein to Ras prevented productive interaction between Ras and GAP as measured by competitive inhibition of GAP-stimulated Ras-GTP hydrolysis (Chuang et al., 1994).

Figure 24

Figure 24: *Comparison of the 3-Dimensional-cartoon structures of the valine-12 and aspartate-12 Ras proteins in the active conformation*

The switch I effector region (residues 30-40) is coloured yellow. Switch II is shown in red with residues 64 and 60, which are important for Raf binding are shown in blue. a) This is valine-12 mutant H-Ras. Notice the absence of hydrogen bonds. b) This is aspartate-12 mutant H-Ras. There are hydrogen bonds formed between the triad of Tyr-32, asp-12 and Gln 61, but also there is an additional hydrogen bond between Tyr-64 and Gln-61 backbone, which increases the rigidity of switch II region. Notice also how residue 61 blocks access to residue 60.


a
Val-12
b
64
61
32
60
Asp-12

Some of these features are summarised in Scheme 1. The different physiological effects of G12D and G12V cannot simply be explained on the basis of different GTPase activity and are, we argue, likely to be due either to weak binding of GTP specifically to G12D and/or to the significantly different conformation of G12D in the region of the g-phosphate of GTP. G12V is continuously activated giving rise to a persistent downstream signal in tumours harbouring this mutation while, in G12D, the downstream signal, though still oncogenic, is less persistent, giving rise to less aggressive tumours. There have to be molecular explanations for the different effects of these two mutations and this analysis represents a start in addressing them.

Scheme 1: Comparison of WT, G12V, and G12D Ras

Wild-type	G12V	G12D
GTP binds strongly	GTP binds strongly	GTP binds weakly
γ-Phosphate of GTP exposed at bottom of trough.	γ-Phosphate of GTP exposed at bottom of trough.	γ-Phosphate of GTP covered in its trough by aspartate side chain.
Switch II is mobile	Switch II is mobile	Switch II is rigid and residue 60, which is important for Raf binding, is obstructed by Gln-61
GAP binds strongly. Ground-state formed.	GAP binds weakly. Ground-state not formed so well.	GAP binds strongly. Ground-state formed.
Transition state formed by R789 of GAP binding in trough.	Also transition-state not formed because R789 of GAP cannot fit in trough. (Val-12 obstructs both.)	Transition-state not formed because R789 of GAP Cannot fit in trough. (Asp-12 obstructs it.)
GTPase hydrolysis rate rate increased by GAP.	GTPase hydrolysis rate not increased by GAP.	GTPase hydrolysis not increased by GAP.

CHAPTER 10. Summary and discussion

In this study, primary adenocarcinomas and their metastatic deposits from 78 patients were examined for Ki-*ras* mutation in codons 12 and 13. In agreement with most other published data using similar techniques (Bos et al., 1987) Ki-*ras* was found mutated in 35% of all carcinomas.

Finkelstein et al. (1993) reported that a G to A transition in the second base of codon 12 is associated with aggressive carcinomas, and Moerkerk et al. (1994) found G to A transitions in either first or second base exclusively in less aggressive (Dukes' B) carcinomas. Here, G to A transitions in carcinomas have been found in all Dukes' stages, with no difference in frequency of G to A transition between metastatic and non-metastatic primary carcinomas. Both Finkelstein et al. (1993) and Moerkerk et al. (1994) found G to T transversions in the second base in Dukes' C carcinomas, though the latter group subsequently reported a preponderance of GTT mutations in Dukes' A and B carcinomas (Span et al., 1996). G to T transversions were observed only in Dukes' C and D carcinomas. It may be significant that the three carcinomas in this study with codon 12 GTT mutations which were originally staged as Dukes' A or B were later re-staged as Dukes' D. These patients with apparently early carcinomas must already have had covert metastatic deposits since they relapsed with liver metastases.

The majority (65%) of carcinomas in this study and in others (Bos, 1989; Bos et al., 1987; Boughdady et al., 1992; Moerkerk et al., 1994; Urosevic et al., 1993) contained only wild-type Ki-*ras*. This and the absence of any difference in Ki-*ras* mutation frequency between primary carcinomas and their metastatic

deposits, and between metastatic and non-metastatic primary carcinomas, indicate that Ki-*ras* mutations are not essential for the attainment of metastatic capacity. Kato et al., (1996) reported that mutations of Ki-*ras* codon 12 in patients with colorectal cancer are less frequent in patients with liver metastases than in patients with no liver metastases (Kato et al., 1996). Together, these observations point to the involvement of genetic factors other than Ki-*ras* mutations in the metastatic process.

As observed by others (Bos et al., 1987; Oudejans et al., 1991; Suchy et al., 1992), this study shows that, if there was a mutation in Ki-*ras*, it was the same mutation in the primary carcinoma and its metastases. On the other hand, populations of carcinoma cells heterogeneous with respect to wild-type versus mutant Ki-*ras* were found both within and between primary and secondary carcinomas, implying that even when a Ki-*ras* mutation occurs in a colon carcinoma it cannot always have been necessary for emergence of the malignant (invasive) phenotype. In 13% of Dukes' C and D patients, Ki-*ras* point mutations were detected in metastases but not in the primary carcinomas. Provided the mutation was not in cells of the primary carcinoma other than those analysed, these findings are consistent, in some cases at least, with a point mutation in Ki-*ras* occurring at one of the later stages of carcinoma progression.

Substantiation of the adverse prognosis associated with the codon 12 GGT→GTT (Gly→Val) Ki-*ras* mutation in colorectal cancer by larger studies might help to identify, for instance, Dukes' A and B patients at greater risk of systemic relapse and hence likely to benefit from adjuvant systemic therapy. Such studies may be compromised if they do not address the issue of *ras* mutational heterogeneity. If *ras*-mutant malignant cells in *ras*-heterogeneous

carcinomas emerge subsequent to malignant transformation, then the *ras* mutation is a progression event, and might be expected to confer a more aggressive phenotype, being additional to genetic changes already conferring the malignant phenotype.

A logical hypotheses can be proposed. It is possible that some colorectal carcinomas are, in fact, polyclonal with respect to the transforming mutations they have acquired. Or, transformed cells are capable of inducing the transformed phenotype in adjacent adenoma cells, even if those cells do not themselves have a full complement of transforming mutations, via paracrine mechanisms or by gap-junctional communication of dominant signals. That neoplastic cells may confer new behaviours on adjacent cells has been shown by experiments in nude mice in which co-injection of metastasis-incompetent cells admixed with metastasis-capable cells leads to the formation of chimeric metastases in which *both* cell types are represented (Moffett et al., 1992).

Another important implication of *ras* mutational heterogeneity relates to therapies directed against *ras* function, such as inhibitors of farnesyltransferase. Transgenic mice expressing v-H-*ras* under mouse mammary tumour virus LTR control develop spontaneous salivary and mammary carcinomas. Farnesyltransferase inhibition can effect complete regression of these tumours without detectable toxicity, but resistance has also been observed (Kohl et al., 1995). Work on farnesyltransferase inhibitors continues but, clearly, the potential of these agents in human cancer will be critically influenced by the phenomena described.

While a strong correlation of mutation of codon 12 to GTT with the more aggressive/advanced (Dukes' C and D) carcinomas was observed, analyses of Ki-

ras in colorectal carcinomas did not reveal the same strong relationships between some other codon 12 mutations and the aggressiveness of the disease described by others (Finkelstein et al., 1993; Moerkerk et al., 1994; Span et al., 1996). It seems unlikely that differences between studies are due to differences (genetic, dietary, etc) between the American (Finkelstein et al., 1993), Dutch (Moerkerk et al., 1994; Span et al., 1996), and Scottish populations of patients since none of these factors would be expected to influence the phenotypic effects of particular Ki-*ras* mutations, although they might cause differences in the frequencies of the various mutations, as has already been noted (Urosevic et al., 1993). The variations between studies may either reflect differences in the mutation rates of other genes encoding proteins that interact, directly or indirectly, with p21 Ras, or the use of different methodologies. ASO hybridization, for example, detects 1 mutant cell in 100 normal cells (Slebos et al., 1992) whereas sequencing and analysis of single-strand conformation polymorphisms are at least 10-fold less sensitive. Publication bias may also be significant.

Survival data on colorectal cancer patients with Ki-*ras* mutations are also controversial. Laurent-Puig et al. (1992) and Kern et al. (1989) found no relation between the occurrence of Ki-*ras* mutations and overall survival. In contrast, Span et al. (1996) found decreased 10-year survival of patients with colon carcinomas harbouring Ki-*ras* mutations, in particular the codon 12 GAT mutation, whereas Benhatter et al. (1993) and I found this mutation to have no significant association with survival. The association found here was between transversions of GGT to GTT in codon 12 and decreased survival. The controversial results between studies are not surprising, most studies including this, contained a relatively small number of patient to reach a conclusive result.

However, a recent more elaborate study of the RASCAL project, which has collected data from 2721 colorectal cancer patients from 22 groups in 13 countries (including data from Finkelstein et al. (1993), Span et al. (1996), but not mine), also came to the conclusion that the codon 12 GTT mutation in Ki-*ras* but not GAT mutation is associated with increased risk of relapse and death (Andreyev et al., 1998). In addition, a similar association between this mutation and poor prognosis has been found for patients with lung cancer (Keohavong et al., 1996).

The role of valine-12 mutant Ras protein in metastasis in animal models is well-established (Bondy et al., 1985; Radinsky et al., 1987; Thorgeirsson et al., 1985). As detailed in Chapter 3, Ras proteins activate a number of intracellular signalling pathways. For example, valine-mutant Ha-Ras stimulates surface membrane ruffling in colon cancer cells (Li et al., 1997) and the expression of matrilysin (a matrix metalloproteinase) (Yamamoto et al., 1995) and angiogenic factors (Rak et al., 1995). Consequently, there are plausible mechanisms by which different *ras* mutants might influence both local carcinoma aggressiveness and the growth of metastatic deposits. However, most, if not all, studies addressing the role of mutant Ras proteins in metastasis *in vitro* and in animal models concentrated on valine-12 mutant Ras and no attempt as yet was made to compare the effects of valine-12 and aspartate-12 mutant Ras on invasion and metastasis in these studies.

The mechanisms whereby different *ras* mutations might influence the aggressiveness of carcinomas are still little understood, although it has long been known that the transforming capacity of Ha-*ras* genes varies with particular codon 12 mutations (Seeburg et al., 1984). Rat fibroblasts transformed with H-*ras* encoding valine in place of glycine display a more fully transformed phenotype

than those transformed with Ha-*ras* encoding aspartate at this position (Seeburg et al., 1984).

In vitro comparison of Rat-1 cells expressing wild type or two different codon 12 activating mutations was undertaken. One of the main conclusions suggested by this study is that Rat-1 cells containing valine-12 mutant Ki-Ras were significantly more invasive *in vitro* than clones expressing aspartate-12 or wild type Ki-Ras. This is consistent with valine-12 Ki-Ras being more aggressive *in vivo.* Increased invasion has been shown to be a direct consequence of valine-12 mutant Ki-Ras because antisense against this particular mutation significantly diminished cell invasion. Although it has to be admitted that to attain more reliable and significant results 10 or more clones of each mutant Ki-Ras should have been compared (in contrast to the 2 clones of each mutation type used here or in the case of GAT-12 clones only one clone has been shown to express GAT-12 Ki-Ras). Unfortunately, this was not practical due to time and funding limitations.

Both valine-12 and aspartate-12 mutant Ki-Ras transform Rat-1 cells equally well. These cells also demonstrate reduced expression of E-cadherin. E-cadherin has been shown to suppress metastasis in animal models (Vleminckx et al., 1991). Moreover, induction of the invasive phenotype by the activated H-*ras* oncogene has been shown to correlate with weak expression of E-cadherin (Chastre et al., 1993). With carcinoma cells, changes in cellular adhesion often reflect a more invasive phenotype. For example, human breast epithelial cells transformed by oncogenic *ras* generally display reduced cell-cell adhesion and increased cell migration (Basolo et al., 1991; Ochieng et al., 1991). However, it is currently unclear how activated Ras proteins alter adhesion junctions but could

involve elevated tyrosine phosphorylation of proteins such as β-catenin and p120 Cas (Kinch M., 1995).

Both mutant Ki-Ras transformed Rat-1 cells demonstrated a reduced expression of β_1 integrin. This is in line with published data showing that Rat cells transformed with *ras* or Rous sarcoma virus were found to have decreased expression of β_1 integrins (Plantefaber and Hynes, 1989). Recently, valine-12 mutant H-Ras was found to negatively affect the integrin activation state (Hughes et al., 1997). The discovery of such a negative feedback loop due to inactivation or reduced expression represents an important connection between decreased matrix adhesion and malignant transformation. Therefore, while anchorage-dependent cells are susceptible to apoptosis when induces to detach from matrix substrate by disruption of integrin mediated cell adhesion (Meredith et al., 1993), transformed cells may overcome the apoptotic pathway by alleviating the requirement for cell adhesion to substratum, thus conferring anchorage independence and initiating the malignant transformation of cells (Ruoslahti and Reed, 1994).

The role of α_6 integrin in invasion and metastasis is well established (Robinovitz 1996). Indeed, the use of functional anti-α_6 integrin antibody was also able to reduce invasion of valine-12 mutant Ki-Ras cells showing an important role of this integrin monomer in *in vitro* invasion.

The difference in invasion capability between the two mutants was also shown to correlate, at least in some clones, with stromelysin-1 production. Increased stromelysin-1 production has been shown to correlate with aggressive tumours *in vivo*, and recently has been shown to play a direct role in facilitating invasion through Matrigel *in vitro* (Lochter et al., 1997). Valine-12 mutated Ki-

Ras has also been shown to increase stromelysin production (LoSardo et al., 1995; Matrisian et al., 1991). It is worth noting, however, that in colorectal cancer stromelysin-1 has been shown to be expressed indirectly by stomal rather than tumour cells (Heppner et al., 1996; Matrisian et al., 1994). Interestingly, stromelysin-1 production was shown to be a consequence of MAPK pathway activation (LoSardo et al., 1995; McDonnell et al., 1994). The reduced ability of aspartate-12 to induce stromelysin-1 production could be due to its reduced ability to bind Raf-1 and stimulate MAPK pathway. Indeed, molecular modelling demonstrates a clear difference in structure between the two mutants where important residues for Raf binding have substantially different conformation or are inaccessible.

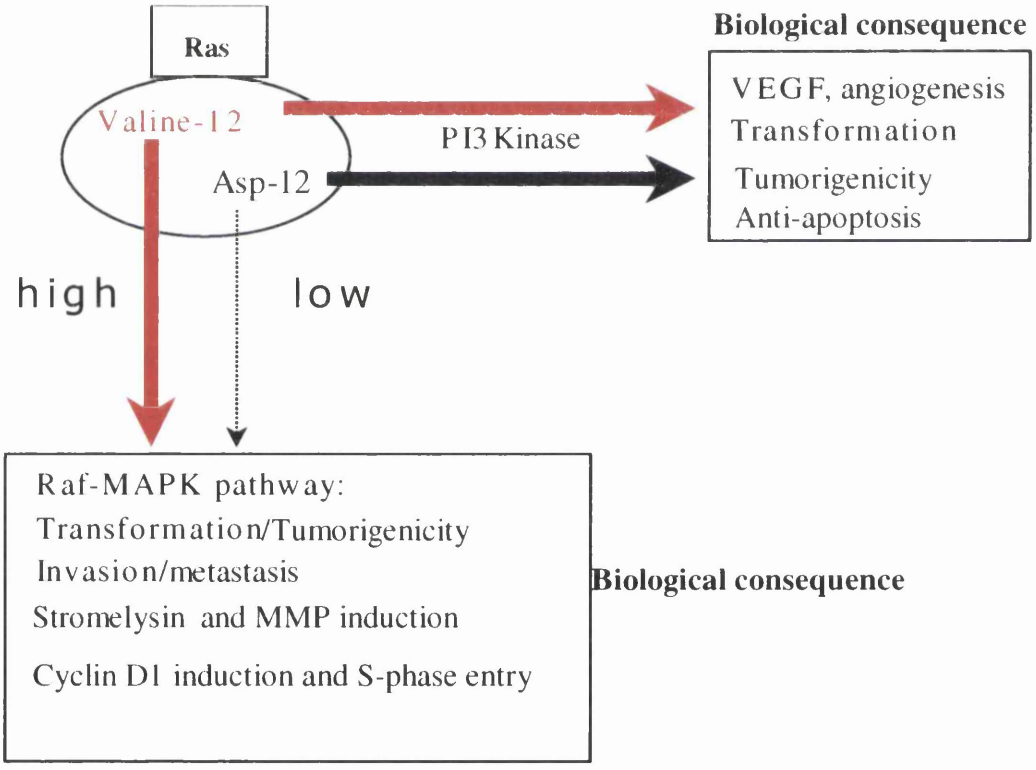
Since it is speculative that the MAPK pathway is not activated by the aspartate-12 mutation, how then could aspartate-12 transform cells? And why is aspartate-12 mutant Ki-Ras selected for in colorectal cancers? Are intriguing questions. The ability of both Ki-Ras mutants to stimulate VEGF production as shown here and by others (Arbiser et al., 1997; Okada et al., 1998; Rak et al., 1995) could partially shed some light on these questions. VEGF production was shown to require PI-3K arm of the Ras pathway (see section 3.5.3). This particular Ras pathway (Ras-PI3K-PKB/Akt) was also shown to be responsible for transformation of cells by R-Ras. Activated R-Ras is unable to stimulate the Raf-MAPK pathway but synergizes strongly with Raf in the transformation of NIH 3T3 cells (Downward, 1996; Saez et al., 1994). It is arguable, based on the ability of activated Ras to induce VEGF production, that in aspartate-12 Ki-Ras, the Ras-PI-3K pathway is fully functional and could give selective advantage for cells harbouring this mutation. However, in valine-12 Ras both the Ras-PI-3K and Ras-

MAPK pathways are fully functional making this particular mutation more oncogenic and associated with more aggressive tumours *in vivo* (Figure 25). This hypothesis is supported by recent work of Webb et al. (1998). The effector domain mutants of oncogenic H-Ras, V12S35 Ras, V12G37 Ras, and V12C40 Ras were tested for their abilities to mediate tumourigenic and metastatic phenotypes in athymic nude mice when expressed in NIH3T3 fibroblasts. The authors show that all mutants displayed comparable tumourigenic properties, but only the mutant that activatess the Raf-mitogen-activated protein kinase kinase (MEK)-extracellular regulated kinase (ERK) 1/2 pathway, namely the V12S35 Ras, induced tumours in the experimental metastasis assay. The authors also show that R-Ras which fails to activate the ERK 1/2 pathway, is tumourigenic but non-metastatic. These results suggest that Ras-mediated tumourigenicity can arise independently of ERK 1/2 activation and that experimental metastasis appears to require constitutive activation of ERK 1/2 pathway. The authors argue that the Ras effector pathways mediating the tumourigenic and metastatic activities can be segregated. Similarly, I argue that aspartate-12 Ki-*ras* mutants are not associated with metastatic phenotype but are still tumourigenic because, on one hand, they fail to sufficiently activate the Raf-mitogen-activated protein kinase kinase (MEK)-extracellular regulated kinase (ERK) 1/2 pathway but on the other hand they activate the PI-3K arm sufficiently. That GAT-12 Ki-*ras* mutants are unable to activate the MAPK pathway, which is associated with cellular senescence and apoptosis in primary cells, probably gives early tumour cells harbouring this particular mutation a selective advantage. This can help to explain why GAT-12 mutation is the most common *ras* mutation in colorectal cancer.

In summary, there is now mounting evidence supporting the notion that some mutations in *ras* are more aggressive than others. *In vivo,* the RASCAL study demonstrated this with a high degree of certainity. *In vitro,* Seeburg et al. (1984) have shown that valine-12 is more transforming than aspartate-12. Recently, Giaretti et al. (1998) have shown that DNA aneuploidy in colorectal adenomas was strongly associated with the presence of GTT-12 transversional mutations in Ki-*ras*, whereas adenomas harbouring GAT-12 transition mutations were associated with low S-phase values. Here, the results not only suggest that different mutations in Ki-*ras* have different biological effects on cells, but a preliminary model illustrating possible reasons for such differences has been introduced. What remains is to verify this mode proposed in Figure 25. This will be discussed in section 12.1.

Figure 25: *A model illustrating probable differences between valine-12 and aspartate-12 mutant Ras proteins in activation of downstream effectors of Ras and their biological consequences*

Valine-12 Ras is thought to activate both the MAPK and the PI-3 kinase pathway with full biological advantages shown in boxes. On the other hand, aspartate-12 is thought to be unable to activate the MAPK pathway (or activates it to a small extent), which is important for invasion and metastasis. However, it can probably still activate the PI-3 kinase pathway giving cells survival advantages such as inhibition of apoptosis, tumourigenicity and the ability to induce angiogenesis
The arrow thickness is proportional to the strength of Ras effectors activation.



SECTION 2

CHAPTER 11. Genetic comparison of advanced primary colorectal tumours and their metastases using Comparative Genomic hybridisation

An important concept that emerged from analysis of Ki-*ras* mutations *in vivo*, is tumour heterogeneity. Equally important is the fact that Ki-*ras* mutations occurred in only 34-38 % of colorectal tumours and in some instances metastases contained only wild type Ki-*ras*. This argues that activated Ki-*ras* is not necessary, in many instances, for metastasis and that there are possibly other mechanisms through which a metastatic phenotype is attained. It was, therefore, rational to use a wide scanning genomic methodology such as CGH in order to answer two main questions. Firstly, what is the extent of the genetic diversity (heterogeneity) between primary colorectal tumours and their synchronous metastases? And secondly, what genetic aberrations, other than Ki-*ras* mutations, are frequent in advanced colorectal cancers?

11.1. ***Optimisation of comparative genomic hybridisation***

CGH is a relatively recent technique developed by Kallioniemi et al. (1992) for analysing whole genomic DNA (Figure 26). The benefits of using such a robust technique is that a single experiment provides an overview of DNA sequence copy number changes, which include deletions, gains, and amplifications. This method is particularly suited to study metastatic colorectal cancer because the "gross" genetic alterations associated with the metastatic process are still poorly defined.

Increased attention to quality control of CGH is essential since experimental variability remains a significant problem (Karhu et al., 1997). Therefore, CGH was carefully optimised by including two controls in every experiment. Firstly, a colorectal cancer derived cell line (Colo320) which has a known amplification of the *Myc* oncogene in the form of double minute chromosomes was used (Schwab et al., 1983) (Figure 27). Secondly, hybridisation of green-labelled normal male DNA *versus* red-labelled normal female DNA and its reverse, red-labelled normal male DNA versus green-labelled normal female DNA were used to show consistent difference in copy number of the X chromosomes between the two genders (Figure 28). In both cases, CGH clearly detected these changes. Moreover, these controls were used in every experiment and unless these controls showed the expected differences the experiment was not considered for analysis.

Figure 26: *Schematic representation of CGH*

Tumour genomic DNA is labeled with biotin (green) and normal genomic DNA is labeled with digoxigenin (red) in a nick-translation reaction or by using random priming. An equimolar amount of both reactions are hybridised to normal male metaphase chromosome spreads and quantitated as described in the methods section. The chromosomes are counter-stained with DAPI and identified by Q-banding patterns.

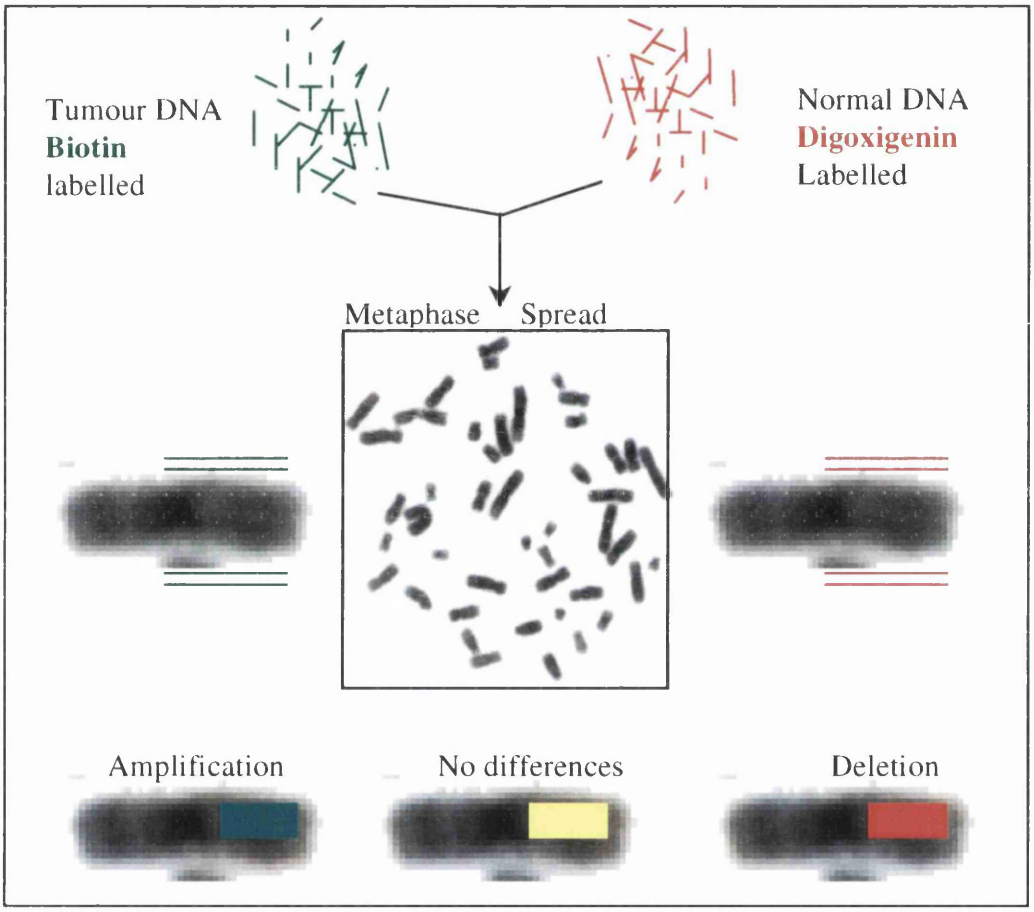
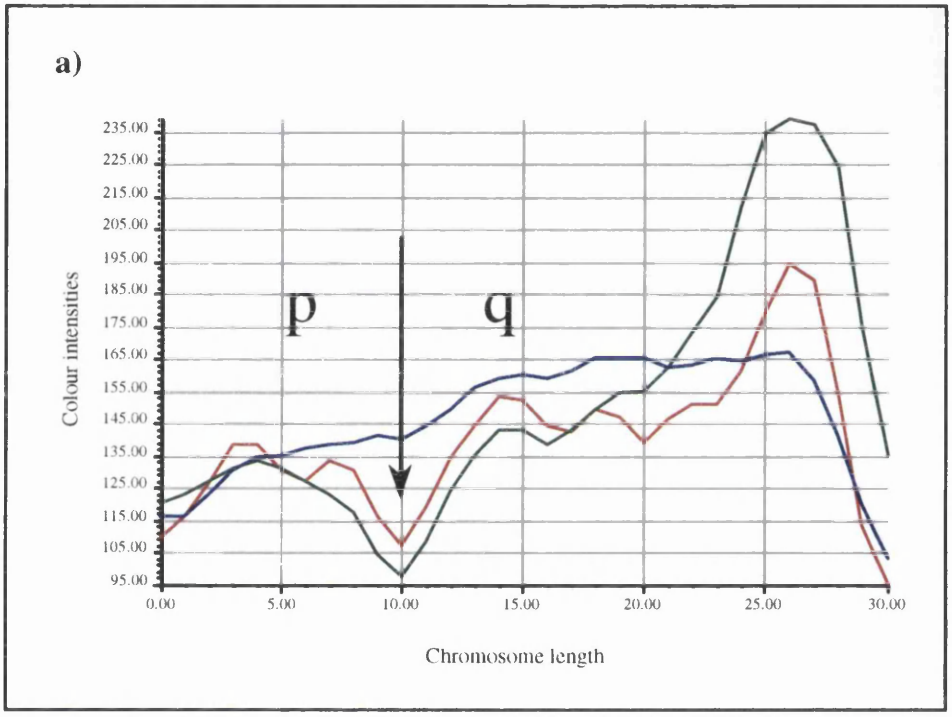
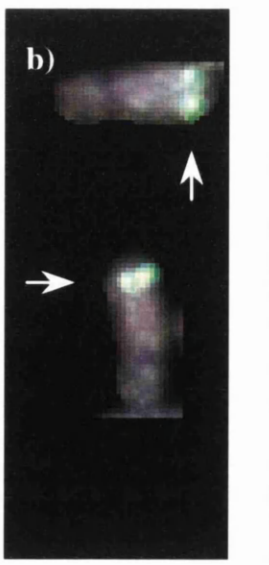


Figure 27: *Detection of c-myc amplification by CGH*

Green labeled DNA from Colo 320 cell line hybridised against normal DNA to normal metaphase chromosomes. a) shows green, red and blue colour intensities along one chromosome 8. The centromeric region is indicated by the arrow.b) Visual representation of two normalised chromosome 8. Arrows indicate the amplified loci on 8q24. c) Average green:red fluorescent intensity profile of chromosome 8 collected from 5 metaphase spreads. Mean ratio is shown as a red line, and the 99% confidence intervals of the mean are shown as black lines. The baseline value that represents the mean ratio of 1.0 is shown as dashed line and the ratio profiles of 0.75 and 1.25 are shown as solid horizontal lines.





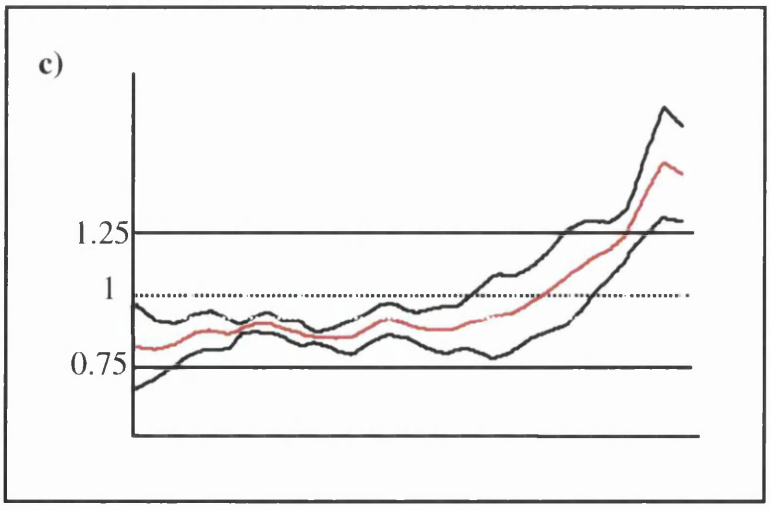


Figure 28

Figure 28: *CGH detection of X-chromosome copy number difference by hybridising normal DNAs from different genders*

A) Normal female DNA labeled green hybridised against normal male DNA labeled red to normal male metaphase chromosomes.

a) Average green:red fluorescent intensity profile of X-chromosome collected from 5 metaphase spreads. Mean ratio is shown as red line, and the 99% confidence intervals of the mean are shown as black lines. The baseline value that represents the mean ratio of 1.0 is shown as dashed line and the ratio profiles of 0.5 and 1.5 are shown as solid horizontal lines.

b) shows green, red and blue colour intensities along one X-chromosome.

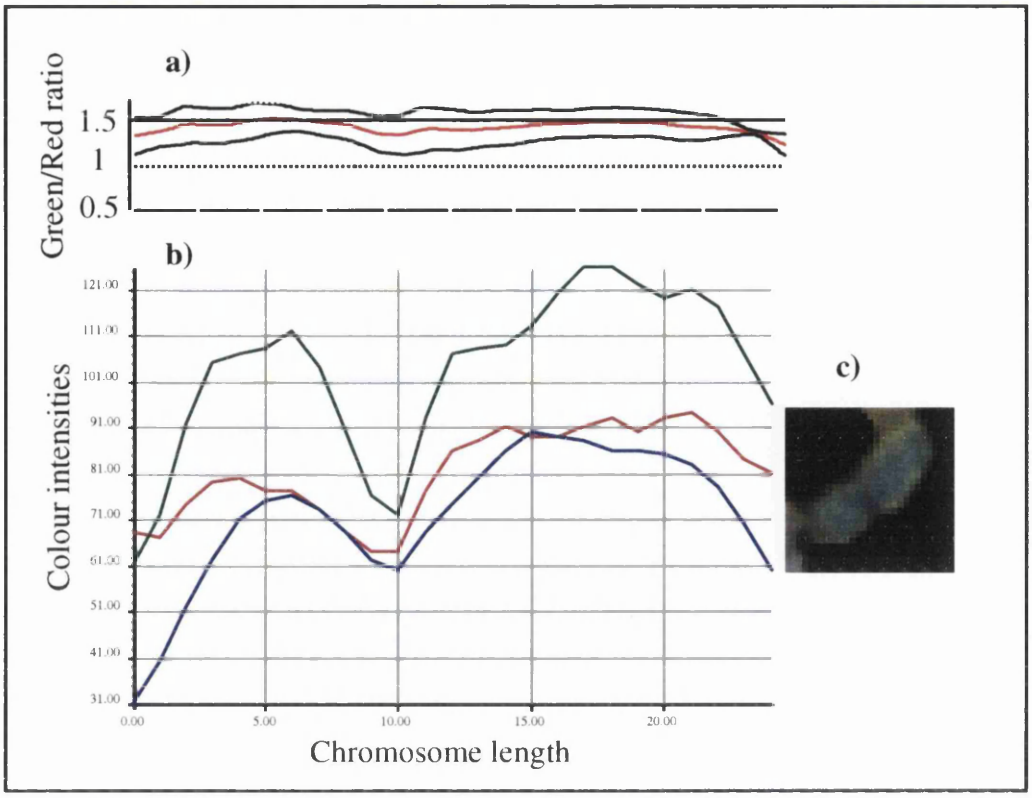
c) Visual representation of the normalised X-chromosome.

B) The reverse of (A), Normal male DNA labeled green hybridised against normal female DNA labeled red to normal male metaphase chromosomes.

a') Average green:red fluorescent intensity profile of X-chromosome collected from 5 metaphase spreads. Mean ratio is shown as red line, and the 99% confidence intervals of the mean are shown as black lines. The baseline value that represents the mean ratio of 1.0 is shown as dashed line and the ratio profiles of 0.5 and 1.5 are shown as solid horizontal lines.

b') shows green, red and blue colour intensities along one X-chromosome.

c') Visual representation of the normalised X-chromosome.


a)
Green/Red ratio
1.5
1
0.5
b)
Colour intensities
121.00
111.00
101.00
91.00
81.00
71.00
61.00
51.00
41.00
31.00
0.00
5.00
10.00
15.00
20.00
Chromosome length
c)

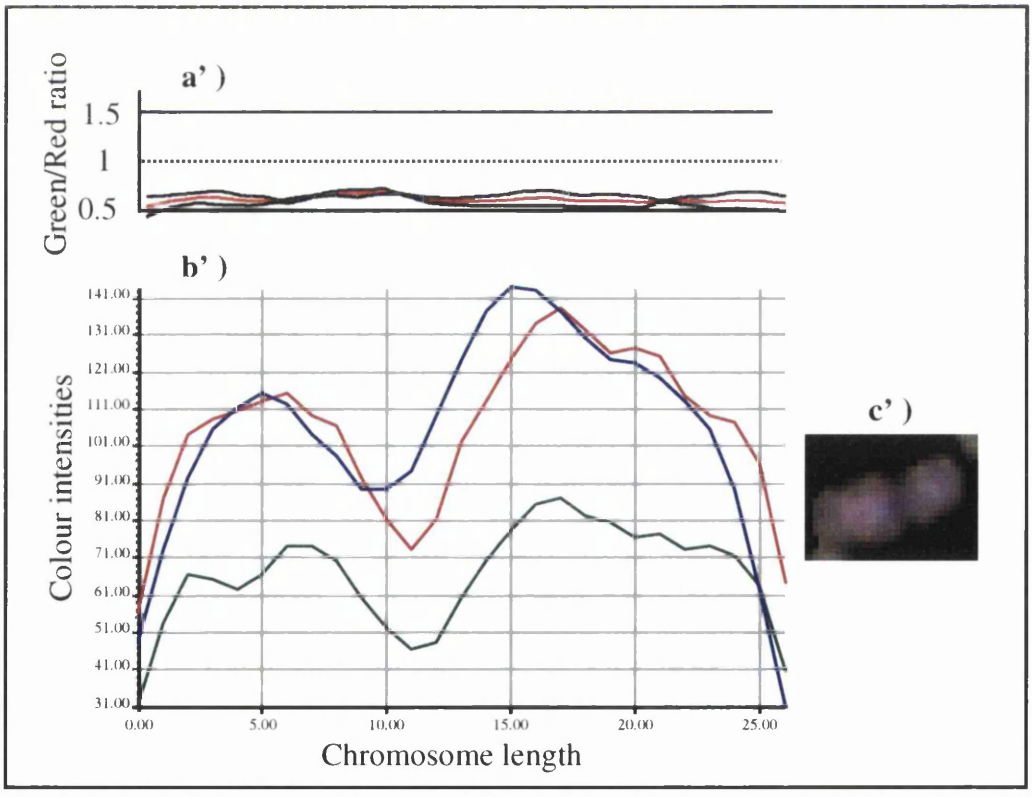
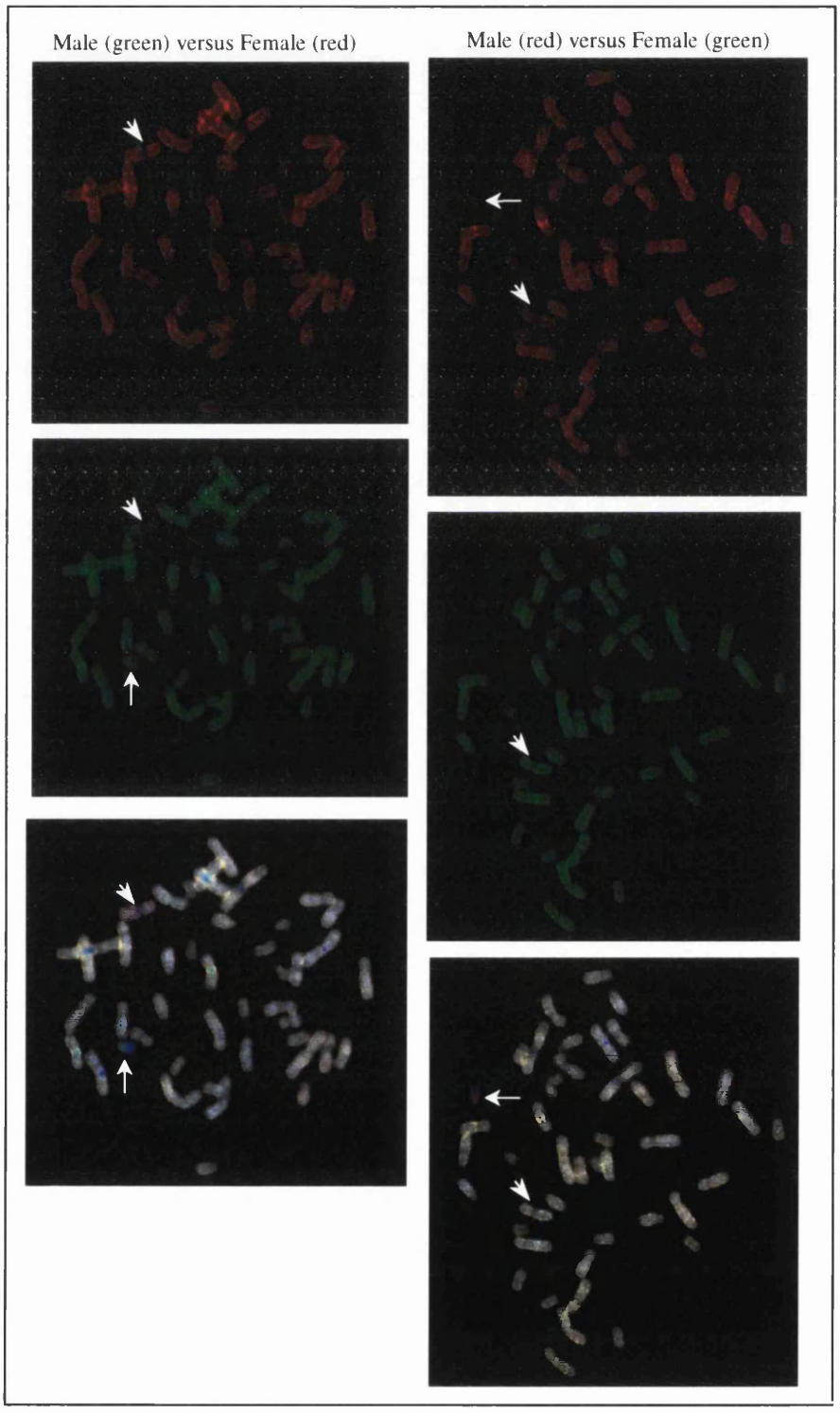

a')
Green/Red ratio
1.5
1
0.5
b')
Colour intensities
141.00
131.00
121.00
111.00
101.00
91.00
81.00
71.00
61.00
51.00
41.00
31.00
0.00
5.00
10.00
15.00
20.00
25.00
Chromosome length
c')

Figure 29: *CGH optimisation by hybridising normal DNA's from different genders*

A) Male DNA labeled green (arrowhead showing the X chromosome and arrow indicates the Y chromosome) hybridised with female DNA labeled red (arrowhead indicating the X chromosome) to normal male metaphase chromosomes.

B) Female DNA labeled green (arrowhead showing the X chromosome) hybridised with male DNA labeled red (arrowhead indicating the X chromosome and arrow showing the Y chromosome) to normal male metaphase chromosomes. See appendix for chromosomal green: red ratio profiles.



11.2. Comparative genomic hybridisation analysis of primary colorectal cancer tumours and their synchronous metastases

11.2.1. Genetic changes in primary and secondary tumours detected by CGH

The extent of the genetic alterations in 12 primary colorectal cancers and their corresponding 14 metastases was assessed. Five patients were classified as Dukes' stage C with only synchronous lymph node metastases, and seven patients were classified as Dukes' stage D with synchronous liver metastases. In two of the patients with liver metastases, lymph node metastases were also available for analysis.

CGH detected extensive genetic alterations in all 12 primary colorectal carcinomas and their metastases (Table 9 and Figure 30). The mean number of genetic aberrations per sample detected by CGH was 9.7 ± 4.6 in primary tumours (range, 1-16) and 11.6 ± 2.9 in metastases (range, 7-18). In nine patients the metastases had more genetic aberrations than their primary tumours, while in three patients the primary tumours had accumulated more genetic aberrations than their metastases. In none of the patients studied were the primary tumours and their metastases either genetically identical or totally different.

Particular chromosomal aberrations were frequent in these advanced colorectal cancers, including increases in copy number of chromosome arms 6p, 7p, 8q, 13q, 16q, 17q and 20q and losses of chromosome 4, 18 and chromosome arm 8p (Table 10).

Table 9: Summary of clinical data and chromosomal copy number changes of 26 tumours from 12 patients with metastatic colorectal carcinoma

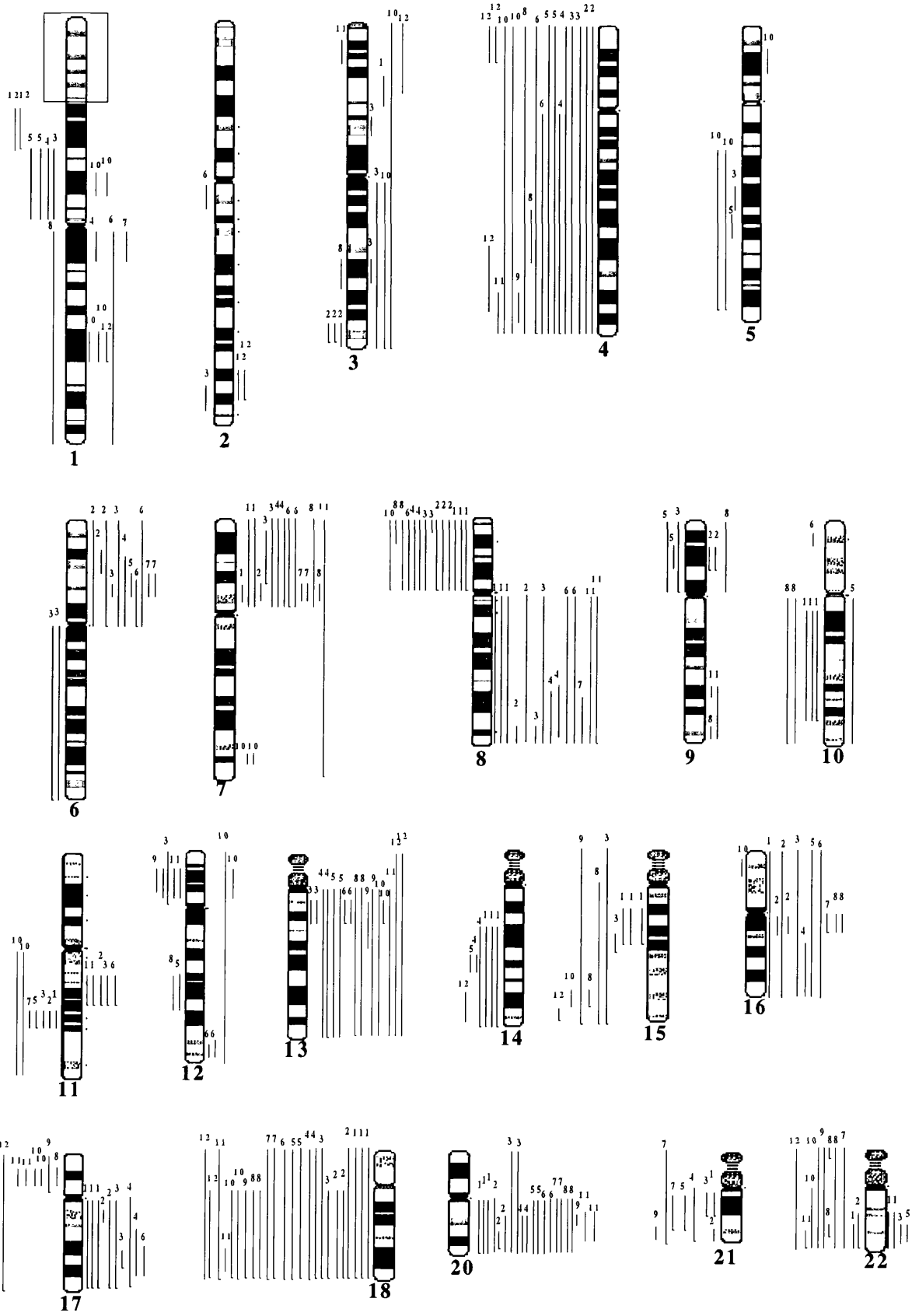
Patient*		Tumour‡	Copy number losses	Copy number gains†	X‡
1	D	P	21q21, 18, 15q21, 14q21-ter, 12p12, 11q23, 10q21-25, 8p.	22q13-ter, **20q**, 17q12-22, 11q14, 9q31, 8q, 7p12.	
		LNM	18, 15q21, 14q21-ter, 12p12, 10q21-25, 8p.	22q13-ter, **20q**, 17q12-22, 16, 11q14, 9q31-ter, **8q24.2-ter**, 7p.	P< 0.001
		LVM	22q13-ter, 18, 15q21, 14q21-ter, 10q21-25, 8p.	**20q13-ter**, 17q12-22, **8q**, **7p**.	
2	D	P	18, 8p, 3q28-ter.	20q, 16q11.2, 6p.	
		LNM	21q22, 18q, 11q23, 8p, 4, 3q28.	**20q13.2**, **17q**, **16**, 11q14, 9p21, **8qter**, **6p22**,	
		LVM	22q12.3-ter, 18q, 8p, 4, 3q28.	**20q12-ter**, **17q12-22**, **16q11.2**, 11q14, **9p21**, 8q, **7p12**, 6p.	P< 0.01
3	D	P	21q21, 18q, 15q21.3, 8p23.2, 6q, 5q21, 4, 2q36.	22q13.2-ter, 20, 17q, 13q13, 8q24.2, **7p21-14**, 6p21.2, 3p14, **3q24**	
		LVM	18,15, 12p, 11q21-23, 9p, 8p, 6q, 4, 1p11-31.	20, 17q23, 16, 13q13, 11q14-14.3, **8q**, **7p**, 6p, **3q**.	P< 0.001
4	D	P	21q, 18, 14q, 8p, 4, 1p11-31.	**20q12-ter**, **17q**, 13q, **8q23-ter**, 7p.	
		LVM	18,14q23, 8p, 4.	**20q12-ter**, **17q21-24**, 16q21-ter,13q,**8q23-24.2**,**7p**,6p21,1q12	P< 0.001
5	D	P	21q21-22.2, 18, 14q23, 12q21, 11q23, 9p21, 5q23, 4, 1p11-31.	22q13.2, **20q**, **16**,**13q**, 6p21.	
		LVM	18, 9p, 4, 1p11-31.	**20q**, **13q**, 10q, 3q24.	P< 0.01
6	D	P	10p14, 8p, 4q.	**20q**, 13q13, 12q24.32, **8q**, **7p**, **6p21.2-11**, 2q12, 1q.	
		LVM	18, 4.	**20q**, 17q22-24, 16, 13q13, 12q24, 11q, 8q, 7p, 6p.	P< 0.01
7	D	P	21q21-22, 18.	**20q**, 16q11.2, 7p12, 6p21, 1q12.	
		LVM	22, 21, 18, 11q21-23.	**20q**, 8q23-ter, 7p12, 6p21.	P< 0.01
8	C	P	22, 18q, 17p12, 15q, 10q, 8p, 4q26-28.	20q, **16q11.2-21**, **13q**, 7p.	
		LNM	22q13.2, 22p11, 18q, 15q25, 12q23, 10q, 8p23.2-22, 4, 3q24, 1q.	**20q**, 16q11.2, **13q**, 9p, 9q34.2, 7p21.	P< 0.001
9	C	P	-	13q13-21.1.	
		LNM	22, 21q22.2, 18q, 17p, 15, 12p12, 4q32-34.	20q12, **13q**.	P=0.4
10	C	P	22, 18q, 17p12, 11q, 8p, 5q14-34, 4.	13q, 12, 7q35, 5p14, 3q, 1p21, 1q31.	
		LNM	22q, 18q, 17p12, 16p13.2, 15q25, 11q, 5q14-34, 4.	13q31, 12p12, 7q35, 3, 1q31.	P< 0.001
11	C	P	18q22, 17p12.	20q12-13.2, **8q**.	
		LNM	22q13.2-ter, 18, 17p12, 4q32-ter, 3p24.	20q12-13.2, 13q, **8q**, 7.	P< 0.05
12	C	P	18q, 4p15.3-ter, 3p21-ter, 1p31-ter.	**13q**, 2q34-36.	
		LNM	22, 18, 17, 15q26.2, 14q31-32, 4p15.3-ter, 4q32-28, 1p31-ter.	**13q**, 2q34-36, 1q31.	P< 0.01

***D, Dukes' stage D; C, Dukes' stage C. ‡P, Primary tumour; LNM, Lymph node metastases; LVM, Liver metastases. †High-level copy numbers are in boldface; ‡X, the probability that shared genetic aberrations in paired specimens are clonally unrelated.**

Figure 30

Figure 30: Ideogram *summary of copy number aberrations involving chromosomes 1 to 22 in 12 patients with metastatic colorectal cancer*

Gains are represented on the right side of the chromosome ideogram and losses on the left. Primary tumours from Dukes' D patients are coloured black and their metastases are shown in green. Primary tumours from Dukes' C patients are coloured red and their lymph nodes metastases are shown in blue. Patient numbers (Table 9) are shown above each line.


1
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17
18
20
21
22

Some, at least, of these genetic changes could be involved in the metastatic process, especially as they were also frequently found in metastases. Many other genetic aberrations were present in the tumours analysed at low frequencies. These, perhaps, represent random events neither necessary nor sufficient to advance the tumour's evolutionary process.

Table 10: Number of patients with the most frequent genetic aberrations occurring either in the primary, or secondary tumours only, or in both

Genetic aberration*	Total number of patients with aberration (n=12)	Primary tumour only (n=12)	Metastases only (n=12)	Primary and metastases (n=12)
4^-	10	0	3	7
$6p^+$	6	1	1	4
$7p^+$	8	0	2	6
$8p^-$	7	2	0	5
$8q^+$	7	0	1	6
$11q21\text{-}23^-$	6	2	3	1
$11q14\text{-}ter^+$	4	0	3	1
$13q^+$	9	0	1	8
15^-	6	0	3	3
$16q^+$	8	2	4	2
$17q^+$	5	0	2	3
$17p^-$	5	1	2	2
18^-	12	0	2	10
$20q^+$	10	0	1	9
22^-	8	0	6	2

*** -, Indicates decrease in copy number; +, increase in copy number; losses and gains could involve all of the chromosome or its arms or parts of its arms.**

11.2.2. Comparison of two Dukes' stages by CGH

A comparison of two different stages of aggressive colorectal cancer could delineate interesting common and different genetic aberrations that explain their different behaviour *in vivo*. Thus, primary tumours and their liver metastases from

seven Dukes' D staged patients and primary tumours and their corresponding lymph node metastases from five Dukes' C staged patients were analysed by CGH.

Some chromosomal aberrations were frequently found in both stages of the disease, including increases in copy number of chromosome arms 7p (8/12), 13q (9/12), 20q (10/12) and losses of chromosomes or chromosome arms 4 (10/12), 8p (7/12), 18 (12/12) and 22 (8/12) (Table 11). These particular changes could have occurred early in the evolution of the disease since they are common to both stages. However, other genetic aberrations seem to be non-randomly distributed between Dukes' C and Dukes' D carcinomas. For example, increased copy number of chromosome arms 6p (6/7 *versus* 0/5) and 17q (5/7 *versus* 0/5) were significantly associated with Dukes' stage D and liver metastases ($p<0.05$) (Table 11), whereas loss of chromosome arm 17p (5/5) was only found in primary tumours and lymph node metastases of Dukes' stage C ($p=0.001$) (Table 11).

Table 11: Frequencies of genetic aberrations in Dukes' C and D patients

Genetic aberration[a]	Dukes' C (n=5)	Dukes' D (n=7)	Fisher's exact test[b]
4 –	5	5	-
8p –	2	5	-
15 -	4	2	-
17p-	5	0	p=0.001
18 –	5	7	-
22 –	5	3	-
6p+	0	6	p=0.007
7p+	2	6	-
8q+	1	6	-
13q+	5	4	-
17q+	0	5	p=0.027
20q+	3	7	-

[a]-, Indicates decrease in copy number; +, increase in copy number; losses and gains could involve all of the chromosome or its arms or parts of its arms.
[b]-, Fisher's exact statistical test is not significant (p>0.05).

11.2.3. Comparison of primary tumours and their metastases

Primary tumours and their synchronous metastases in individual patients frequently revealed the same chromosomal changes at both sites. Such changes included increases in copy number of chromosome arms 6p, 7p, 8q, 13q, 17q and 20q and loss of chromosomes 4,18 and chromosome arm 8p (Table 12), indicating

Table 12: Frequencies of the most common genetic alterations in primary colorectal cancers and their metastases

Genetic aberration*	Primary tumour (n=12)	Secondary tumour (n=14)
4^-	7	11
$6p^+$	5	6
$7p^+$	6	9
$8p^-$	7	7
$8q^+$	5	9
$11q21\text{-}23^-$	3	4
$11q14\text{-}ter^+$	1	5
$13q^+$	8	9
15^-	3	7
$16q^+$	4	7
$17q^+$	3	7
$17p^-$	3	4
18^-	10	14
$20q^+$	9	12
22^-	2	8*

***-, Indicates decrease in copy number; +, increase in copy number; losses and gains could involve all of the chromosome or its arms or parts of its arms. * indicates p=0.051**

close clonal relationships between primary and secondary lesions. In an attempt to quantitate the degree of clonal relationship, a mathematical model, described previously (Kuukasjarvi et al., 1997) was used. This model, estimates the probability that shared genetic aberrations in paired specimens are not shared by chance alone. Eleven of 12 patients had a high probability of a close clonal

relationship between primary and secondary carcinomas (Table 9). In patient 9, the clonal relationship probability by this model was low, so that the shared genetic gain was likely to be attributable to chance alone. In no case analysed were the metastases genetically identical to their primary tumours. In all patients, the metastases seem to have acquired new genetic aberrations not found in their primary tumours. For example, in patients 1, 2, 7, 9, 11 and 12, all chromosome 22 or part of its q arm was underrepresented only in the metastases (Table 9). Similarly, in patients 2 and 11, increased copy number of chromosome arm 7p and in patient 4 increased copy number of chromosome arm 6p was detected only in the metastases (Table 9, Figure 31 and 32). Furthermore, some of these genetic changes found only in the metastases seem to be frequent and not just random genetic events (Table 10), suggesting their importance in the metastatic process. Interestingly, reduced copy number of chromosome 22 was mainly found in metastases ($p=0.051$) (Table 12)

The presence of different genetic lesions between the primary tumours and their metastases not only indicates genetic heterogeneity between the two lesions, but may also point to the presence of genetic heterogeneity within primary tumours. This is best illustrated by patients 1 and 2 (Table 9), where both the primary tumours and two different metastatic lesions (lymph node and liver) were available from the same patients for CGH analysis. In patient 1 the genetic aberrations found in lymph node and liver metastases differed to such an extent that they are likely to have evolved separately from the original 'founder' clone (Figure 32). In patient 2, the lymph node and liver metastases were similar genetically to each other and so may have evolved from the same clone in the primary tumour. However, they differed from the primary clone analysed, thus

again suggesting the existence of more than one clone of cells within the primary tumour (Figure 32).

The same conclusion, that the primary tumours used in this study are heterogeneous, can be deduced from the comparisons of the genetic similarities and differences between each primary tumour and its metastasis. Interestingly, lymph nodes metastases seem to have accumulated substantially more chromosomal aberrations than hepatic metastases. On average, lymph nodes metastases have a median of six more changes than the corresponding primary tumours, while liver metastases have a median of only one more change. Indeed, two patients (1 and 5) have hepatic metastases with many fewer chromosomal changes than their corresponding primary tumours (Table 11).

Figure 31

Figure 31: *Examples of genetic gains and losses from patient 4 primary tumour and its liver metastasis detected by CGH*

Average green:red fluorescent intensity profiles collected from at least 5 metaphase spreads and corresponding chromosome ideograms are shown. The primary tumour profiles are shown on the left, and its liver metastasis profiles are shown on the right. Mean ratios are shown as thick lines, and the 99% confidence intervals of the mean are shown as thin lines. The baseline value that represents the mean ratio of 1.0 is shown as dashed lines and the ratio profiles of 0.75 and 1.25 are shown as solid horizontal lines.

The normalised three-colour image of the primary tumour from the same patient is illustrated in Figure 32 for visual support. Chromosome numbers are given on the left.

The primary tumour shows losses of chromosomes or chromosome arms 4, 8p, 18, 21q and gains of chromosome arms 7p, 8q23-ter, 13q, 17q, 20q12-ter. The liver metastasis shows losses of chromosomes or chromosome arm 4, 8p, 18 and gains of chromosome arms 7p, 6p, 8q23-24.2, 13q, 17q21-24, 20q12-ter. * Chromosome arm 6p copy number is increased in the liver metastasis only, while 21q is only lost in the primary tumour.

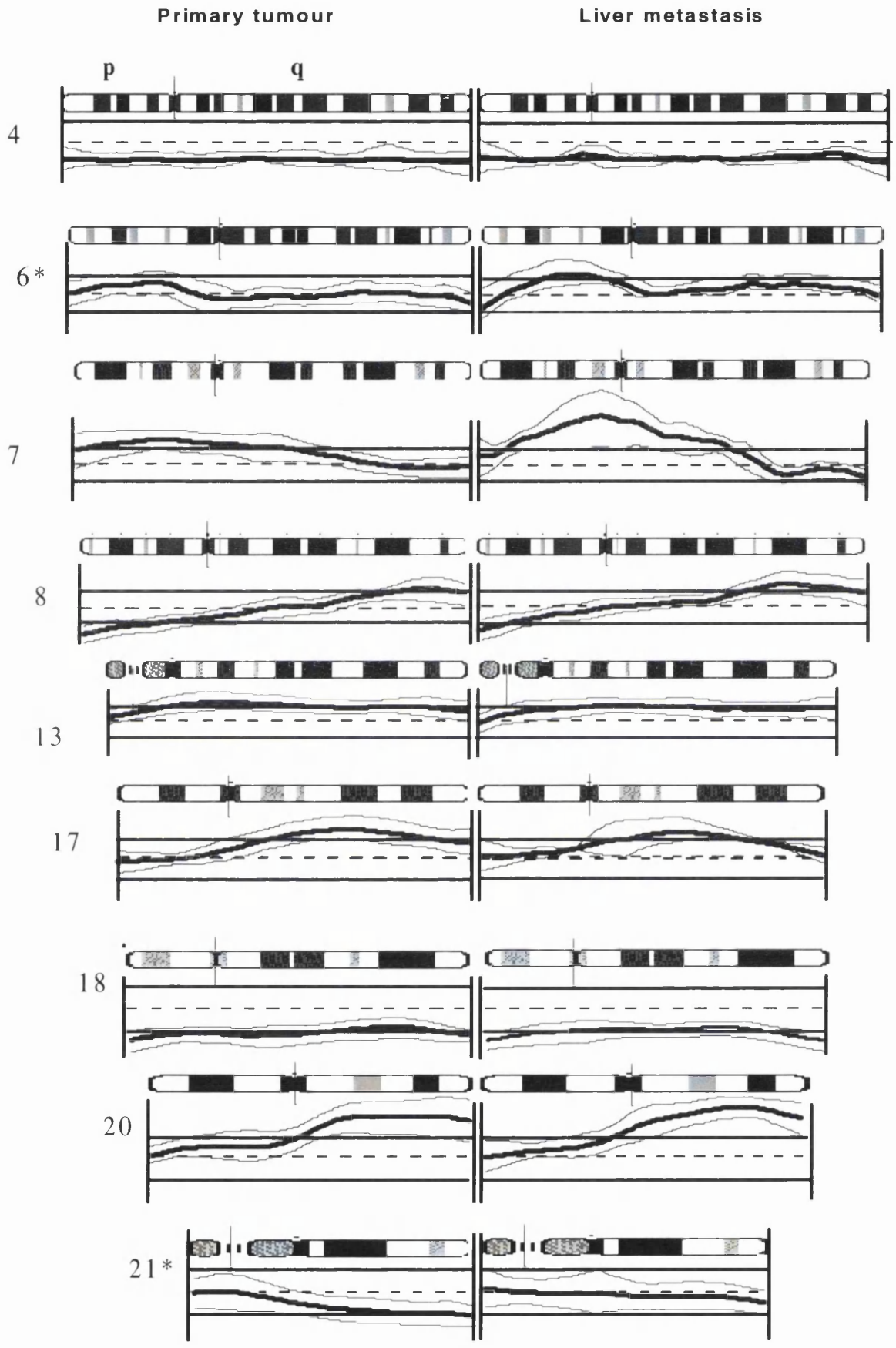

Primary tumour
Liver metastasis
p
q
4
6*
7
8
13
17
18
20
21*

Figure 32: *Representative CGH images from patient 4 (Table 9) primary tumour DNA*

A) Normal DNA labelled with digoxigenin. B) Tumour DNA labelled with biotin. C) DAPI stain of the chromosome spread. D) Normalised image.

Deletions or decreased chromosomal copy number are stained red, and amplifications or increased chromosomal copy number are intensely stained yellow/green. Arrowheads represent specific genetic aberrations with + indicating amplifications or increased copy number and - indicate deletions or decreased chromosomal copy number. The profiles for the normalised image is shown in Figure 31.

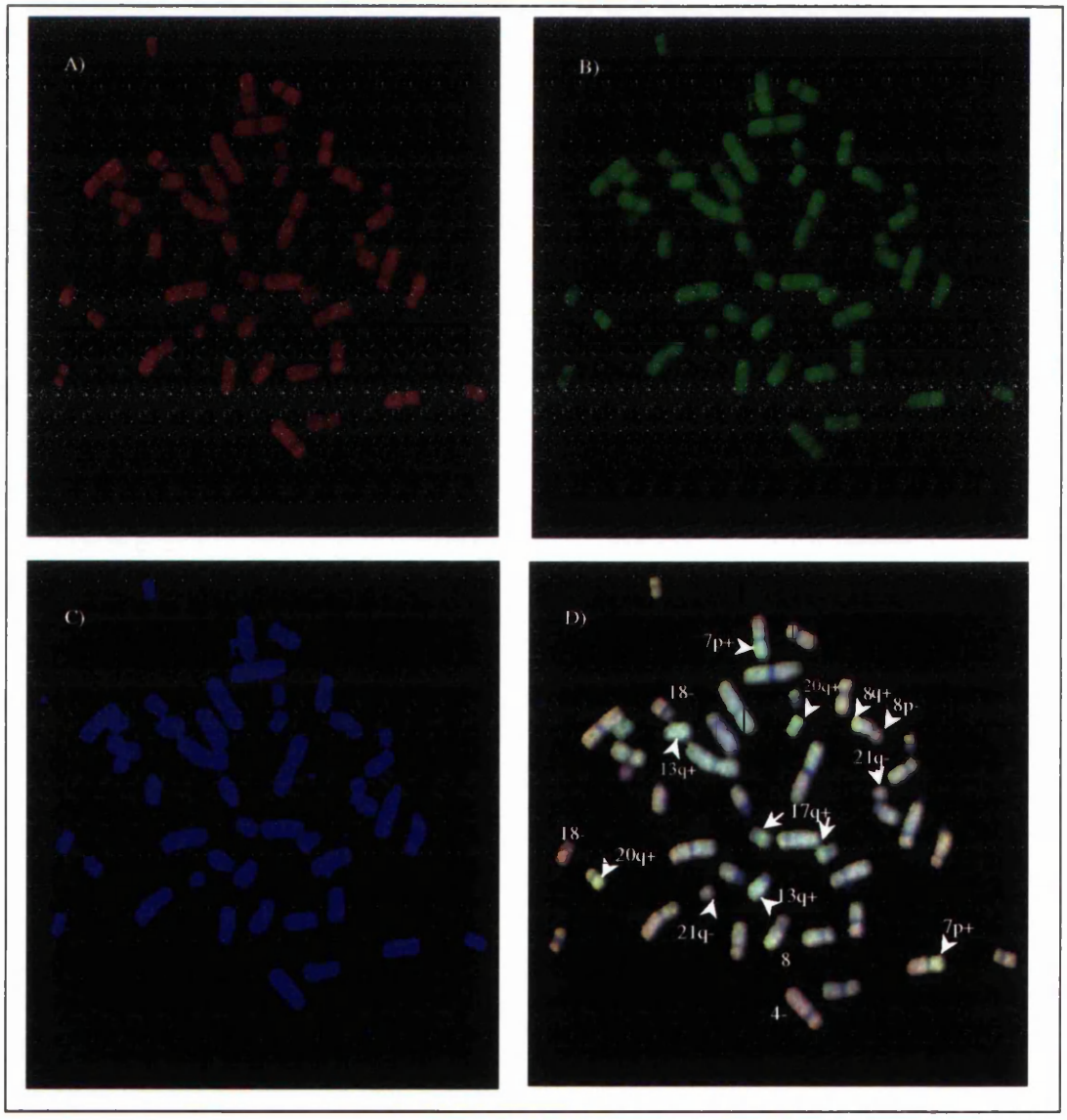
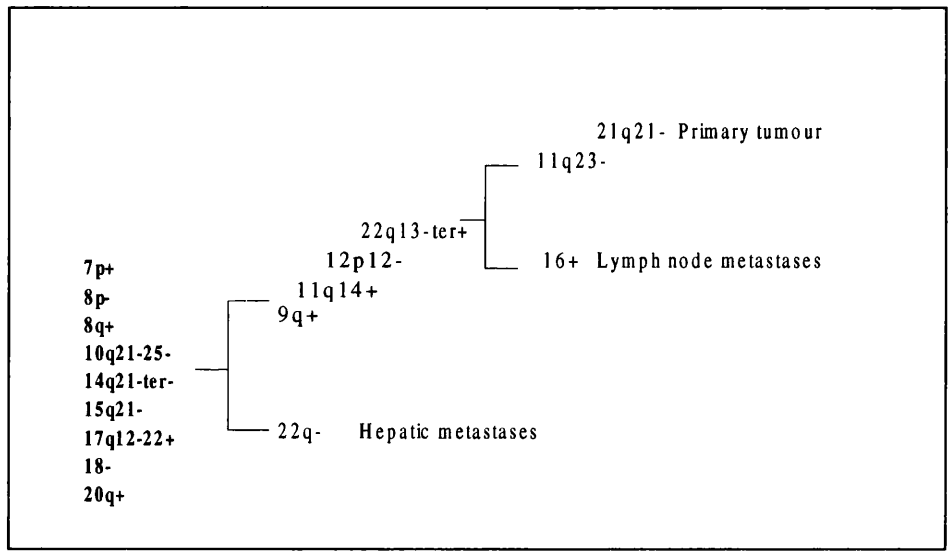


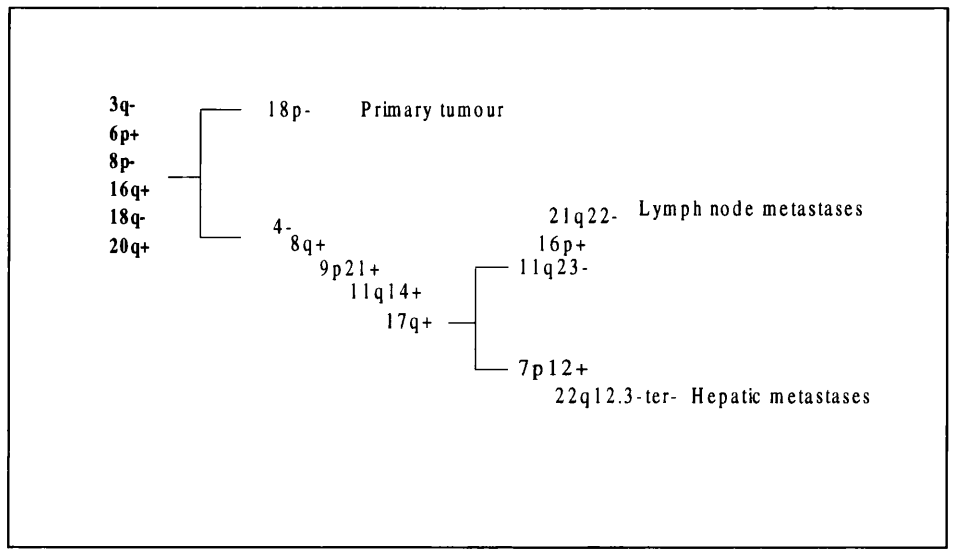
Figure 33: *Clonal relationships between primary and metastatic carcinoma in lymph node and liver for two patients with metastatic colorectal cancer*

Common genetic aberrations between all tumour lesions are shown in bold. The order in which the genetic aberrations are shown to occur between branch points is arbitrary. - indicates deletion or loss, while + indicates amplification or gain.

Patient 1



Patient 2



11.3. ***Summary and discussion***

The progression of adenoma to colorectal carcinoma is accompanied by accumulation of many genetic changes (Fearon and Vogelstein, 1990). Recently, Mertens et al. (1997) assessed the distribution of chromosomal gains and losses in published karyotypes from 11 tumour types including 333 cases of colorectal carcinomas. Frequent gain of chromosomes 7, 8q, 13 and 20 and losses of 1p, 5q, 8p, 14p, 17p, 18 and 22 were found. In another study, Ried et al. (1996) used comparative genomic hybridisation to identify chromosomal imbalances in adenomas and carcinomas of patients with colorectal cancer. Carcinomas were found to have multiple chromosomal aberrations involving gain of chromosomes 1, 13 and 20 and chromosome arms 7p and 8q, whereas chromosome 4 and chromosome arms 8p and 10q were frequently underrepresented. This study showed that the transition of adenoma to carcinoma is characterized by the emergence of multiple chromosomal aberrations. Such studies, clearly, define the genetic imbalances found in colorectal carcinoma and point to the accumulation of non-random genetic aberration patterns that lead to progression of adenomas to carcinomas. However, the relevance of these and other genetic alterations to evolution of a metastatic phenotype has not yet been defined. The progression of colorectal carcinoma to metastatic disease remains poorly studied, and has been restricted to genes thought to be involved in the metastatic process, despite the fact that metastasis, particularly to the liver, is one of the major causes of death from colorectal cancer.

The study of metastasis in colorectal cancer would be informative, since clinically the disease is subdivided into stages with different clinical and pathological behaviours *in vivo*. Comparing genetic changes in the primary tumours and their corresponding metastases from the same patients could reveal subtle genetic differences between the lesions, thereby pinpointing important

genetic events that could predispose to metastatic dissemination. Also, the analysis of paired samples from the same patient make it possible to assess the degree of clonal divergence and genetic heterogeneity, which could have immense importance not only in understanding the biology of metastasis, but also in its treatment.

This study was designed to assess the extent of genetic similarities and differences between primary colorectal carcinomas and their corresponding metastases, and to investigate whether additional genetic aberrations are associated with progression of colorectal carcinoma beyond the primary tumour.

The use of CGH, a broadly scanning genome method, combined with analysis of paired tumour samples from the same patients, have facilitated our search for molecular cytogenetic markers that may indicate regions of the genome involved in progression and metastasis of colorectal cancer *in vivo*. Previous studies relied on animal models and LOH techniques to study tumour progression (Dove et al., 1995). Although these studies were very valuable in advancing our understanding of the metastatic process, they gave a restricted view of genomic changes involved in human colorectal cancer progression.

This CGH analysis shows that certain genetic alterations are especially common in metastatic colorectal carcinoma. These changes include gains of chromosome arms 7p, 8q, 13q and 20q and underepresentation of chromosome 4, 18, chromosome arms 8p, and 22q. Thus, a chromosome profile for metastatic colorectal cancer that can be used to identify and set priorities in selecting genes for further study has been constructed.

Previous analysis using karyotyping (Mertens et al., 1997) or CGH (Ried et al., 1996) have also shown that most of these genetic aberrations are frequent in

colorectal cancer. The results, therefore, are concordant with the published data regarding genetic alterations in colorectal cancer. However, the previous studies did not focus on a particular stage of the disease, which could underestimate the importance of some aberrations to particular Dukes' stages. This study shows that both Dukes' C and D stages of the disease share many genetic changes (Table 11 and Figure 34), some of which are probably involved in invasion, since invasion is probably an essential step in metastasis to both lymph nodes and liver. For example, chromosome arm 20q was frequently gained in both lymph node and liver metastases. Interestingly, chromosome arm 20q is the location of the gene for the enzyme matrix metalloprotease-9 (MMP-9 or gelatinase B) (Linn et al., 1996), which degrades collagen type IV, a major component of basement membranes. Increased MMP-9 levels have been shown to be associated with metastatic colorectal cancer (Zeng et al., 1996). Also, chromosome arm 7p gain was frequently found (8/12) in primary tumours and/or their metastases. Chromosome arm 7p is known to harbour epidermal growth factor receptor (*EGFR*) and T-cell tumour invasion and metastasis-1 (*TTIM1*) genes (Kondo and Shimizu, 1983). Increased EGFR expression has also been found in advanced colorectal tumours (Dukes' C and D) and those exhibiting vascular and lymphatic invasion (Kluftinger et al., 1992). Somatic cell fusion studies have shown that the invasive capabilities of hybrid cells depended on the presence of chromosome fragment 7p12-cen (Collard et al., 1987; Habets et al., 1994).

Decreased copy number of chromosomes 4, 18 and 22 was also very frequent in both Dukes' stages. Putative tumour suppressor genes have been localized to 18q21: *DCC*, deletions of which have been correlated with colon cancer progression (Cho and Fearon, 1995), and two members of the *SMAD* gene

family, *SMAD2* and *SMAD4*, which are mutated in a proportion of human colon cancers (Riggins et al., 1997). Interestingly, it has recently been shown that over-expression of *SMAD4* is associated with p21 induction and growth inhibition of carcinoma cells (Hunt et al., 1998).

To date, no convincing tumour suppressor genes or oncogenes have been mapped to chromosome 4. Though it has been shown that chromosome 4 is involved in cellular senescence *in vitro* (Ning and Pereira-Smith, 1991). Deletions or decreased copy number of this chromosome could therefore be an advantage in the selection of immortal cells. Also, in agreement with the data presented, LOH involving 22q has been shown to be frequent in colorectal cancer, and correlates with metastatic colorectal carcinoma (Yana et al., 1995).

Increased copy number of chromosome arms 6p and 17q were only found in Dukes' stage D tumours and liver metastases. This has not been previously shown to be frequent in colorectal cancer, perhaps due to the fact that very few studies have analysed the most advanced colorectal cancer and even fewer have analysed the most advanced colorectal cancer. To date, most cytogenetic studies concentrated on either earlier stages of colorectal cancer progression or made no attempt to correlate genetic aberrations with progression markers such as Dukes' staging. Chromosome 6p harbours the gene encoding vascular endothelial growth factor (Wei et al., 1996), a promoter of tumour angiogenesis. Over-production of VEGF has been associated with distant metastatic recurrence in lymph-node-negative patients with colorectal cancer (Ishigami et al., 1998; Kang et al., 1997). Chromosome arm 17q contains the *ERBB2* gene (Popescu et al., 1989), which is highly expressed in bone metastases from breast and gastrointestinal cancers, and its expression to be correlated with advanced cancers (Pantel et al., 1993).

Notably, Yang et al. (1997) have shown that liver metastasising primary colorectal tumours and their liver metastases expressed significantly higher levels of c-erbB-2 mRNA than non-metastasising primary tumours. Moreover, c-erbB-2 mRNA expression significantly predicted the development of liver metastases in tumours that had not metastasised to lymph nodes. The authors argue that *ERBB2* gene may play an important role in the development of metastasis in colorectal cancer and its mRNA expression may have important implications for deciding which patients with lymph node negative disease require chemotherapy. In addition, by using an amplotype-PCR approach on 12 primary colorectal tumours and their metastases, a recent study has shown that loss of chromosme 4 and gain of chromosome 6 were significantly more frequent in metastases (Malkhosyan et al., 1998).

The data presented here show that advanced colorectal cancer is clonally diverse. Indeed, this CGH analysis suggests that heterogeneity is not only present between the primary tumours and their metastases but could also be present within primary tumours themselves. The results also suggest that metastases accumulate genetic aberrations additional to those found in the primary tumours. Whether these additional changes are underepresented in the primary tumours but are manifestations of the clonal evolution of metastatic cells (Korczak et al., 1988) or have evolved in the metastases themselves, is not clear. Nevertheless, the data indicate that additional common genetic aberrations could be important in the acquisition of a metastatic phenotype (Fearon and Vogelstein, 1990).

The genetic heterogeneity between primary tumours and their metastases demonstrated here not only supports the previous observation regarding Ki-*ras* heterogeneity, but also reveals its extent in advanced colorectal cancer. Similarly,

LOH studies demonstrated significant heterogeneity within early carcinomas arising within colorectal adenomas (Boland et al., 1995). Previous CGH studies involving primary breast carcinomas and their corresponding metastases (Kuukasjarvi et al., 1997; Nishizaki et al., 1997) have demonstrated extensive heterogeneity within the primary tumours and between the primary tumours and their metastases, to the extent that, in some patients, the metastases genetically differ almost completely from their paired primary tumours. In my case, however, the primary tumours and their metastases often shared many common genetic aberrations. This indicates that clonal divergence and genetic instability in colorectal cancer are not as extensive as those seen in some breast cancers.

The finding that lymph node and liver metastases, even those from the same patients, do not always show similar genetic aberrations indicates that the mechanisms involved in haematogenous dissemination to distant organs differ from those required for spread to regional lymph nodes. The marked genetic differences between Dukes' C (lymph node metastasis) and Dukes' D (liver metastasis) tumours suggest that the former are not always simply an earlier stage of the latter and, thus, in some instances at least, they are distinct forms of the disease (Figure 34). Indeed, it is not uncommon in clinical practice to find patients with haematogenous metastases without the involvement of their lymph nodes (Dworkin et al., 1995).

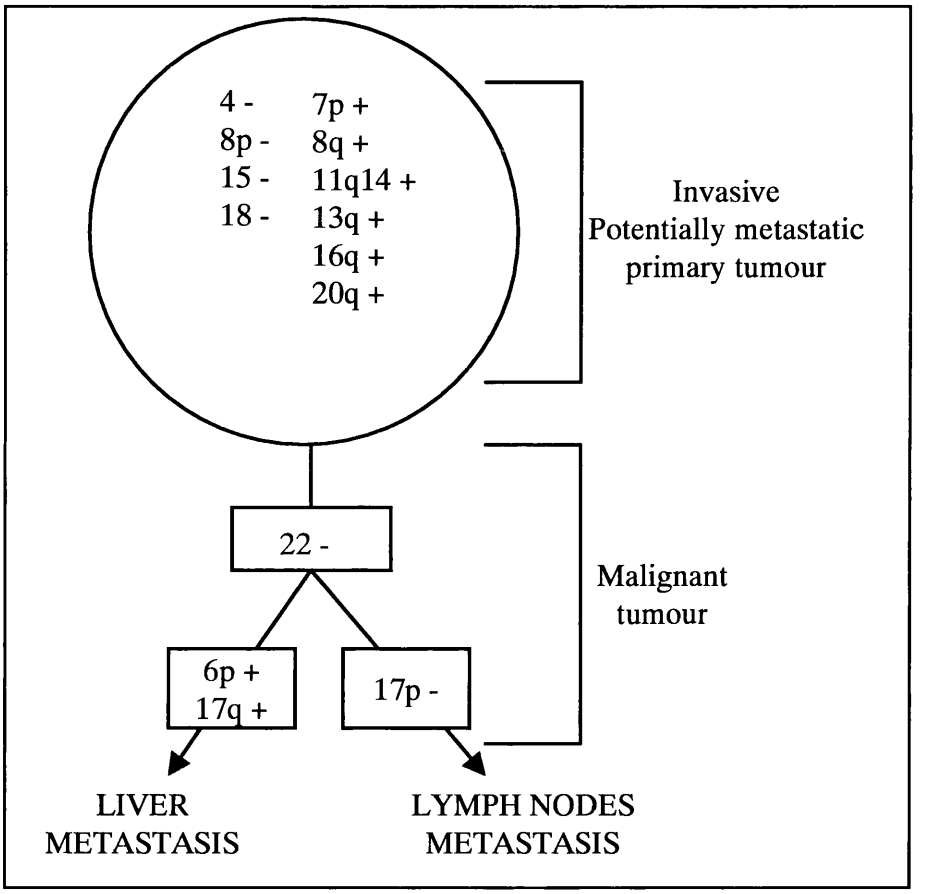
Through the use of a wide-scanning genome methodology, I have described a number of genetic aberrations associated with metastatic colorectal cancer, albeit in large chromosomal regions which must contain many possible target genes, both known as well as those yet to be discovered. The generation of such aberrant chromosome profiles is valuable for guiding future research. The

data suggest that chromosomes 4, 6, 7, 8, 13, 17, 18, 20, and 22 are all worthy of further analysis with more focused techniques. They also stress the importance of correlating genetic aberrations with progression markers such as Dukes' staging to avoid missing critical events in the metastatic process. In addition, the analysis of primary tumours and corresponding metastases has proved valuable in delineating genetic mechanisms that might underlie cancer progression through clonal evolution.

Figure 34: *A model representing genetic aberrations involved in the metastatic process in colorectal cancer*

Common genetic aberrations between Dukes' C and D (4-, 8p-, 15-, 18-, 7p+, 8q+, 11q14+, 13q+, 16+ and 20q+) could represent early genetic events important in the acquisition of invasive phenotypes since invasion is required for successful metastasis in both stages. Further genetic aberrations such as loss of chromosome 22 seen mainly in metastases of Dukes' C and Dukes' D patients and chromosome arm 17p found only in lymph nodes metastases could give cells the capability to colonize regional sites.

The addition of further genetic aberrations such as the increase copy numbers of chromosome ams 6p, 17q representing gain of function, could give cells survival advantages in colonizing distant sites such as the liver.



GENERAL DISCUSSION

CHAPTER 12. GENERAL DISCUSSION

Functionally, it is useful to divide the results in this thesis into two parts. The first part deals with genetic aberrations associated with colorectal cancer progression, while the second part gives an insight into genetic heterogeneity in colorectal cancer.

To assess the genetic aberrations associated with progression of colorectal cancer, mutation analysis of the Ki-*ras* gene and CGH were used. Mutation analysis of codons 12 and 13 of the Ki-*ras* gene in 78 patients with colorectal cancer has shown that valine-12 (GTT) mutation in codon 12 to be associated with aggressive colorectal tumours and with early mortality of patients harbouring this particular mutation. None of the other codon 12 and 13 mutations showed such correlation. Interestingly, the dramatic effect of valine-12 mutation on survival was masked when the analysis of survival included all the other possible codon 12 and 13 mutations. This result was not surprising since Ki-*ras* mutations, other than valine-12, were not particularly associated with aggressive tumours. However, these results stress the importance of separately correlating or stratifying each type of Ki-*ras* mutations with survival data to avoid misinterpreting the "real" role of Ki-*ras* mutations in colorectal cancer progression.

It is worth noting that this study, as with the majority of other studies, included a relatively small number of patients and when the results were stratified further each class of Ki-*ras* mutations contained too small a number of patients to be of any significant reliability. However, the recent study of Andreyev et al.

(1998) compiled from the work of many investigators, which included a total number of 2721 patients, gives the results obtained in this thesis significant support in that when specific mutations were evaluated, only valine-12 mutations were associated with increased risk of recurrence and death. However, contrary to my findings, it has shown that the Ki-*ras* mutations are not associated with any Dukes' stage and that the presence of Ki-*ras* mutation increased the risk of recurrence and death. Nevertheless, it is evident that mutations of Ki-*ras* by themselves are neither necessary nor sufficient for tumour formation or metastatic progression since only 30-50 % of colorectal tumours contain mutated Ki-*ras* gene and as I have shown, in some cases at least, the metastases could form without the need for mutationally activated Ki-*ras*.

It was therefore of some importance to search for genetic aberrations, other than Ki-*ras* mutations, that could be associated with metastatic colorectal cancer. The choice of a wide-scanning methodology such as CGH was essential for two reasons. First, very little is known to date about the "gross" or overall chromosomal aberrations in advanced colorectal cancer and secondly, metastasis is a complex process involving multiple genetic and epigenetic events. The use of a wide-scanning methodology could shed more light on the significance of the interactions of multiple genetic aberrations present within each tumour sample and their combined importance in the metastatic process.

CGH analysis of 12 patients with colorectal cancer pinpointed genetic aberrations, albeit large areas of chromosomal aberrations, frequently associated with advanced colorectal cancer. The results obtained from CGH analysis of these tumours have been summarised previously. To date, this represents the first study to directly compare advanced primary colorectal tumours with their synchronous

metastases. Other studies using CGH concentrated on genetic events associated with the progression of adenomas to carcinomas (Ried et al., 1996; Meijer et al., 1998) or CGH and Karyotypic analysis of primary colorectal tumours without any attempt to stratify primary tumours into different stages (Nakao et al., 1998; Mertens et al., 1997). Although the results presented here are remarkably similar to the results presented in the above studies, direct comparisons of the results is unfair since the comparison would be between tumours of different Dukes' stages. To date, there is only one other study that restricted the analysis to Dukes' D colorectal tumours and compared the genetic composition of primary tumours to their synchronous metastases, albeit using a different approach known as amplotyping (Malkhosyan et al., 1998). The agreement in chromosomal aberration between the results presented here and Malkhosyan et al. (1998) study indeed is striking. Nevertheless, the data presented here are based on a small number of patients and further larger studies should shed more light on the nature and extent of genetic aberrations in advanced colorectal cancer.

It should be mentioned that the results I present do not necessarily prove that the chromosomal aberrations found in advanced colorectal cancer are associated with poor prognosis or that early colorectal tumours do not possess these aberrations; they merely state the reportoire of genetic aberrations found in advanced colorectal cancer. The stratification of tumours into different Dukes' stages has important implications on the study of genes involved in tumour progression since currently the majority of studies either make no attempt to classify primary tumours into different stages before analysis and are clumped together as "primary tumours" or patients with occult metastasis are erroneously included in studies as "early tumours". This in turn can partly explain the wide

variation in results obtained from different studies on the genetics of tumour progression and stresses the importance of choosing the appropriate tumour material to avoid masking genetic events that are essential for the metastatic process.

Regarding the other part of the thesis, namely tumour heterogeneity, the evidence for and causes of tumour heterogeneity were dicussed in Chapter 4. In this section, I will discuss the relevance of my findings to tumour heterogeneity in general.

The data obtained from mutational analysis of codons 12 and 13 of the Ki-*ras* gene show that the presence of a Ki-*ras* heterogeneity within primary carcinomas from single sections was not infrequent. For example, some primary tumours contained areas of cells with mutated Ki-*ras* gene, while adjacent areas from the same section contained cells with wild-type Ki-*ras*. There are at least two mechanisms by which such heterogeneity could have arisen. One mechanism involves the evolution of the two clones from separate stem cells (polyclonality). The other mechanism could simply reflect genetic instability in cancer where Ki-*ras* mutant clones have arisen from previously established cancerous clone(s) containing wild type Ki-*ras* gene. Thus, the presence of genetic heterogeneity in a tumour by itself does not necessarily indicate a polyclonal origin but it merely indicates that a developed cancer can be composed of more than one clone. Giaretti et al. (1996), using FACS analysis, has confirmed the presence of Ki-*ras* heterogeneity in colorectal cancer. However, while Shibata et al. (1993) found evidence of Ki-*ras* heterogeneity in adenomas, different areas within colorectal carcinomas were found to be homogenous for Ki-*ras* mutations. These discrepencies in results between different investigators are not uncommon in

cancer research since they usually reflect the effects of different methodologies, tumour materials used and personal interpretations of the presented data. For example, Shibata et al. (1993) have used a small number of patients (seven carcinomas) whose tumours have been pre-selected to contain Ki-*ras* mutations. This pre-selection step can be misleading because it excludes from the analysis tumours containing a small proportion of Ki-*ras* mutants. Also, in these "special" cases, mutant Ki-*ras* gene might have been a necessary genetic aberration for progression into carcinomas. Thus, finding Ki-*ras* homogenously mutated in different parts of a tumour does not necessary indicate that the tumour is not heterogenous for other markers, but rather could suggest a role for Ki-*ras* mutations in progression in some tumours. Indeed, the authors found loss of the normal p53 allele to be variable within the single cancer analysed and detected evidence, albeit in a small number of cases, of tumour heterogeneity with respect to the Ki-*ras* locus.

These observations were unfortunately attributed to contamination by normal cells or dismissed from the argument. In my case, the majority of primary tumours and their synchronous metastases contained the same mutation indicating the importance of Ki-*ras* mutations in tumour progression in some patients. A similar argument would also apply to the study of Boland et al. (1995). The authors findings that deletions involving 5q, 17p and 18q were homogeneously found in carcinomas could indicate the importance of these genetic aberrations in tumour progression and so can not be used as markers for tumour heterogeneity. This argument raises an important point about tumour progression in that there appear to be different mechanisms by which tumours progress. For example, those that require mutated Ki-*ras* gene for their progression, which appear

homogeneous for that particular aberration (but can give the false impression of monoclonality or homogeneity in tumours), and tumours that require genetic aberrations other than mutated Ki-*ras* for their progression (the use of Ki-*ras* mutations in these tumours is a better indicator for the presence of tumour heterogeniety since they are not required for tumour progression).

Another caveat of particular relevance to the study of genetic heterogeneity in tumours is the stage at which the tumour is analysed. The analysis of advanced tumours could be misleading in term of the extent of genetic heterogeneity, since early heterogeneous tumours may become apparently homogeneous because of domination of tumours by a growing clone under strong selective pressures, to a point where the minor clones become undetectable. Although the use of tissue microdissection could alleviate this problem, the numbers of sites sampled and the size of patches used remain poorly standardised and potential sources of experimental errors.

The comparison of primary tumours and their synchronous metastases from the same patients by CGH revealed that both tumours share many common genetic events and that metastases, in some instances at least, not only accumulated additional genetic aberrations but contained other genetic defects that were not found in their primary tumours. This and other evidence suggests that primary tumours are heterogenous in their genetic composition and that, in some instances, the metastatic clone(s) could be a minor component in the genetic composition of primary tumours and, thus, could be easily masked in analysis. On the other hand, these additional or other genetic aberrations found only in metastases could have evolved within the metastases themselves. Nevertheless, tumour heterogeneity should be addressed whenever certain genetic event(s) are

proposed as a prognostic marker for cancer progression or whatever clinical therapy is offered to patients. An important concept that has arisen from this work is that in, at least some patients, metastases to liver do not necessarily have accumulated more genetic aberrations than metastases to lymph nodes and that the genetic aberrations leading to liver or lymph nodes metastasis are significantly different. Thus, the genetic evolution of the two Dukes' stages could be different and not necessarily consequential (Figure 35).

Metastasis is a complex process and the relevance of the genetic aberrations detected in this study to the evolution of metastatic clones will ultimately depend on thorough understanding of the effects of these changes at the molecular and cellular levels. This work represents a preliminary step in the ongoing war against cancer. As a pathologist, the work has offered me a unique privilege to realize the complexity of the metastatic process and the immense struggle that the clinicians and scientists together have to endure in the battles ahead.

12.1. ***Future work***

The result obtained in this work show valine-12, rather than aspartate-12, Ki-Ras protein to be associated with more aggressive tumours *in vivo* and cells expressing it with more invasive phenotype *in vitro*. To explain this different behaviour, a hypothesis, which suggests that valine-12 but not aspartate-12 Ki-Ras is able to bind Raf-1 or activate MAPK pathway, is proposed. This hypothesis is testable, and could be very rewarding indeed.

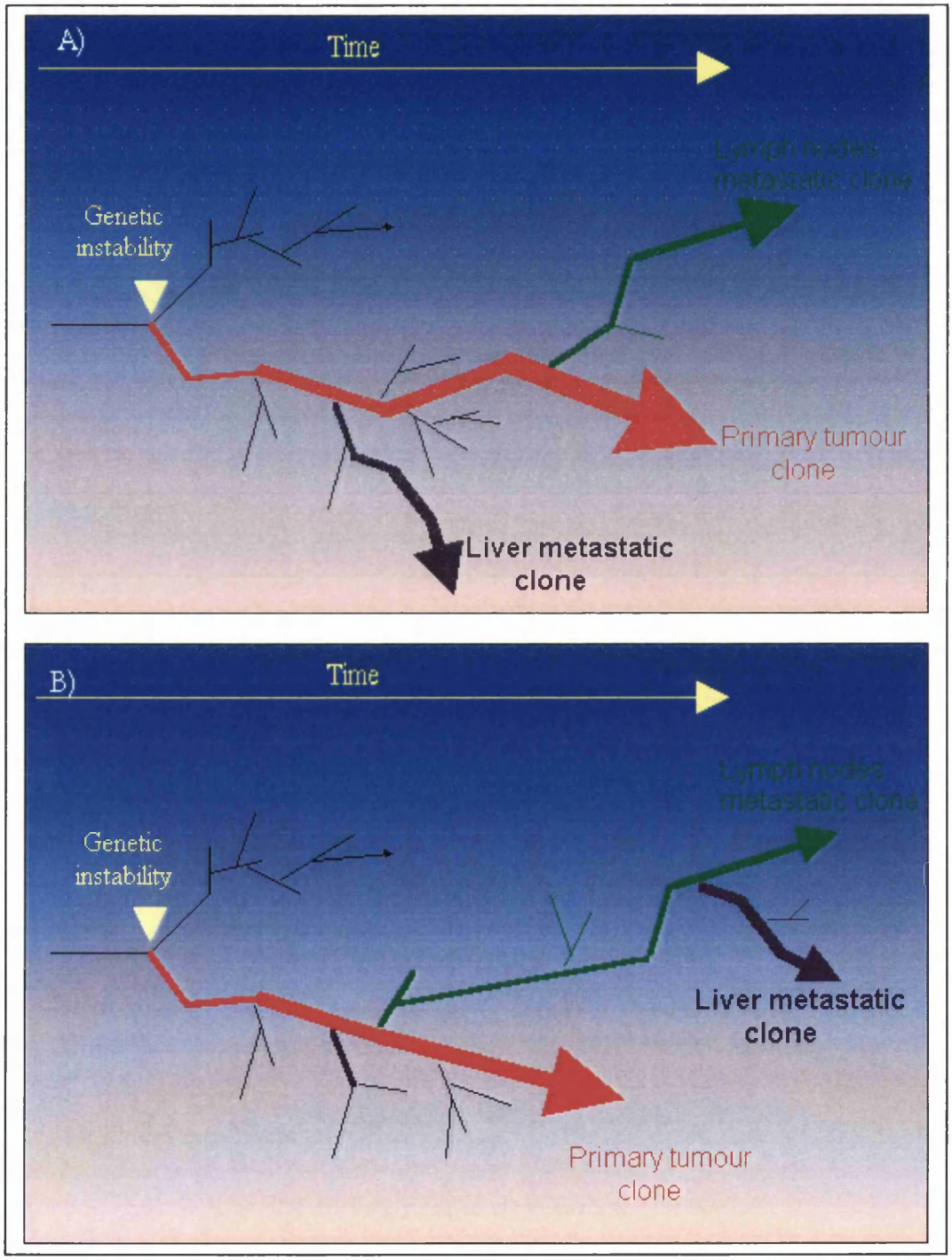
The results obtained using CGH also show certain chromosomal aberrations to be frequent in advanced colorectal cancer. I propose that future work should focus on narrowing the areas of interest using probes targetted against interesting genes within these areas. In particular, microarrays technology could be of immense benefit if properly applied to this type of work. Some genes of interest are listed in Table 13. It has to be noted, however, that the chromosomal aberrations found contain a large number of genes and that chromosomal areas found to be amplified or increased in copy number could potentially contain defective or deleted genes as well.

Table 13: A summary of interesting genes for further persuit

Chromosome	Current genes of interest
6p	*VEGF, P21, Cyclin D3, BAK, PIM-1, CDC5L*
7p	*EGFR, PDGFA, TTIM1*
8q	*c-MOS, c-MYC, FAK*
9p	$P16^{INK4}$, $P14^{ARF}$
11q	*Cyclin D1, BCL1, FGF4, FGF3, EMS1*
13q	*DP1*
17p	*P53*
17q	*ERBB2, FGF1*
18q	*SMAD2, SMAD4, DCC*
20q	*AIB, BTAK, c-SRC, MMP-9*
22	*P300*

Figure 35: *A simplified schematic representation of clonal evolution in colorectal cancer*

Clonal evolution is represented rather like a tree with new clones branshing from it. The thick red arrow represents the major clone in the primary tumour. A) Represents a scenario where by liver metastasis clones arise independently and at a different time from clones destined to colonise lymph nodes. B) Represents another scenario in which liver and lymph nodes metastatic clones evolve jointly but diverge at a later stage in the genetic evolution sequence.



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