A Genetic Study of Liver Metastasis from Primary Colorectal Cancers and Uveal Melanomas

by

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DECLARATION

I hereby declare that no part of this thesis has been previously submitted in support of any other degree or qualification, at this, or any other university or institute of learning.

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ABBREVIATIONS

5-FU	5-fluorouracil
ADP	adenosine diphosphate
APC	adenomatous polyposis coli
APS	ammonium persulphate solution
ATM	ataxia telangectasia
ATP	adenosine triphospahte
B-CLL	B cell – chronic lymphoid leukaemia
bp	base pair
CCD	(cooled) charged couple device
cDNA	complementary DNA
CEA	carcinoma embryonic antigen
CGH	comparative genomic hybridisation
CIN	chromosomal instability
CNS	central nervous system
COMS	Collaborative Ocular Melanoma Study
CT	computed tomography
DAPI	4'-6-Diamidino-2-phenylindole
DCC	deleted in colon cancer
DLM	died with liver metastases
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOP-PCR	degenerated oligonucleotide primer – PCR
dTTP	deoxy-thymine triphosphate
DUK	died of unknown causes
DUR	died of unrelated causes
dUTP	deoxy-uracil triphosphate
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGF-R	EGF-receptor
FAP	familial adenomatous polyposis
FISH	fluorescent *in situ* hybridisation
FITC	fluoroscein isothiocyanate
GAP	GTPase activating protein
GDP	guanosine diphosphate
GI	genetic imbalance
GTP	guanosine triphosphate
H&E	haematoxylin and eosin
HNPCC	hereditary non-polyposis colorectal cancer
IRD	infra-red dye
LFU	lost to follow-up
LOH	loss of heterozygosity
MAMA	monoallelic mutation analysis
MCC	mutated in colon cancer
MCR	mutation cluster region
Min	multiple intestinal neoplasia

MMP	matrix metalloproteinase
MMR	mismatch repair
MOM-1	modifier of Min
MRI	magnetic resonance imaging
mRNA	messenger RNA
MSI	microsatellite instability
MSI-H	MSI – high level
MSI-L	MSI – low level
NER	nucleotide excision repair
NICE	National Institute for Clinical Excellence
NIH	National Institutes for Health
NSAID	non-steroidal anti inflammatory drug
OCT	optimum cutting temperature (compound)
PCR	polymerase chain reaction
PHA	phyto-haemagglutinin
PBS	phosphate buffered saline
PBSe	phosphate buffered saline-EDTA
RB	retinoblastoma
RER	replication error
RNA	ribonucleic acid
SKY	spectral karyotyping
SSC	standard saline citrate
SSCT	standard saline citrate-Tween 20
SSCTM	standard saline citrate-Tween 20-Marvel™
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEMED	N, N, N', N', -tetramethylethylenediamine
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
ULS	Universal Linkage System
UV	ultra violet
VEGF	vascular endothelial growth factor
VHL	Von Hippel-Lindau
YAC	yeast artificial chromosome

SUMMARY OF THESIS

Cancer is a multifactorial disease, with the development of metastases being a major cause of morbidity and mortality. Several molecular pathways are thought to be involved in the tumourigenesis of colorectal cancer and possibly metastasis. The aim of this research was to explore aspects of these pathways and relate genetic changes to clinicopathological variables and outcome, with the goal of ascertaining novel genetic abnormalities predictive of metastasis. A second cancer, uveal melanoma was also studied as a comparison, as these cancers invariably metastasise to the liver, in which abnormalities of chromosomes 3 and 8 have already been shown to be predictive of liver metastasis and hence a poor prognosis. Fluorescent *in situ* hybridisation (FISH) analysis of fresh-frozen uveal melanomas using alpha-centromeric probes for chromosomes 3 and 8 confirmed a significant association between genetic imbalance and the presence of liver metastasis and reduced survival. Difficulties were encountered with using FISH for the analysis of chromosomal abnormalities in formalin-fixed paraffin-embedded samples of colorectal cancers and their liver metastases. Therefore, comparative genomic hybridisation (CGH) was utilised for the genome-wide analysis for regions of chromosomal amplification and loss. The technique was partially successful; however problems were encountered in obtaining analysable target metaphase slides, this was partly overcome by the manufacture of target slides in-house. The analysis of microsatellite instability (MSI) using polymerase chain reaction (PCR) was more successful with the presence of MSI being associated with the presence of solitary metastases, but interestingly not with an improved prognosis. Finally, the collection of a single paired fresh-frozen sample of a colorectal cancer and its metastasis enabled all the techniques to be applied, thus showing that prospective collection and analysis is not only feasible but would allow the clinical significance of genetic abnormalities to be assessed more accurately.

CHAPTER 1 – INTRODUCTION

CONTENTS

One in three people will develop a cancer during his or her lifetime, with one in four dying as a consequence of the disease (Cancer Research Campaign, 1999). Worldwide, cancer is the third most common cause of death, after death from infectious diseases and diseases of the circulatory system (Franks, 1997).

1.1 NEOPLASIA

Neoplasia literally means "new growth," and the new growth is termed a neoplasm. The term neoplasm is used interchangeably with the term "tumour," which was originally applied to the swelling caused by inflammation and cancer is the common term for all malignant tumours. It should be noted that the term neoplasia reflects both benign and malignant tumours the only absolute criterion is the ability of malignant tumours to invade surrounding tissue and to colonise distant sites (metastasis), (Cotran et al., 1989). The best available definition of a neoplasm is one that was suggested by Sir Rupert Willis (1952),

"A neoplasm is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and which persists in the same excessive manner after the cessation of the stimulus which has evoked the change."

Analysing this definition in functional terms, it becomes clear that there are at least four types of disturbance of cell behaviour inherent within it:

1) A disturbance in cell proliferation.
2) A disturbance in cell differentiation.
3) A disturbance in the relationship between cells and their surrounding structure.
4) A disturbance in normal programmed cell death (apoptosis).

There is no single cause for malignancy, but the influence of a number of environmental and genetic events play a part in the malignant transformation. Evolution through natural selection is a concept that applies to many biological systems (Darwin, 1859). It can also be applied to cancer development, where a multi-step process involves the selection of a number of somatic mutations and therefore clones of cells (Nowell,

1976; Vogelstein and Kinzler, 1993; Klein, 1998). A number of reports also reveal a distinctive pattern of genetic alterations in different tumours, implying that the carcinogenic process may proceed through a variety of genetic pathways (Shackney and Shankey, 1997). The extent to which different alleles are favoured in the selection process is totally dependant on the surrounding milieu, with different genes and phenotypes being selected for, by environmental variations (Breivik and Gaudernack, 1999). There are many types of malignant tumours occurring in man, which are causally related to environmental factors. Such factors may be chemical, physical or viral. The genetic influence in malignant transformation is by the interplay of so-called cancer genes. A cancer gene is any gene sequence contributing directly to neoplastic change.

1.2 CANCER GENETICS

There appears to be four groups of genes, at present, that are thought to contribute to neoplastic change and tumour progression by constitutive activation, mutation or deletion. These are oncogenes, tumour suppressor genes, DNA repair genes and apoptotic genes

However, as with most biological systems, there is some functional overlapping among these categories.

1.2.1 Oncogenes

Oncogenes in general have a positive effect on tumour development and tend to be highly conserved "household" genes involved in a variety of cell signalling pathways, coding for growth factors, growth factor receptors or signal transduction proteins. Others code for transcription factors that when activated as a result of mutations, retroviral insertions, chromosomal translocations or gene amplifications, result in cell growth and division. Examples of oncogene activation include, *c-myc*, which becomes activated when an immunoglobulin locus is inserted next to it, resulting in Burkitt's lymphoma (Teich, 1997) and the formation of the Philadelphia chromosome, which results in the formation of a new protein the product of the fusion of *BCR* and *c-abl* genes as a result of chromosomal translocation in chronic myeloid leukaemia (Rowle, 1973).

1.2.2 Tumour Suppressor Genes

Tumour suppressor genes conversely tend to negatively regulate cell growth and whose absence can lead to tumourigenesis. It has been known for sometime that the fusion of normal cells to malignant cells, results in the suppression of the malignant phenotype, as long as the chromosome complement derived from the normal parent is maintained (Klein, 1987). Further information was derived from studies of retinoblastoma, a malignant tumour of the eye. There are two clinical forms of retinoblastoma, one of which is sporadic (always unifocal and unilateral) and the other hereditary (often multifocal and/or bilateral). Knudson (1971) proposed that the development of any retinoblastoma was the consequence of two mutations within a retinoblast, the "two-hit" hypothesis. One of these mutations was inherited while the other was acquired spontaneously during the child's development. Comings (1973) proposed that the Knudson's two genetic targets could be the two alleles of a single gene, whose product negatively regulated cellular growth. Thus, a germ line mutation in one retinoblastoma (*RB*) allele was followed by a somatic loss of the remaining gene, as a result of chromosome loss, mutation, deletion or mitotic crossing over, resulting in tumour formation (Cavenee, 1983). Other examples of tumour suppressor genes include: *p53*; *p16* and *p15*, cyclin dependant kinase inhibitor genes; Von Hippel-Lindau disease gene (*VHL*) and E-cadherin gene, although many more exist (Klein, 1998; Kok et al., 1997).

1.2.3 DNA Repair and Apoptotic Genes

A number of genes exist which act either to repair DNA or are involved in programmed cell death (apoptosis). There are those that directly repair DNA, such as those involved in nucleotide excision repair (NER) and DNA mismatch repair (MMR), (e.g. *hMSH*, *hMLH*), but also those that act as cell checkpoint genes, thus allowing DNA to be repaired prior to progression through the cell cycle (e.g. *hBUB1*, *p53*), (Janin, 2000).

Genes which influence apoptosis include *p53*, *bcl-2* and *v-abl*. The protein product of *bcl-2* acts by interacting with the protein products of *BAX*, *BAD* and several other genes, which act in concert to either accelerate or inhibit apoptosis (White, 1996).

The onset of cancer is generally initiated by mutations in normal (wild-type) cellular genes. These mutations develop as a result of uncorrected errors in DNA replication e.g. after exposure to physical or chemical carcinogens. The gene function can either be activated or inactivated by these mutations. These mutations may arise as a result of several mechanisms, the process being termed "genetic instability" (Lengauer et al., 1998)

1.3 GENETIC INSTABILITY

It is generally accepted that cancers arise as a result of mutations in genes that control cell genesis and cell apoptosis (Lengauer et al., 1998). Mutations in these genes may arise as a result of alterations in chromosome number, chromosome translocations, subtle sequence changes or gene amplifications.

1.3.1 Alterations in Chromosome Number

Alteration in chromosome number involves losses and gains of whole chromosomes (aneuploidy). For example, the complete loss of chromosome 3 in uveal melanomas is associated with liver metastasis (Prescher et al, 1990; Sisley et al., 1990). Cytogenetic studies have shown that the majority of cancers have lost or gained chromosomes, so called "chromosomal instability" (CIN), (Reichman et al., (1981). However, these cytogenetic studies may actually underestimate the true extent of such changes, and the loss of heterozygosity (LOH), where either the paternal or maternal allele is lost, is commonplace in the majority of tumours (Kinzler and Vogelstein, 2002). Even in instances where the chromosomes themselves appear to be unaffected, the true genetic status may vary as the loss of one allele is often accompanied by the duplication of the remaining allele, thus leaving a cell with a normal karyotype but an abnormal allelotype (Vogelstein et al., 1989).

Genes that when altered that could lead to CIN include those involved in: chromosome condensation; sister chromatid adhesion; kinetochore structure and function; centrosome/microtubule function and dynamics; and cell "checkpoint" genes which monitor progression of the cell through the cell cycle (Lengauer et al., 1998). One type of checkpoint, the "DNA-damage checkpoint", prevents cells with DNA damage from entering mitosis. DNA damage could have arisen as a result of: base mismatches

by DNA polymerase; exogenous mutagens (such as ultraviolet light); endogenous mutagens (such as oxygen free radicals); or from incomplete repair. Chromosomes containing damaged DNA could segregate abnormally because sister chromatids may still be connected to each other by abnormal DNA-DNA or DNA-protein links (Lengauer et al., 1998). Several genes have been implicated in DNA-damage checkpoint control, including ataxia telangectasia mutated (ATM), the ATM-related gene (*ATR*), *BRCA 1* and *BRCA 2* genes, and *p53* (Lane, 1998). A second checkpoint effectively targets cell division itself, the "spindle checkpoint," ensuring that chromatids do not separate until they have aligned along the mitotic spindle. A disruption at this checkpoint could lead to daughter cells receiving an abnormal complement of chromosomes (Cahill, 1998). Potential spindle checkpoint genes mutated in colorectal cancer include *hBUB1* (Cahill, 1998). Another cause of aneuploidy, involves abnormal numbers of centrosomes and although abnormal numbers of centrosomes have been detected in breast, lung, prostate, colon and brain cancers, specific genes responsible for centrosome formation have not yet been found (Doxsey, 1998).

1.3.2 Chromosome Translocations/Rearrangements

These alterations can be seen on cytogenetic analysis as fusions of different chromosomes or of normally non-contiguous segments of a single chromosome. This can have the result of fusing two genes together, thus leading to the activation of a normally quiescent gene or the inactivation of an active gene. In addition, the loss of large proportions of chromosomal material can also occur during the complicated rearrangements that take place during translocation. These deletions can often be seen as loss of heterozygosity at the molecular level.

Two patterns of chromosome translocation are seen in human cancers, the complex type and the simple type. The complex type involves many random translocations not only between tumours, but also between individual cells and can also result in the gain and loss of chromosome material. The simple type is characterised by a distinctive rearrangement of chromosome segments, and is found in certain lymphomas and rare sarcomas (Le Beau and Rowley, 1986), and also in chronic myeloid leukaemia with the Philadelphia chromosome, where the *c-abl* gene on chromosome 9 is joined to the *BCR* gene on chromosome 22 (Rowle, 1973).

1.3.3 Subtle Sequence Changes

These involve either base substitutions, or the insertion or deletion of nucleotides resulting in either translational errors or the premature cessation of protein synthesis. For example, missense mutations in the K-*ras* gene occur in over 50% of colorectal cancers (Fearon et al., 1993). There are three separate mechanisms involved in the repair mismatched bases, one involves the detection and replacement of mismatched bases by DNA polymerase and the others are nucleotide excision repair and DNA mismatch repair. As yet no consistent pattern of defects of DNA polymerase has been seen in tumours (Lengauer et al., 1998).

Nucleotide excision repair (NER) is primarily involved in repairing damage caused by exogenous mutagens. Defects in NER are notably seen in patients with xeroderma pigmentosum, who develop skin tumours in response to exposure to ultraviolet light (Cleaver, 1968).

The mechanism of DNA mismatch repair involves the correction of mismatched and/or unmatched bases, which arise after DNA replication, or occasionally secondary to mutagens (Jircny, 1998). Defects in DNA mismatch repair enzymes usually results in the non-correction of mismatched bases, particularly in long base repeating tracts known as microsatellites, thus the genetic instability produced is also termed "microsatellite instability" (MSI), (Jircny, 1998).

1.3.4 Gene Amplifications

These are seen as homogenously stained regions or double minutes on cytogenetic analyses, and usually represent multiple copies of growth promoting genes (Lengauer et al., 1998). Gene amplifications tend to occur in late stage cancers and tend to affect a few specific genes, notably, genes that may be important in the metabolism or inactivation of certain chemotherapeutic agents. The mechanism of gene amplification is largely unknown, although inactivation of *p53* may play an important role (Yin et al., 1992). Examples of specific gene amplifications include, the amplification of N-myc that is seen in approximately 30% of advanced neuroblastomas (Seeger et al., 1985) and that of *HER2/neu* gene in breast cancers, which is also thought to have prognostic significance (Pegram et al., 1998).

Mutations in genes do not necessarily imply a tumourigenic state, as these mutations may initiate apoptosis or a selective growth advantage may not be induced. For mutations to produce a tumourigenic state, the mutation must either initiate growth and clonal expansion at a rate greater than its surrounding normal cells, or apoptosis is not induced until the cell has replicated several generations more than its normal counterparts.

1.4 OVERVIEW OF CANCER TUMOURIGENESIS

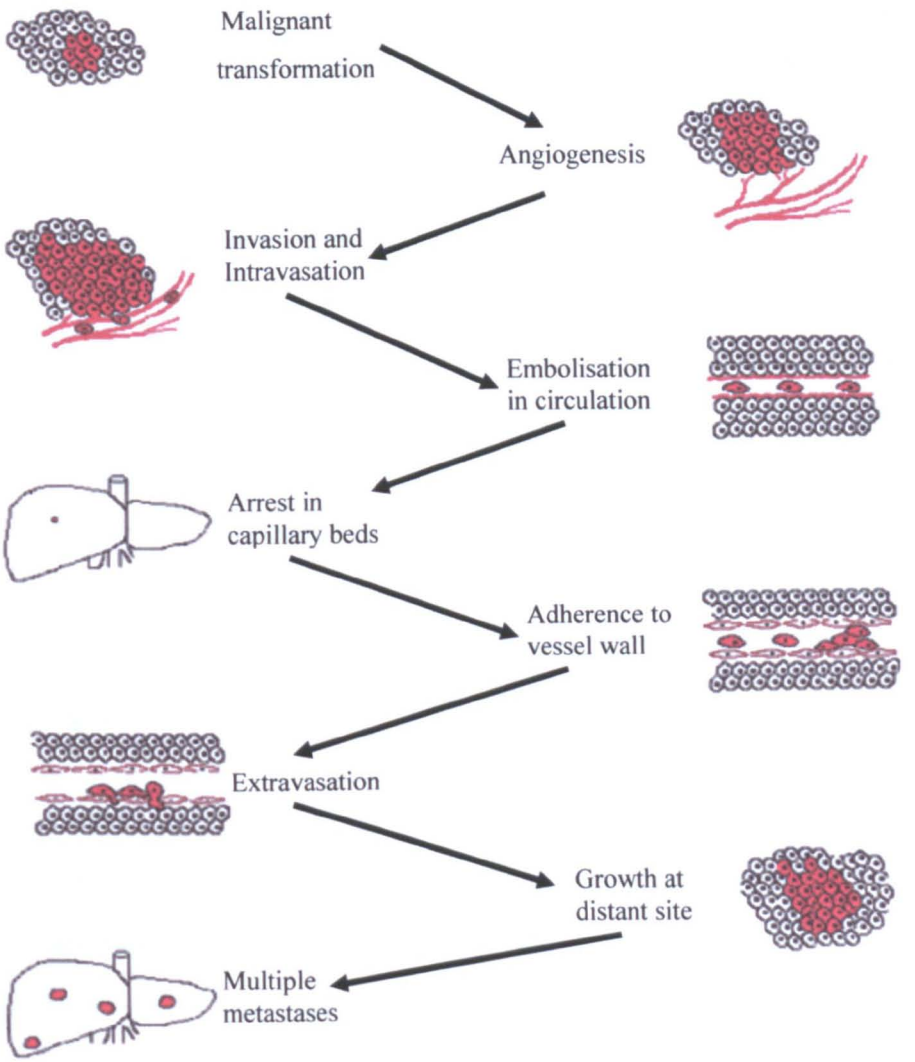
In summary, several key events may need to occur in order for a neoplasm to arise: genetic instability; activation of oncogenes; inactivation of tumour suppressor genes; defects in DNA repair mechanisms; functional loss of cell cycle checkpoint control; mutational or regulatory changes in genes that protect cells against apoptosis. However, as with any biological system, there are exceptions; leukaemias and lymphomas may require a smaller number of changes than solid tumours, whereas retinoblastoma appears to only require two mutational losses (Lengauer et al., 1998).

Unfortunately, for many patients the primary tumour is not the prime cause of death, the development of metastases poses the major cause of morbidity and mortality in patients with cancer, with symptoms including cachexia, anorexia, apathy and pain as a result of bone or liver metastases.

1.5 METASTASIS

Metastasis describes the spread and growth of a tumour from its original (primary) site to a distant (secondary) site via the blood stream (haematogenous metastasis), the lymph vessels (lymphatic metastasis) or transcoelomic (through the pleural or peritoneal spaces). There are several steps in the metastatic process: invasion into adjacent structures; intravasation into blood or lymphatic vessels; arrest of the liberated cancer cells at a receptive site; extravasation out of the vessel and into the surrounding tissues and for growth to occur angiogenesis must take place (neovascularisation) to supply the growing tumour with nutrients (figure 1.1).

Figure 1.1 Diagrammatic representation of the metastatic cascade (adapted from Fidler, (1990)).



At each of these steps the cancer cell must have the ability to complete each step of the metastatic cascade. The cancer cell does not necessarily have to be very efficient in completing each step but must be able to do so eventually, in order to proceed to the next step. Therefore, inefficiency can be expressed as the death of large numbers of cancer cells entering each step of the metastatic cascade, or inefficiency may manifest as the length of time required completing one or another of the metastatic process, and so may explain why some tumours metastasise more readily than other tumours (Weiss, 1990).

1.5.1 Invasion

There appears to be two processes involved in the penetration of tissues by cancer. Firstly, the proliferation of cancer cells causes them to expand along the pathways of least resistance and secondly, the active locomotion of cancer cells through the tissues, which involves attachment of the cancer cell to the basement membrane, degradation of the connective tissue, and finally migration (Liotta et al., 1991).

The degradation of the basement membrane involves the release of enzymes e.g. the metalloproteinases, a family of metal-dependant endopeptidases (Stetler-Stevenson, 1990) which include enzymes with degradative activity for interstitial collagen, type IV collagen, type V collagen, gelatin and proteoglycans, as well as the release of certain enzyme inhibitors, such as tissue inhibitor of metalloproteinases (TIMPs) which can inhibit interstitial collagenase, stromelysin and type IV collagenase (Khokha et al., 1989). This is because maintenance of the normal basement membrane is a continuous process of degradation and synthesis. Therefore, a reduction in the synthesis of connective tissue by normal cells could also result in cancer cell invasion (Adams et al., 1982). Studies by Segain et al. (1996), suggest that the expression of gelatinase by fibroblasts is induced to a greater extent by cell lines derived from the primary tumour as compared to metastases, suggesting that the primary tumour not only induces degradation of the surrounding connective tissue to aid metastasis but the expression of gelatinase by the surrounding fibroblasts is then switched off once the tumour cells have reached their metastatic site and thus enabling adhesion; or, a specific subclone of cells induce gelatinase in the primary tumour which enable the release of a metastatic subclone of cells. The expansion of the tumour itself could also cause pressure atrophy

of the surrounding tissue, thus allowing the spread of cancer cells (Gabbert et al., 1987). Neutrophils are known to play an important role the inflammatory response, releasing proteolytic enzymes and generating oxygen free radicals, thus providing another mechanism in which to facilitate the spread of cancer cells (Glaves, 1983).

In the case of colorectal cancers, metastatic efficiency appears to be greater in cells cultured from the deep part (serosal surface) of the tumour as compared to cells cultured from the superficial part (mucosal surface) of the tumour (Jass et al., 1989; Danova et al., 1995; Inomata et al., 1998). This could be explained by differing phenotypes (subclones) between the superficial and deep parts of the tumour (Kim et al., 1991) or, the fact that intravasation and spread is facilitated by a rich lymphatic and vascular system, which is, absent superficial to the muscularis mucosa (Fenoglio et al., 1973). Talbot et al. (1980), showed that invasion of the thick walled extramural veins located on the serosal surface of the bowel, was associated with a poorer prognosis and the frequent development of liver metastases.

1.5.2 Intravasation

This usually involves the active migration of cancer cells into the lumen of lymphatic and blood vessels, in a similar manner to leukocytes (de Bruyn and Cho, 1982). Migration may be facilitated by certain motility aiding factors, such as MRP1, which appears to be highly expressed in primary tumours as compared to its metastases (Cajot et al., 1997). Another method could be tumour erosion into lumen of these vessels as a consequence of pressure necrosis (Ouichi et al., 1996).

1.5.3 Arrest of Cancer Cells

During haematogenous metastasis, cancer cells become arrested in the microvasculature of many organs. The majority of cancer cells will arrest in the first microvasculature system they encounter, which in the case of colorectal cancers, this could be the liver, via the portal system. Studies have shown that the site for major cancer cell death is in these microvasculature systems (Weiss, 1990). Cell death within the microvasculature system is biphasic, the majority of cells being destroyed within 5 minutes of entering the system and then the remaining survivors over a variable length

of time. The rapid phase of post intravasation cancer cell death may occur as a result of mechanical stress placed on the cell as they pass through the microcirculation (Weiss and Dimitrov, 1986). The slow phase of post intravasation cancer cell death involves destruction by the host defence system (Weiss et al., 1989). However, Chambers et al. (2000), using *in vivo* video-microscopy studied the outcome of fluorescently labelled cancer cells in the microcirculation of a variety of murine tissues and found that rather than being destroyed, the vast majority of cancer cells survived arrest and deformation, and then went on to extravasate into the tissue.

Arrest of cancer cells at certain sites is probably enhanced by the expression of cell surface receptors, which enhance the arrest of the cancer cell and allow the cells to survive the host defences once they have arrested. For example, cell adhesion molecules such as ganglioside GM2, oligosaccharides (sialyl le structures, (Ono et al., 1996; Sato et al., 1997; Yamada et al., 1997)), β1 integrins, bcl-2 (Skopelitou et al., 1996), EGF-R (Parker et al, 1998), CEA (carcinoma embryonic antigen), (Kim et al., 1997) and CD44 (Guo et al, 1994) may enhance colorectal cancer cell arrest and survival within the liver (Singh et al., 1997).

1.5.4 Extravasation

There appears to be two different extravasation mechanisms, the first involves the active migration of cancer cells in a similar way to leukocytes and the other, follows intravascular growth, when after the tumour reaches a certain size it bursts through and out of the vessel (Crissman et al., 1985). Movement of tumour cells into the liver could be further aided by the fact that the liver sinusoids are heavily fenestrated and so allowing an easier passage of metastatic colon cancer cells (Fukumura et al., 1997). The intravascular growth of the tumour itself may induce an inflammatory reaction, which may weaken the vessels adventitial tissues and thus aid extravasation (Weiss et al., 1989). As stated previously, Chambers et al. (2000) propose that the rate-limiting step is not arrest and extravasation but post-extravasation events, suggesting that nearly all cancer cells, be they highly metastatic or not, can arrest, deform and extravasate into the surrounding tissue, but only cells with the genotype and phenotype to divide and continue to grow, can survive in these tissue beds.

1.5.5 Neovascularization

The growth of tumour emboli beyond 0.1-0.2cm^3 requires the growth of blood vessels into the tumour, as the nutritional requirements of the cells will not be satisfied by the diffusion of nutrients alone (Carlsson et al., 1979). Work by Folkman (1989) has established that angiogenesis is necessary for both primary and metastatic tumour growth, indicating the higher the vascularity of the primary tumour, greater is the chance of distal metastases. In both uveal melanoma and colorectal cancer, increased vessel density has been associated with the development of metastases (Folberg et al., 1993; Rummelt et al., 1995; Takahashi et al., 1995). The secretion of angiogenic factors, such as VEGF appears to aid angiogenesis (Brown et al., 1993; Tokunaga et al., 1998), but the process of angiogenesis is also controlled by anti-angiogenic factors, such as angiostatin and the modulation of these various factors may provide a suitable point of action for anti-metastatic therapy (Warren et al., 1995; Cherrington et al., 2000).

1.6 OVERVIEW OF METASTASIS

It has long been observed that certain tumours show an organ-specific pattern of metastasis. Breast cancers preferentially metastasise to the bone, liver, brain and lung and the incidence of liver metastases from primary cutaneous melanoma and ocular melanoma is greater than metastases to any other single organ, even though these tumours do not directly drain into the liver (Pickren et al., 1982). In 1889, Stephen Paget proposed the "seed and soil" hypothesis of metastasis, stating, "when a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil." This is most probably due to specific cancer cell ("seed")-target organ ("soil") interactions allowing and facilitating invasion, intravasation, arrest, extravasation, neovascularisation and growth at a distant site (Fidler and Hart, 1982; Fidler, 1990, 1995; Radinsky, 1995; Radinsky and Ellis, 1996). Ewing 40 years later challenged Paget's "seed and soil" hypothesis, claiming that metastasis occurred solely as a result of the arrest of the majority cancer cells in the first microvasculature system they come across, which in the case of colorectal cancer would be the microcirculation of the liver. However, studies by Sugarbaker (1981), concluded that common regional metastases could be attributed to anatomic or mechanical considerations, such as

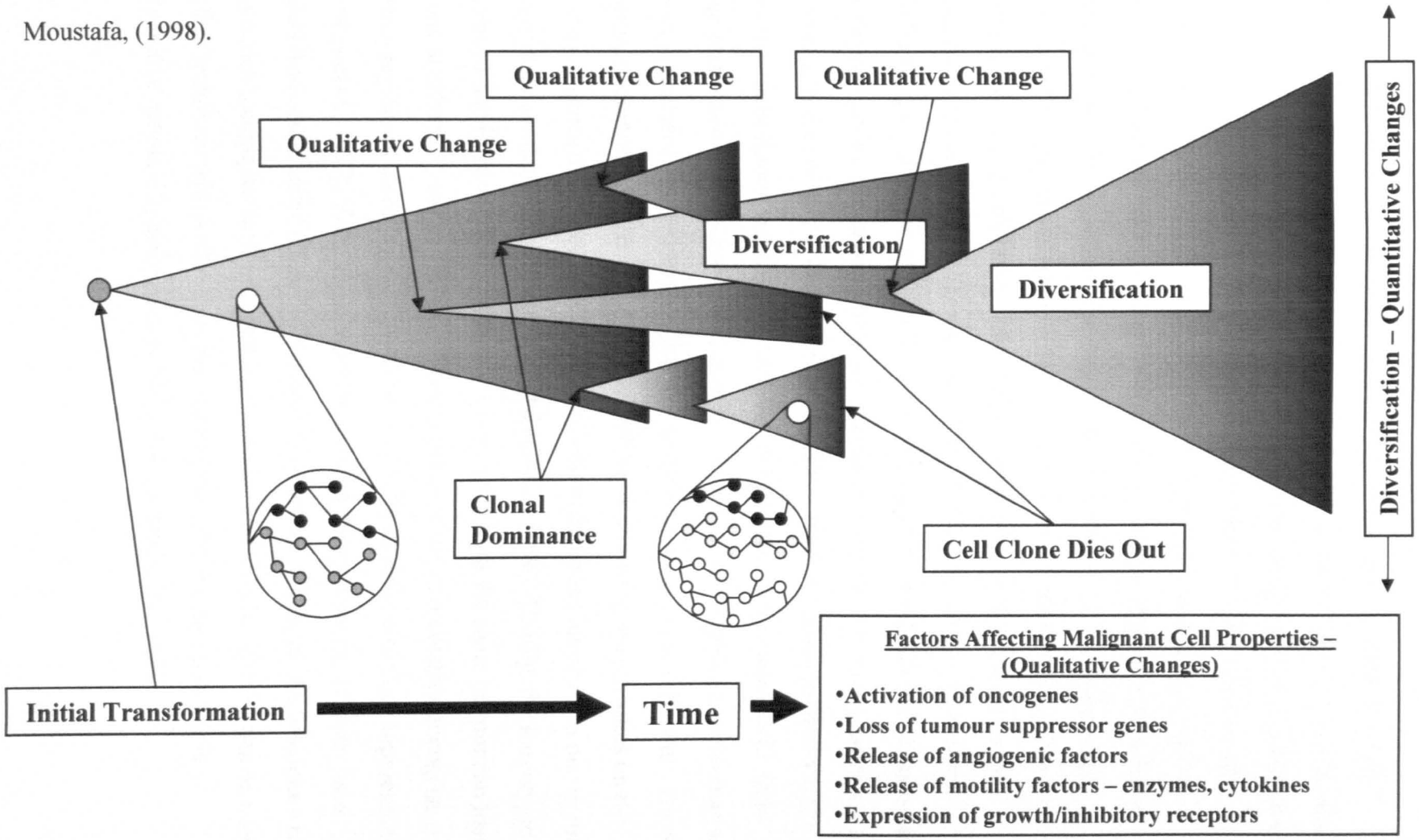
efferent venous circulation or lymphatic drainage to regional lymph nodes, but that distal organ colonisation by metastatic tumour cells from numerous types of cancers established their own patterns of site specificity. It is interesting to note that cells from hepatic metastases show different karyotypes and phenotypes when compared to lymph node metastases even from the same patient, suggesting that site-specific interactions relevant to the selection of various clones from the same cancer (Fidler and Kripke, 1977; Gregoire et al., 1993; Singh et al., 1997; Nicolson and Moustafa, 1998).

It is known that intra-tumoural heterogeneity exists with different subclones of cells, each with a slightly different phenotype and possibly genotype (Fidler and Hart, 1982; Kim et al., 1991; Danova et al., 1995; Katsura et al, 1996). Nowell proposed a genetic theory of tumour evolution in 1976, where there is sequential selection of variant subclones being undertaken by either genetic changes within the metastatic cascade or by local host defence systems, mechanical and/or biological (figure 1.2). Tollenaar et al. (1997) provided evidence that clonal divergence seemed to take place during the transition from adenoma to carcinoma, where several different phenotypes were seen, but only one would be required to produce metastases. The outcome of metastasis is also dependant on the cell interacting with the local microenvironment in order to avoid the host immune response, vascularise and hence grow (Radinsky, 1995; Fidler 1995; Singh et al., 1997). The newly formed metastasis may then progress to metastasise itself (Fidler, 1990).

Variations in metastatic potential is not necessarily dependant on increased aneuploidy (Frankfurt et al., 1984; Jass et al., 1989; Lind et al., 1992), but requires only that genes which aid metastasis are switched on and that those genes which suppress metastasis (at any stage in the metastatic cascade) are switched off (Schirmacher, 1985; Klein and Klein, 1985).

It is unlikely that there is a single metastatic gene, but the interactions of multiple metastasis-related genes occur. Studies have shown that when cells derived from metastases were injected into nude mice, they were more likely to produce metastases than those cells derived from the primary tumour (Morikawa et al., 1988; Adachi et al, 1999).

Figure 1.2 Diagrammatic representation of tumour progression: Waves of clonal divergence adapted from Nicolson and Moustafa, (1998).



1.6.1 Metastatic Genes

Cancer metastasis is a highly complex process and as such is unlikely to be controlled by a single gene. It is more plausible that a number of genes may be either activated or inactivated (Sobel, 1990). These genetic changes may occur permanently or transiently within the genome (Radinsky and Ellis, 1996). Several different approaches have been taken in the search for genes involved in metastasis. One approach uses techniques to identify genes that are differentially expressed in cells with different metastatic properties. Differential ("plus-minus") screening of cDNA libraries constructed from metastatic and non-metastatic cells of the same tumour type, relying on the premise that the metastatic phenotype is associated with a change in the transcription of various genes (Dong et al., 1995; Cajot et al., 1997). Subtractive hybridisation, involves the physical removal of sequences of mRNA which are common to both populations of metastatic and non-metastatic cells, thus isolating mRNA which is different in the two populations (Lee et al., 1996). A third technique involves the use of reverse transcription and the polymerase chain reaction to compare cDNA from paired metastatic and non-metastatic cells in a search for either genetic loss (putative tumour suppressor gene) or over-expression (putative oncogene) in cells with different metastatic potentials (Fidler and Radinsky, 1996). A second approach relies on the isolating of genes that encode for proteins of known functions involved in one or more steps of the metastatic cascade (Fidler and Radinsky, 1996). Thirdly, the karyotypic analysis of metastasising and non-metastasising cells from the same tumour can identify regions of chromosomal gains, which may predict the site of a putative oncogene, whereas regions of chromosomal loss may predict for a putative tumour suppressor gene (Mertens et al., 1997). Karyotypic analysis involves the preparation of metaphase spreads from short term cultures of the tumour under investigation and provides a basis from which techniques such as fluorescence *in situ* hybridisation (FISH) can be used. Another technique which allows for the examination of the entire genome is comparative genomic hybridisation (CGH), (Kallioniemi et al., 1992).

1.7 COLORECTAL CANCER

Colorectal cancer is the third leading cause of cancer deaths worldwide every year, after lung cancer and stomach cancer (Cancer Research Campaign, 1999). In the United Kingdom colorectal cancer was the second commonest cause of death due to cancer accounting for nearly 16,000 deaths in both men and women in 1994, with a reported incidence of approximately 29,000 (Office for National Statistics, 2000).

1.7.1 Aetiology

It is now generally accepted that most colorectal cancers arise from the progression from an adenoma to a carcinoma (Muto et al., 1975). There are numerous aetiological factors, environmental and genetic, all interacting to initiate and promote the formation of an adenoma into a cancer (Wilmink, 1997).

1.7.1.1 Environmental Factors

A number of investigators have proposed that a diet high in fat (especially saturated fats), protein, calories, red meat and alcohol, and low in fibre, dietary fruit and vegetables, calcium, folate and other micro-nutrients are associated with an increased incidence of colorectal cancer (Haenszel and Kurihara, 1968; McKeown-Eyssen and Bright-See, 1985; Armstrong and Doll, 1975; Jacobs 1988; Vargas and Alberts, 1993). Several studies have shown a positive association between alcohol intake and colorectal cancer, possibly by stimulating mucosal cell proliferation and by activating pro-carcinogens (Boutron and Faivre, 1993; Kune and Vitetta, 1992). Interestingly, aspirin is thought to have a protective effect against the development of colorectal cancer (Thun et al., 1991; Smalley et al., 1999) however, not all epidemiological studies have substantiated this claim (Gann et al., 1993).

Smoking has also been linked with the genesis and development of adenomas, with data directly linking the duration and the amount of smoking to the size of adenomas (Giovannucci et al., 1994a, 1994b; Giovannucci and Martinez, 1996).

The presence of inflammatory bowel disease is also thought to play a role in the development of colorectal cancer with patients with ulcerative colitis having an increased risk, especially if they were young and have extensive disease at diagnosis

(Ekbom et al., 1990a). The risk in Crohns disease although initially less certain (Glotzer, 1985), also appears to be associated with an increased risk of colorectal cancer, particularly if there is extensive colonic involvement (Ekbom et al., 1990b).

1.7.1.2 Genetic Factors

Genetic factors were thought to play an important role after several cancer families were found. Studies divided these familial colorectal cancers into two groups, those characterised by the presence of multiple colorectal polyps (polyposis) and those without polyposis, hereditary non-polyposis colorectal cancer (HNPCC).

There are several types of polyposis syndromes, which include; familial adenomatous polyposis coli (FAP), Peutz-Jeghers syndrome, Cronkkite-Canada syndrome and hyperplastic polyposis. FAP is an autosomal dominant inherited disorder, in which individuals are affected with hundred to thousands of adenomatous polyps, although only one to two ever become malignant. The average age of diagnosis of polyposis is 27 years of age, with the median age of cancer in these patients at 39 years. The incidence of FAP in the United States is 1 in 5000 to 10,000 although only 1% of all colon cancers occur in these patients because of the practise of prophylactic colectomy. A variant of FAP is Gardner syndrome, a rare autosomal dominant disease, where as well as the colonic manifestations there are also soft tissue tumours, osteomas, dental abnormalities and congenital hypertrophy of the retinal pigment epithelium (Gardner, 1951; Gardner and Plenk 1952; Gardner 1962). Another variant is Turcot's syndrome, a rare autosomal recessive disease, which is characterised by the presence of CNS tumours as well as a predisposition to colorectal cancer.

HNPCC also displays an autosomal dominant inheritance pattern and is characterised by the early onset of colorectal cancers, which are predominantly right-sided and with multiple primary sites (synchronous and metachronous). There is also a predisposition to developing carcinoma of the ovary, endometrium, stomach, small bowel, ureters and renal pelvis (Lynch and Smyrk, 1996). The syndrome is diagnosed according to the Amsterdam criteria, where at least three relatives have had colorectal cancer, affecting two or more generations, one a first degree relative of the other two and diagnosed before the age of 50 years, all in the absence of FAP (Vasen et al., 1991). HNPCC accounts for around 2-3% of all colorectal malignancies and of which there are two subtypes described; the Lynch I and Lynch II syndromes. Lynch syndrome I, or site-

specific colorectal cancer is characterised by an early age of onset, on average 45 years and a predilection to proximal colon cancers (70%). In Lynch syndrome II or cancer family syndrome, frequent carcinomas in other organs occur in addition to this (Lynch et al., 1985, 1993).

The majority of colorectal cancer cases are sporadic with only 3-5% occurring in those with well-characterised inherited diseases. However, studies have shown that relatives of those with colorectal cancer have a higher than average lifetime risk of colorectal cancer. Therefore, a person may have a genetic predisposition to colorectal cancer, but environmental factors may initiate and/or promote tumourigenesis. Primary prevention therefore involves the identification of genetic and environmental factors that are involved in the aetiology of colorectal cancers.

1.7.2 Clinical Course

Approximately 60-70%% of all colorectal cancers are located within the rectum, recto-sigmoid or sigmoid colon, 5% within the descending colon, 12% within the transverse colon and 22% within the ascending colon (Mortensen et al., 1992). Clinical symptoms depend on the site of the tumour. Left sided tumours tend to present with prominent features of disturbance of bowel function such as malaena, diarrhoea and constipation, whereas, right-sided tumours tend to present with non-specific symptoms of weakness, weight loss and unexplained anaemia (Forrest et al., 1991). All colorectal tumours spread by direct extension into adjacent structures and also by metastasis via the lymphatic and blood vessels. Sites of metastatic spread include the regional lymph nodes, liver, lung, bones and occasionally the brain (Dayal and DeLellis, 1989).

Diagnosis relies on a careful history and examination, proctoscopy and sigmoidoscopy, faecal occult blood tests followed by either a colonoscopy or a double contrast barium enema. If the cancer is particularly large and especially if it is a rectal cancer, other imaging techniques may be used such as magnetic resonance imaging (MRI), computed tomography (CT) and/or transrectal ultrasonography, in order to assess the size of the tumour and to see if it is invading adjacent structures (Mortensen et al., 1992).

Useful blood tests include the measurement of CEA, which is raised in 19-40% of patients with early tumours and almost 100% of patients with large metastatic tumours

and has also proven to be a useful indicator of recurrence. However, raised levels of CEA may also be produced by cancers of the lung, breast, ovary, urinary bladder and prostate, and also in a variety of non-neoplastic disorders, such as alcoholic cirrhosis, pancreatitis and ulcerative colitis (Haier et al., 2000).

1.7.3 Treatment

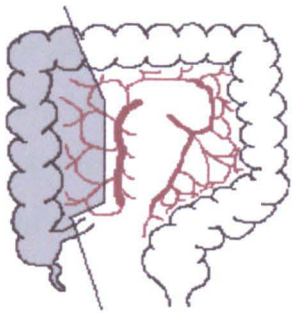
The treatment options considered for a patient with colorectal cancer is dependant on the patient's clinical state and any co-morbid disease, the site of the tumour and the clinical stage of the disease as outlined in appendix I. Essentially, the treatment offered to a patient, must be in the best interests of the patient with due consideration of any morbidity or mortality as a result of the therapy.

1.7.3.1 Surgery

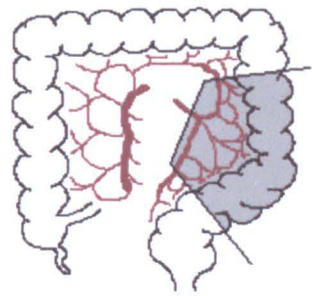
Surgery is the only curative treatment option for colorectal cancer. The cancer and involved bowel is excised together with the regional lymph nodes (which usually lie adjacent to the major blood vessels). The length of bowel resected is dependent on the site of the cancer and the blood supply to that part of the bowel (figure 1.3). If there are multiple tumours within the colon, a total colectomy may be performed, with the small bowel being anastomosed to the rectum. If tumours are also present in the rectum, a pan-proctocolectomy is performed to excise the entire large bowel. This may be required for patients with FAP. For small polyps, a colonoscopic polypectomy may be all that is required, as long as the stalk of the polyp is clear of malignant invasion (Bond, 1993; Winawer et al., 1993; Byers et al., 1997).

Local recurrence rates for colorectal cancer are dependant on a number of factors including tumour involvement of either the circumferential or resection margins and on surgical technique, this is more so in the case of rectal cancer, where recurrence rates vary from 4-40%, depending on the operating surgeon. For rectal cancers, the technique of total mesorectal excision (where there is meticulous dissection outside the mesorectal fascia, with conservation of the nerve supply to the bladder and genital organs), offers the lowest local recurrence rates (Heald et al., 1998).

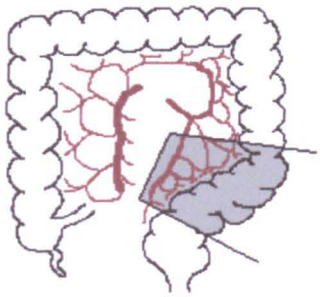
Figure 1.3 The various surgical resections that can be performed for colorectal cancer (adapted from Mortensen et al., 1992).



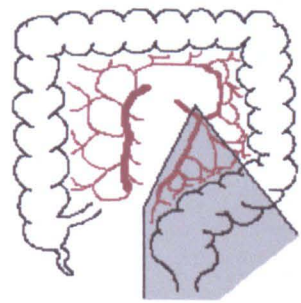
Right hemicolectomy



Left hemicolectomy



Sigmoid colectomy



Abdomino-perineal resection

1.7.3.2 Chemotherapy

Chemotherapy may be given either pre-operatively (known as neo-adjuvant chemotherapy) or post-operatively (adjuvant chemotherapy). Currently, adjuvant chemotherapy is only offered to patients with stage III (Dukes C) colorectal cancers, comprising of a combination of 5-fluorouracil (5-FU) with either levamisole or leucovorin (National Institutes of Health, 1990; Wolmark et al., 1993). Adjuvant chemotherapy has also been given via the hepatic portal vein in an attempt to reduce the occurrence of liver metastases, however a randomised trial by the Swiss Group for Clinical Cancer Research revealed no survival differences between systemic 5-FU versus hepatic portal vein infusion (Laffer et al., 1998). The use of adjuvant chemotherapy in patients with stage II (Dukes B) cancers remains controversial, but a recent large meta-analysis study claimed a survival advantage of 2% when surgery alone

was compared with surgery plus 5-FU/leucovorin (International Multicentre Pooled Analysis of B2 Colon Cancer Trials (IMPACTB2), 1999).

In stage IV (Dukes D) cancers, distant metastases are already present and chemotherapy has been administered either systemically or via a hepatic artery infusion in an attempt to be curative, however, there have been no consistent improvement in survival. Chemotherapy with 5-FU has been used palliatively, given either as a continuous infusion or in bolus doses (Moertel, 1994). Newer agents such as Irinotecan (CPT-11), a topoisomerase-I inhibitor and Tomudex, a specific thymidylate synthase inhibitor have recently been licensed for use in patients with metastatic disease, who have either not previously received chemotherapy or are refactory to 5-FU therapy (Rothenberg et al., 1996; Conti et al., 1996; Cunningham, 1998; Von Hoff, 1998).

Chemotherapy is not without side-effects, such as nausea, vomiting, diarrhoea, mucositis, neutropaenia, hair loss and malaise but, chemotherapy has demonstrated improved survival and quality of life when compared to supportive care alone (Scheithauer et al., 1993).

1.7.3.3 Radiotherapy

Short-term radiotherapy (over five days) has yielded excellent results in terms of reducing local recurrent rates after surgery for small rectal cancers, while long-term radiotherapy has primarily been used to down size large rectal tumours (Marijnen and van de Velde, 2001). However, its main role has been in palliating recurrent tumours (Forrest et al., 1991). Some studies have shown that either neo-adjuvant or adjuvant radiotherapy reduces the incidence of local recurrence by up to 50%, though some researchers claim that radiotherapy may be compensating for imperfect surgery (Willett et al., 1993; Schild et al., 1997). Radiotherapy is not without its side-effects, which include erythema to the skin, small bowel radiation enteritis, small bowel obstruction and perforation. The growing trend is for radiotherapy to be used for large tumours only.

1.7.3.4 Biologic Therapy

Newer treatment modalities are currently being developed such as immunotherapy and radio-immunotherapy. The use of monoclonal mouse antibodies to colorectal cancer (such as MOAB 17-1A, which is an antibody directed to an epithelial cell surface glycoprotein present on normal and malignant cells), has been used successfully in the

laboratory setting and clinical trials are currently on-going (Colacchio, 1997; Riethmuller et al., 1998; Pazdur et al., 1999). Radio-immunotherapy with radioactive iodine bound to anti-CEA monoclonal antibody and autologous tumour vaccines may also provide clinicians with other options in their armaments (Benson, 1996).

1.7.4 Histopathology

A single layer of epithelial cells lines the colon and rectal surfaces. These arise from 4-6 stem cells, which are situated at the base of crypts. These stem cells give rise to a population of absorptive cells, mucus secreting goblet cells or neuroepithelial cells. These cells then migrate from the base of the crypts towards the apex, differentiating en route, where at the apex the cells slough off with the passage of faeces. This journey takes 3-6 days (Lipkin et al., 1963).

Normally, the rate of genesis of these cells equals that of cell loss at the luminal surface. If however, the rate of genesis exceeds that of loss, then a tumour arises. This early tumour usually appears as a small elevated button or as a small polypoid mass. These epithelial tumours tend to produce polyps. These polyps can be divided into non-neoplastic or neoplastic depending on whether they have malignant potential, however there is some overlap between the two.

Generally, non-neoplastic polyps include: hyperplastic polyps, which histologically are composed of well formed glands and crypts lined by well differentiated epithelial cells, and as stated have no malignant potential, but a small proportion of these with adenomatous foci may undergo neoplastic transformation; hamartomatous polyps, which represent developmental malformations resulting in lesions, which consist of essentially normal mucosal components but which are arranged abnormally, malignant degeneration is extremely rare but can occur; inflammatory polyps, which occur in patients with long standing inflammatory bowel disease, predominantly in ulcerative colitis and rarely Crohns disease and lymphoid polyps, which occur as a result of mucosal protrusions secondary to reactive hyperplasia of the mucosal and submucosal lymphoid tissues (Dayal and DeLellis, 1989).

Neoplastic polyps include a group of adenomas, which can be divided histologically into tubular, tubulo-villous and villous adenomas. They are differentiated on the basis of

the majority tissue present, whether it is tubular or composed of finger like papillae (villous type).

There is a definite increase in the risk of cancer from tubular to tubulo-villous to villous type adenomas (Day and Morson, 1978). Increasing malignant potential also relates to the size of the polyp, and increases from 1% if it is less than 1cm in diameter to 10% if it is between 1-2cm in diameter, and finally up to 45% if it is greater than 2cm in diameter (Muto et al., 1975; Day and Morson, 1978). Carcinoma *in situ*, i.e. without invasion of the underlying fibrovascular core or submucosa, of the colon is present in about 10% of villous adenomas, with frank invasive carcinoma in an additional 25-40% (Coutsoftides et al., 1979). As the tumour increases in size it eventually extends to encircle the lumen, which can take up to one to two years. The deeper layers are invaded slowly, and so for a long time the tumour tends to remain superficial.

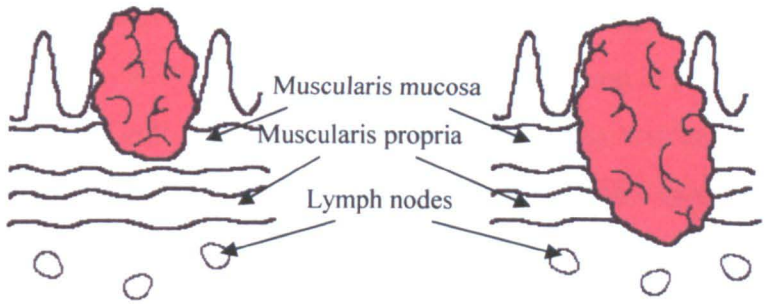
Ninety-five per-cents of all colorectal carcinomas are adenocarcinomas, many of which produce mucin. Commonly this mucin is secreted within the gland lumen or within the interstitium of the gut wall. This secretion of mucin is thought to aid the extension of the cancer by dissecting the gut wall layers and thus leading to a poorer prognosis (Bresalier et al., 1998).

1.7.5 Prognosis

Various factors influence survival after surgery, but the most important factors which determine prognosis are the stage (the extent of spread), tumour-free surgical margins, lack of lymphatic/blood vessel invasion and the grade (the level of differentiation) of the cancer (Hobday and Erlichman, 2001).

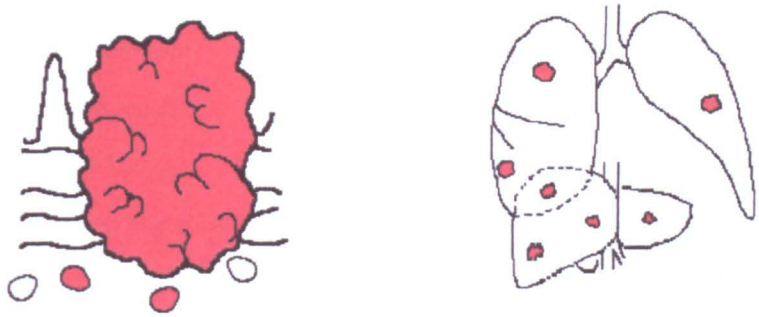
Currently the most reliable indicator of prognosis is the stage of the disease. Staging can only be performed once the cancer has been excised and analysed by a pathologist. The characteristics that form the basis of the staging system are the degree of penetration of the tumour through the bowel wall, the presence or absence of lymph node metastases and the presence or absence of distant metastases, such as liver or lung metastases (figure 1.4). There are three widely used staging classifications, the Dukes, the Astor-Coller modified Dukes staging and the TNM classification (American Joint Committee on Cancer, 1997), (see appendix II).

Figure 1.4 Pathological staging of colorectal cancer (adapted from Dayal and DeLellis, 1989).



Stage 1 – T1 or T2, N0, M0
(Dukes A)

Stage II – T3 or T4, N0, M0
(Dukes B)



Stage III – Any T, N1-3, M0
(Dukes C)

Stage IV – Any T, any N, M1
(Dukes D)

Generally, the 5-year survival rates, when corrected for age is between 70-90% for Dukes' A and B cancers, 30-40% for Dukes' C cancers and less than 20% for those with distant metastases. The development of complications, particularly perforation or obstruction, adversely affects survival (Mortensen et al., 1992). However, if all patients including those with inoperable cancers are taken into account, the five-year survival rate is between 35-49%, which is particularly disheartening in view of the potential for cure, if the disease is diagnosed early (Dayal and DeLellis, 1989).

1.8 GENETICS OF COLORECTAL CANCER

The clonal nature of cancer is that it is derived from a single cell. That cell has a growth advantage over its adjacent cells, as a result of a mutation in its genetic make-up. With successive cycles of cell division, further mutations occur, conferring further growth advantages for individual cells and also conferring abilities that enable them to metastasise and grow at distant sites (Nowell, 1976).

The clonal nature of cancer as proposed by Nowell (1976) was examined in colorectal cancers by Vogelstein et al. (1987). They looked at the X chromosome in these cancers. A female has two X chromosomes, one derived from her mother and the other from her father. In any given cell only one of the X chromosome is active i.e. either the maternal or paternal X chromosome. When looking at normal colonic mucosa, inactivation of either chromosome is distributed equally throughout the population, however, in the case of benign or malignant tumours, there was a monoclonal pattern of X inactivation. This was consistent with other cytogenetic studies performed on many carcinomas (Martin et al., 1979; Reichmann et al., 1981) and adenomas (Mitelman et al., 1974; Reichmann et al., 1985).

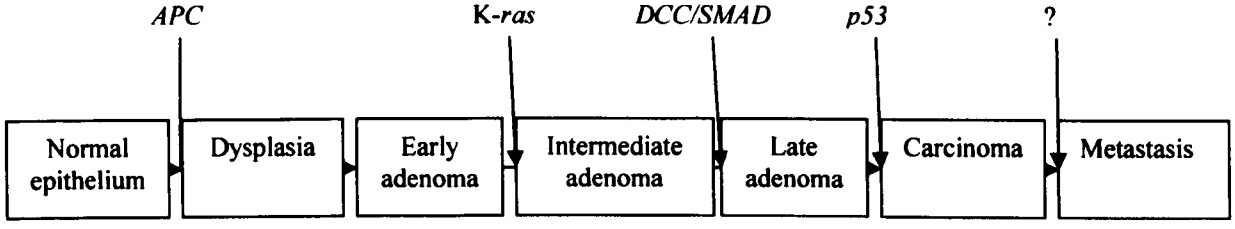
It is generally accepted that there is a step-wise progression from adenoma to carcinoma, involving the accumulation of a number of genetic mutations. There appears to be two pathways in colorectal cancer tumourigenesis, the first involves gains and losses of chromosome segments (chromosomal instability), accounting for approximately 90% of all colorectal cancers and the other involving mutations of DNA mismatch repair genes (microsatellite instability), (Vogelstein et al., 1988; Aaltonen et al., 1993; Peltomäki et al., 1993; Ionov et al., 1993; Thibodeau 1993). However, there may be a third pathway with alterations in the level of DNA methylation resulting in genomic instability (Jones and Gonzalgo, 1997; Breivik and Gaudernack, 1999).

1.8.1 Chromosomal Instability Pathway of Colorectal Cancer Tumourigenesis

The chromosomal instability pathway as proposed by Vogelstein et al. (1988), involves the sequential accumulation of several mutations: the loss or mutation of the *APC* gene on chromosome 5q; mutation of K-*ras*; the loss of *DCC* on chromosome 18q

and the loss or mutation of *p53* (figure 1.5). It also appears that accumulation, rather than the order of genetic mutations is important (Fearon and Vogelstein, 1990).

Figure 1.5 The chromosomal instability pathway of colorectal cancer tumourigenesis (adapted from Fearon and Vogelstein, 1990).



1.8.1.1 Chromosome 5q: The *APC* gene

Chromosome 5q losses were reported to occur in 20-50% of colorectal cancers, but also of interest is the fact that loss of chromosome 5q sequences occurred not only in large cancers but also in small benign colorectal tumours, suggesting that the inactivation of this tumour suppressor gene occurred as an early event (Vogelstein et al., 1988). Another line of evidence came from the examination of patients with familial adenomatous polyposis coli (FAP), where loss of 5q was visible on cytogenetic analysis (Herrera et al., 1986).

The area of alteration was a small region on chromosome 5q 21 (Bodmer et al., 1987; Leppert et al., 1987). Four genes were mapped to this region *MCC*, *TB2* (*DP1*), *SRP 19* and *APC* (Joslyn et al., 1991). The adenomatous polyposis coli (*APC*) gene is found to be mutated in the germ line of FAP patients and in some sporadic cancers (Nishisho et al., 1991). Most mutations occur in the central region of the *APC* gene, called the mutation cluster region (MCR), and usually result in COOH-terminally truncated proteins. Mutations in the first or last thirds of the *APC* gene are associated with a milder form of polyposis, termed attenuated polyposis (*AAPC*), which is associated with fewer polyps and a later time of onset (Spirio et al., 1993). Conversely, mutations in the central mutation cluster region are associated with a more severe phenotype, with thousands of polyps, an earlier age of onset and extra-colonic manifestations such as osteomas and retinal lesions (Polakis, 1995). The majority of mutations (60%) occur in

the mutation cluster region, which only accounts for 10% of the entire coding region. Almost all the mutations, of which there are 737 mutations recorded so far, are either nonsense mutations (30%) or frame-shift mutations (68%), resulting in a truncation of the APC protein (De Vries et al., 1996; Beroud and Soussi, 1996). Approximately 80% of patients with FAP have been shown to have mutations of the *APC* gene. To determine the cause of FAP in the remaining 20%, Laken et al., (1999) used monoallelic mutation analysis (MAMA) to determine independently the status of each of the two *APC* alleles. They found that there was reduced expression from one of the two alleles suggesting that complete loss of both alleles is not necessarily required and that there may be an additional gene involved in FAP patients, such as *APCL* located on 19p13.3 which is a homolog of *APC* (Nakagawa et al., 1998; van Es et al., 1999).

The importance of the *APC* gene in tumourigenesis comes from evidence collected from chimeric mice which produce a truncated APC protein and who develop on average 30-50 intestinal tumours by the age of 90 days, and are known as Min (multiple intestinal neoplasia) mice (Moser et al., 1990). Homozygous mice for the mutation at the *APC* allele invariably die in utero, while those heterozygous for the mutation develop polyps from the third week onwards. Analysis of these polyps by Oshima et al., (1995), showed that these polyps consisted of a microadenoma surrounded by normal intestinal epithelium. The analysis of the dissected microadenoma revealed loss of the wild-type allele with preservation of the mutant allele. Dietrich et al., (1993) also found that a gene located on chromosome 4, was able to modify the number of polyps formed and was named *Mom-1* (modifier of Min-1). Halberg et al., (2000) also found that the combination of *APC* and *p53* mutations in Min mice leads not only to an increased number of polyps but also to an increased malignant potential of these polyps.

The wild-type *APC* is thought to be primarily involved in apoptosis (Morin et al., 1996). Immunohistochemical analysis indicates that the APC protein is located on the basolateral membrane and levels increase as the cell migrates from the base of the crypt upwards (Miyashiro et al., 1995). The APC protein is also thought to play an integral part in the intra- and inter-cellular signalling pathway and has several functional domains, some of which act as binding and degradation sites for β-catenin. Beta-catenin is also involved in cell-to-cell adhesion, and plays an important role in the activation of E-cadherin (Rubinfeld et al., 1993). With the ability of the APC protein to bind to β-catenin, it may also inhibit the β-catenin/TCF regulated transcription of certain genes

such as c-myc (Morin et al., 1997; He et al., 1998). Another function of the wild-type APC protein is its involvement in chromosome segregation by binding to, and promoting the assembly of microtubules (Munemitso et al., 1994; Smith et al., 1994).

1.8.1.2 The *Ras* Oncogene

The first *ras* genes were identified as the transforming components of the Kirsten and Harvey rat sarcoma virus genomes. Later, other *ras* genes were found. Three very closely related proteins in the Ras family, H-Ras, K-Ras, and N-Ras were found to be the primary regulators of cell growth and are frequently mutationally activated in tumours, although K-Ras appears to be involved in malignancy much more frequently than either H-Ras or N-Ras (Bos et al., 1987; Forrester et al., 1987; Bishop, 1991).

The activated Ras protein hydrolyses GTP to GDP, and so may be involved in the transduction of signals (Roussel, 1998). It is also thought to act on the cytosolic serine/threonine protein kinase, Raf, where the activated Raf kinase phosphorylates another enzyme MAP (mitogen activating protein) kinase, which in turn acts on several target proteins some of which are involved in gene regulation. However, the Raf/MAP kinase pathway is not the only pathway in altering gene expression and there are probably others (Neer, 1995; Pawson, 1995).

Recently, the gene responsible for neurofibromatosis type 1 (*NF1*) was found to have GAP activity (Martin et al., 1990). GAP's are negative regulators of *ras* and so if the healthy allele of *NF1* is lost, *ras* cannot be switched off (Haubruck and McCormick, 1991). In 50% of colon cancers there is no *ras* gene mutation but in a few there was a mutation of *NF1* (Li et al., 1992).

1.8.1.3 Chromosome 18q: The *DCC* and *SMAD* genes

While 17p is the most common region of allelic loss in colorectal cancer, chromosome arm 18q is the site of the second most common region, lost in approximately 70% of cases (Vogelstein et al., 1988). There are several tumour suppressor genes located on 18q. The first to be described was that for the *DCC* (deleted in colon cancers) gene located on chromosome 18q21.3 (Fearon et al., 1990). The protein sequence of DCC is very similar to that of neural cell adhesion molecules that are involved in cell-to-cell adhesion (Cho et al., 1994). Loss of *DCC* may allow increased motility of the cells and thereby enhancing metastasis (Fearon et al., 1990;

Goi et al., 1998). Studies by Jen et al., (1994) and later by Shibata et al., (1996) found that colorectal cancers with either a loss of *DCC* gene or a loss of DCC expression had a worse prognosis in terms of liver metastasis, as compared to cancers which expressed DCC. However, Gotley et al., (1996) found no cases of complete loss of DCC expression in either the primary or metastatic colorectal cancer.

Interestingly, approximately 90% of pancreatic carcinomas also show allelic loss at 18q. Hahn et al., (1996) reported the identification of a putative tumour suppressor gene on chromosome 18q21.1, which was termed *DPC4* (for homozygously deleted in pancreatic carcinoma, locus 4). The gene *DPC4* coded for a protein which was very similar to the Drosophila Mad ('mothers against decapentaplegic') protein and the Caenorhabditis elegans Sma protein, both of which were implicated in signal transduction by members of the IGF-β family, in these organisms (Sekelsky et al., 1995; Hoodless et al., 1996). Derynck et al., (1996) proposed a revised nomenclature incorporating the Sma and Mad roots to form SMAD. DPC4 was designated as SMAD4. Subsequently other SMAD proteins were found to exist, all sharing a certain degree of homology. These were termed SMAD1, SMAD2, SMAD3 and SMAD5 encoded for by genes situated on chromosomes 4q28, 18q21.1 (approximately 3Mb proximal to SMAD4), 15q21-22 and 5q31 respectively (Riggins et al., 1996; Eppert et al., 1996; Nakao et al., 1997). All are involved in the signal transduction of the transforming growth factor-beta (TGF-β) family of cytokines, which also include activins, inhibins, bone morphogenetic proteins and Müllerian-inhibiting substance (Zhu et al., 1998). Members of the TGF-β family exert a wide range of biologic effects including regulation of cell growth, differentiation, matrix production and apoptosis. TGF-β suppresses growth in normal cells, however, the action of TGF-β is further complicated because it can also increase the growth and metastatic potential of colon cancer cells (Huang et al., 1995). They also play an important role in mouse embryogenesis, determining tissue differentiation and body plan (Waldrip et al., 1998). Zhu et al., (1998) reported that disruption of the *SMAD3* gene in mice resulted in the mice developing large colorectal neoplasms, which metastasised to the lymph nodes. Bruno et al., (1998) showed that SMAD5 played a vital role in the signalling pathway by which TGF-β inhibits the proliferation of human haematopoietic progenitor cells.

1.8.1.4 The *MCC* gene

The *MCC* (mutated in colon cancer) gene was identified on 5q 21 several months before the *APC* gene (Kinzler et al., 1991; Lindgren et al., 1992). Although there were several reported incidence of mutation in 6% of sporadic colorectal cancers, there were no mutations seen in 90 FAP kindreds (Nishisho et al., 1991). Therefore, the role of the *MCC* gene in colorectal cancer tumourigenesis appears to be limited.

1.8.1.5 Chromosome 17p13.1: The *p53* gene

Certain DNA viruses can induce tumour formation. In particular, the SV40 DNA virus causes tumour formation through the activity of a protein called large T antigen. In order to understand the mechanism by which large T antigen induces oncogenesis, attempts were made to detect the host cell protein to which large T antigen binds. Immunoprecipitation of large T antigen from cells infected by SV40 revealed a host cell protein of 53 kDa (known as p53) in addition to large T antigen itself (Lane and Crawford, 1979).

The *p53* gene was originally thought to be a proto-oncogene, partly because of its increased expression on tumours and because it was apparently able to transform rat embryo fibroblasts in collaboration with *ras* oncogenes (Eliyahu et al., 1984). However, subsequent studies in colorectal cancers indicated that the wild-type *p53* gene was a tumour suppressor gene and not a proto-oncogene. The studies in colorectal tumours consistently showed losses of 17p (Baker et al., 1990). The common region of deletion was mapped to 17p 13.1 (Isobe et al., 1986).

Drawing on Knudson's "two-hit" hypothesis, it was hypothesised that loss of 17p 13.1 removed one copy of the suppressor gene, while the remaining copy was mutant. The majority of somatic mutations of the *p53* gene were single base substitutions (point mutations) resulting in a replacement of one of the amino acids (missense mutation). There are several sites at which mutations can occur, so called "hot spots" and this normally causes a loss of function (Nigro et al., 1989; Malkin et al., 1990; Hollstein et al., 1991). Normal p53 protein exists as a tetramer and binds to specific DNA sequences and thus regulates the transcription of genes (Kern et al., 1991). One of the genes that p53 protein regulates is the *p21* gene (*WAF1/CIP1*) (El-Deiry et al., 1993), which codes for a protein that inhibits cyclin-dependant kinases, which control entry into the cell cycle (Waldman et al., 1995). This is not the only pathway that controls cell cycle and

there are several others where p53 protein acts to control genes involved in cell proliferation, by either acting to induce apoptosis or arresting the cell in the G1 phase, which may optimise the time available for DNA repair within the cell (Clarke et al., 1993). The interaction between certain DNA tumour virus proteins, such as the E1B protein of adenoviruses and the E6 protein of papilloma viruses can also result in the inactivation of many of the functions of the p53 protein, hence mimicking the effects of a mutation in the gene (Oliner et al., 1992).

Germline mutations in the *p53* gene are responsible for the Li-Fraumeni cancer syndrome, characterised by an increased risk of early breast cancer, childhood sarcomas and other neoplasms. Carriers of a mutated *p53* gene also have an increased risk of developing early cancers, with the likelihood of a cancer developing, increasing to nearly 90% by the time they are 65 years old (Malkin et al., 1990). Interestingly, *p53* is not required for the normal development of the embryo in mice, but these mice are more susceptible to tumour formation (Donehower et al., 1992). Also worthy of note, is that cancers without functional p53 protein are resistant to DNA damaging chemotherapeutic agents, as the cell continues to divide and does not undergo apoptosis, such as Wilm's tumour and B-CLL. Tumours that have low proportions of *p53* mutations such as testicular tumours are more sensitive to DNA damaging chemotherapeutic agents.

It is notable that the genes identified in colorectal tumours affect almost all the cellular compartments; *APC* in the cytoplasm, *ras* at the cell membrane and *p53* within the nucleus, and that they also have similar sites of action, such as on TGF-β. Suggesting that normal cells have evolved several levels of cellular protection and that many of these protective mechanisms must be disassembled before a cancer can develop (Kinzler and Vogelstein, 1998). Even when malignancy does occur, the subclones continue to evolve, developing varying degrees of metastatic capability, together with radiation and drug resistance (Nowell, 1976). It must be noted that not all tumours have abnormalities of *p53* or *APC* and not all have *ras* mutations, it can therefore be assumed that there are also other pathways involved in colorectal tumourigenesis (Kinzler and Vogelstein, 1998).

1.8.2 Microsatellite Instability Pathway of Colorectal Cancer Tumourigenesis

Microsatellites are short tandemly repeated base sequences that occur randomly throughout the genome and may be of varying sizes both between cells and individuals (Bocker et al., 1997). A microsatellite sequence is defined as two or more bases, which are repeated e.g. CACA. Microsatellites may be either monomorphic and show no variation between individuals or polymorphic, showing a variety of sizes. Very occasionally, the length of a microsatellite changes between one cell and its offspring after cell division, which has been ascribed to a slippage by DNA polymerase during DNA replication (Frayling, 1999). Slippage is where one DNA strand rides over its complementary partner and is more likely to happen in regions of repetitive sequences probably because DNA polymerase is less able to process through these regions (Strand et al., 1993). When this alteration is found somatically in tumour cells, as compared to normal tissue cells, it is termed replication error. Thus, tumour cells showing changes in microsatellite lengths are termed replication error positive (RER+). Genes involved in the detection and repair of these replication errors are termed DNA mismatch repair genes. Mutations in these DNA mismatch repair genes lead to microsatellite instability (MSI), thus the terms replication error positive and microsatellite instability can been used interchangeably. The presence of MSI has been further categorised into MSI-H (when MSI is present in more than 30% of microsatellites examined) and MSI-L (<30% of microsatellites examined), (Boland et al., 1998). This generalised genomic instability may affect cancer-related genes and result in tumour formation (Aaltonen et al., 1993; Peltomäki et al., 1993; Thibodeau 1993; Ionov et al., 2000; Janin, 2000). Genes that could possibly be inactivated due to frame-shift mutations include those with long microsatellite tracts such as *TGFβ receptor II* gene (where TGFβ is known to inhibit growth and induce apoptosis), the *BAX* gene (which also induces apoptosis) and the *APC* gene (Janin, 2000).

Microsatellite instability has been found in up to 90% of tumours of the hereditary non-polyposis colorectal cancer (HNPCC) syndrome (Aaltonen et al., 1993) and in nearly 20% of sporadic colorectal cancers (Thibodeau et al., 1993; Aaltonen et al., 1993; Kim et al., 1994; Rüschoff et al., 1995). The incidence of HNPCC is around 2-3% of all colorectal malignancies (Evans et al., 1997) and are characterised by early onset of colorectal cancer (usually before the age of 50 years), location of tumours in the right

side of the colon, and an increased risk of developing tumours in other organs such as the uterus, stomach, bladder, small intestine and ovary (Marra and Boland, 1995). Sites where there is a rapid turnover of cells and where cells are sloughed off as a result of normal physiological processes (Peltomäki and de la Chapelle, 1997). Janin (2000), proposes that the loss of the remaining wild-type allele of an MMR gene in RER+ cancer prone individuals occurs many times in a number of cells at sites where there is a continuous proliferation of cells, the ensuing genetic instability then leads to the accumulation of numerous mutations in a number of genes, which can then either lead to carcinogenesis or early senescence and apoptosis. The mutated cells, which have escaped apoptosis and have not been sloughed off, could then continue to mutate at an explosive rate, forming the so-called "aggressive polyp". This hypothesis could explain why several cases of advanced cancers have occurred in patients less than three years after normal colonoscopic screening (assuming these polyps were not missed at the initial screening). However, the small bowel also has a rapid turnover rate of mucosal cells and yet the risk of small bowel tumours is only 1% compared to the risk of colorectal cancers in hereditary non-polyposis cancer syndrome, which is close to 80% (Aarnio et al., 1995). This may be explained by the differing luminal environments at the two sites, where differences in water and potential carcinogen contents could affect the selection of these mutated cells (Janin, 2000). The effect of environment could also explain why the majority of endometrial cancers, in HNPCC kindreds, occur in pre-menopausal women as compared to post menopausal women as the hormone dependant endometrium proliferation, leads to an increased risk (Aarnio et al., 1995). Conversely, the absence of childhood tumours could be explained by a more efficient induction of apoptosis in young stem cells, which may become less efficient with increasing age (Janin, 2000).

The microsatellite instability found in colorectal cancers is very similar to that found in yeast, where mutations in DNA mismatch repair (MMR) genes cause a 100-700 fold increase in microsatellite instability. The first human MMR gene *MSH2* (*MutS* homologue 2) was so called because of its close similarity to the MMR homologue, *MutS*. The second major locus found to be mutated in HNPCC was identified as *MLH1* (*MutL* Homologue 1). Other MMR genes have also been identified: *PMS1* and *PMS2*, so called because mutations in these genes cause aberrations in the post-meiotic segregation of chromosomes (Baker et al., 1995), and *GTBP/MSH6* (G:T mismatch

binding protein). The human homologue of these yeast genes, are represented by *hMSH2*, *hMLH1*, *hPMS1* and *hPMS2* which are located on chromosomes 2, 3, 2 and 7 respectively (Nicolaides et al., 1994; Papadopoulos et al., 1994). Liu et al., (1996) found that the majority (90%) of microsatellite instability found in HNPCC patients was associated with mutations of *MSH2* and *MLH1*, a very small percentage are associated with mutations of *PMS1* and *PMS2* and none with mutations of *GTBP/MSH6*. Although no mutations of *GTBP/MSH6* were found in colorectal cancers from patients with HNPCC, endometrial cancers showing microsatellite instability from HNPCC kindred do show mutations in *GTBP/MSH6* (Wijnen et al., 1999). Another DNA mismatch repair gene, *MSH3* (*MutS* homologue 3), located on chromosome 5q, although not directly implicated in HNPCC, has been found to be mutated in some colorectal cancers from HNPCC patients (Akiyama et al., 1997).

The tumours that develop with microsatellite instability are usually situated in the right side of the colon, have a near diploid chromosomal constitution and are thought to be less aggressive in terms of growth, rate of relapse or recurrence, and metastases as compared to the tumours associated with loss of heterozygosity for genes (Lothe et al., 1993; Thibodeau et al., 1993; Bubb et al., 1996; Myrhoj et al., 1997). These tumours are also less likely to have loss of heterozygosity on 5q, 17p and 18q (Thibodeau et al., 1993; Kim et al., 1994).

DeWeese et al., (1998) reported that mouse embryonic stem cells from mice carrying mutations in either one or both alleles for *MSH2*, displayed an increased survival following protracted exposures to low level ionising radiation as compared to wild-type embryonic stem cells. The increased survival was attributed to a failure to execute apoptosis efficiently, when a defect in DNA mismatch repair was present. There is also preliminary evidence that the presence of MSI might also confer increased resistance to the cytotoxic effects of alkylating agents of the types used in cancer chemotherapy (Fink et al., 1996; Carethers et al., 1999).

1.8.3 Methylation as a Pathway for Colorectal Cancer Tumourigenesis

Primordial germ cells and embryonic stem cells can progress through the cell cycle and divide without any detectable levels of DNA methylation (Lei et al., 1996). However, once differentiation begins, DNA methylation is essential for the cell's

viability (Baylin et al., 1998; Okano et al., 1999) and appears to play an important role in stabilising the active (euchromatic) and inactive (heterochromatic) DNA regions (Bird, 1992).

It is known that in colorectal cancer cells there is generalised hypomethylation of the genome. In normal cells, about 80% of the 5^{1}-CG-3^{1} dinucleotides are methylated and it is thought that methylation plays a role in gene expression and chromosome condensation (Bird, 1992). In mammalian cells, DNA methylation occurs at the 5-position of cytosine within the CpG dinucleotide with approximately 70% of all CpG dinucleotides being methylated. The distribution of these CpG dinucleotides is not random within the genome, but appears to be concentrated in small genomic regions called "CpG islands," and which appear to reside within or near the promoter regions of various genes, where methylation apparently impairs the ability of transactivating factors to bind and initiate gene expression (Goel et al., 2001). Conversely, the same mechanism has been found to silence tumour suppressor genes in a variety of tumours (Herman et al., 1998). This distinct pathway, involving transcriptional silencing in selected genes in cancer has been termed CpG island methylator phenotype (CIMP), and is distinct from age-related methylation which has been observed in gene promoters as a function of age (Toyota et al., 1999).

The majority of tumour cells have less methylation than normal cells but, in conjunction with this generalised hypomethylation is a region specific gain of methylation within the CpG islands, which is associated with an increase in activity of the DNA methyltransferases (Baylin et al., 1991; Laird and Jaenisch, 1994; Jones, 1996). Genes which can become silenced by CpG island hypermethylation, include; the retinoblastoma gene, the Von Hippel-Lindau gene, the E-cadherin gene and the cyclin dependant kinase inhibitor genes, *p16* and *p15* (Baylin et al., 1998). Thus, alterations in the level of methylation in genes can either activate normally silenced genes or silence normally active genes (Baylin et al., 1998; Robertson and Wolffe, 2000).

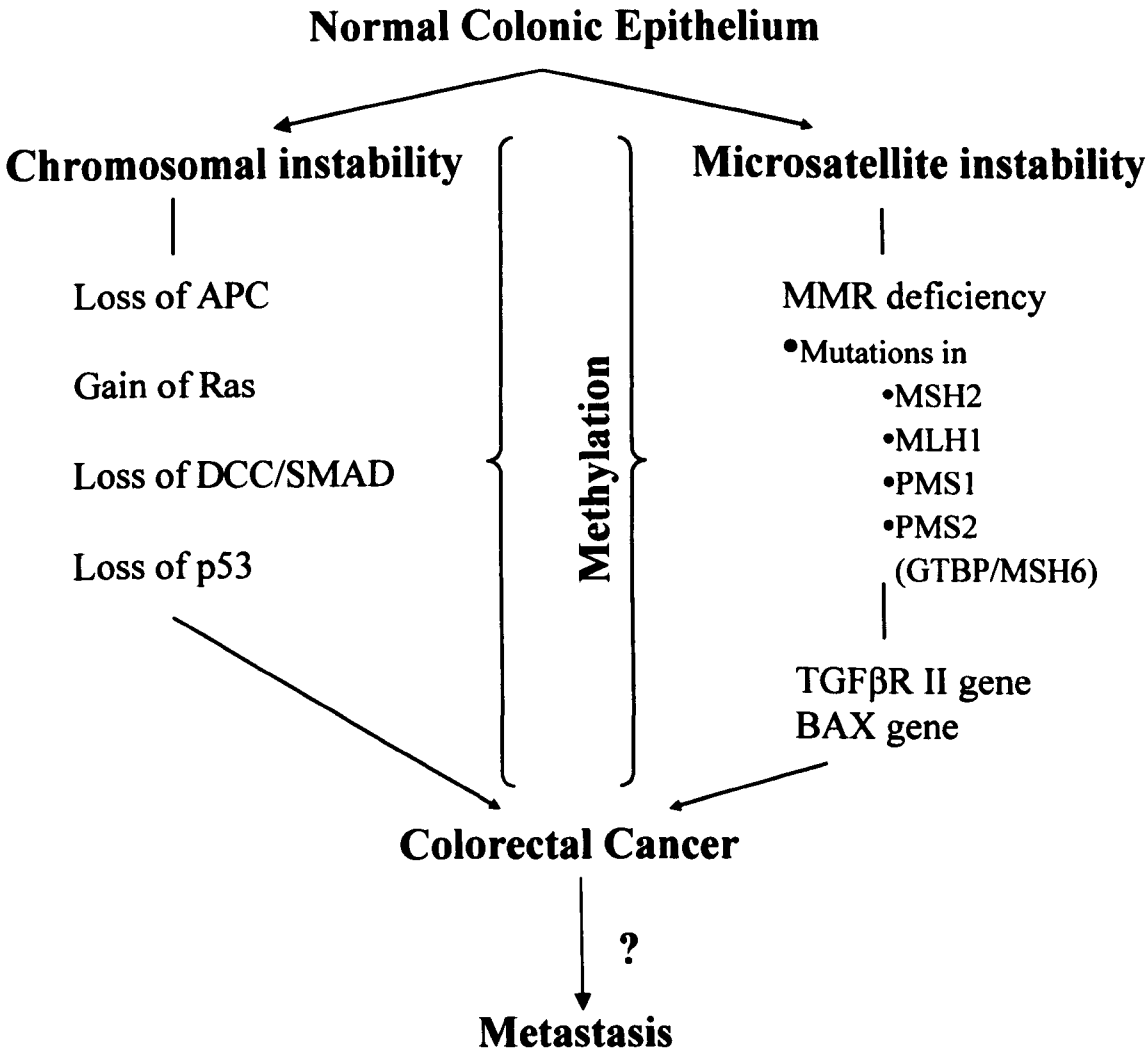
A correlation between microsatellite instability and aberrant DNA methylation has also been recently highlighted, with a strong association between CpG island methylation and MSI being reported in sporadic colorectal cancers (Ahuja et al., 1997; Viegl et al., 1998; Miyakura et al., 2001; Hawkins et al., 2002). Yamamoto et al. (2002), analysed both hereditary (from HNPCC patients) and sporadic colorectal cancers for aberrant DNA methylation, and stated that 53% of the sporadic colorectal cancers with

high-level microsatellite instability (MSI-H) also exhibited CpG island methylation whereas only 23% of the HNPCC tumours did so. Concluding that the differential methylation within these CpG islands, may be associated with divergent developmental pathways in hereditary and sporadic colorectal cancers, despite both exhibiting similar MSI-H phenotypes.

1.8.4 A Genetic Model for Colorectal Cancer Tumourigenesis

Thus, the process of colorectal tumour evolution is probably the interaction of several mechanisms; chromosomal instability, microsatellite instability, methylation and probably others, as yet not described (figure 1.6).

Figure 1.6 A genetic model for colorectal cancer tumourigenesis.



1.9 COLORECTAL CANCER METASTASIS

Colorectal cancers can metastasise to a variety of organs. Sites of metastatic spread include the regional lymph nodes, liver, lung, bones and occasionally the brain (Weiss et al., 1986). Liver metastases are present in 15-25% of patients at the time of diagnosis of colorectal cancer and another 20% of patients will develop liver metastases following resection of the primary tumour (Ballantyne and Quin, 1993; Fong et al., 1996). The median survival time of patients with liver metastases from colorectal cancer, and receiving no treatment for these metastases, is less than 1 year (Ballantyne and Quin, 1993). Thus, metastases represent a significant cause of morbidity and mortality in patients with colorectal cancer.

1.9.1 Diagnosis of Liver Metastases

In the case of colorectal cancer, prospective studies indicate that serial serum carcinoma embryonic antigen (CEA) assays are the most effective screening method for the detection of subsequent metastases, with 85-90% of patients with liver metastases having an elevated CEA (Kemeny et al., 1982). Investigations prior to any therapy include a detailed history and examination, CT scan of the chest, abdomen and pelvis and regional imaging of the liver with magnetic resonance imaging (MRI) and possibly angiography (Fong et al., 1996).

1.9.2 Treatment of Metastases

Several modalities exist for the treatment of liver metastases; liver resection surgery, systemic chemotherapy, hepatic artery chemotherapy of the liver, cryoablation, microwave coagulation therapy, radiation therapy, chemo-embolisation, ethanol injection and laser photocoagulation (reviewed in Geoghegan and Scheele, 1999; Shibata et al., 2000). Although all of these therapeutic methods may offer some palliative benefit, none of them provides satisfactory effectiveness in the control of liver metastases as compared to liver resection performed in selected patients. Thus, liver resection remains the treatment of choice, if feasible (Shibata et al., 2000).

1.9.2.1 Chemotherapy and De-arterialisation

Chemotherapy may be administered either systemically or regionally directly to the liver. Recently, the National Institute for Clinical Excellence, (2002), published recommendations on the use of chemotherapy in patients with colorectal cancer metastases confined to the liver, when shrinkage of the metastases might permit potentially curative surgery, recommending 5-fluorouracil (5-FU)/folinic acid and oxaliplatin as first line combination therapy and irinotecan as second line monotherapy. However, multidrug resistance is found in virtually all colorectal cancers and appears to be an inherent property of colonic mucosa cells (Fong et al., 1996). The exact mechanism of 5-FU inactivation is not completely understood, but future trials of chemotherapeutic agents may incorporate biologic studies of cancer cells in the hope of identifying cancer cells resistant to 5-FU and so direct therapy using other chemotherapeutic agents (Leichman et al., 1995).

As the liver metastasis grows it begins to derive most of its blood supply from the hepatic artery as compared to the hepatic portal veins, whilst the normal hepatocytes derive their blood supply mostly from the portal circulation (Breedis and Young, 1954). Thus, hepatic artery chemotherapy would preferentially target the growing liver metastases. However, hepatic artery chemotherapy is not without significant complications and as yet has not been shown to improve survival as compared to systemic chemotherapy (reviewed in Geoghegan and Scheele, 1999). Similarly, occlusion of the hepatic artery should have a greater effect on the tumour than the normal liver. Three main approaches have been employed; embolisation, hepatic artery ligation and intermittent occlusion of the hepatic artery using an implanted intra-arterial balloon (the use of intermittent occlusion is an attempt to prevent the development of a collateral circulation from the portal vein circulation). Only a few studies have been undertaken investigating the usefulness of de-arterialisation, all with inconclusive results (reviewed in Fong et al., 1996).

1.9.2.2 Cryoablative and Microwave Coagulation Therapy

Rapid freeze/thaw of tissues results in cellular crystal formation and is associated with significant cellular damage and cell death. This cytotoxic effect is the basis of cryoablative therapy of liver metastases (Hass and Taylor, 1948). It appears to be a safe and effective procedure in experienced centres, however, complications can arise such

as; intra-operative hypothermia, cardiac dysrhythmias, cracking of the ice ball resulting in catastrophic haemorrhage, pleural effusions, liver abscesses, bilomas/bile fistulae, myoglobinuria resulting in acute renal failure, cryoshock (a phenomenon similar to septic shock, but without any evidence of infection) and death (Seifert et al., 1998). Its efficacy as a curative treatment has yet to be established.

Microwave coagulation therapy was first developed in the late seventies, primarily for use in liver surgery as a method of cutting and coagulating the bleeding surface simultaneously (Shibata et al., 2000). Its use in the treatment of colorectal cancer liver metastases is only recent and its efficacy has yet to be established. However, it has been used successfully in the treatment of hepatocellular carcinoma (Hamazoe et al., 1995). Shibata et al., (2000) reported a prospective study comparing microwave coagulation therapy with liver resection surgery and found similar survival rates in both groups, concluding that microwave coagulation therapy was equally effective in the treatment of multiple metastases and also associated with less blood loss. Further prospective large-scale studies will need to be undertaken to verify their results.

1.9.2.3 Surgery

Surgical resection however, remains the treatment of choice for liver metastases and as yet remains the only modality that offers any chance of a cure (Fong et al., 1996; Harmon et al., 1999).

Several surgical liver resections are possible as well as isolated segmentectomy (Blumgart, 1994), but curative resection is dependant on the complete removal of the tumour with clear margins of at least 1cm (Shirabe et al., 1997; Cady et al., 1998).

A number of factors are involved in influencing prognosis in patients undergoing a liver resection. Age, gender, primary tumour grade and primary tumour location are not major factors. Factors associated with a poor prognosis includes; symptomatic clinical presentation, extensive disease affecting more than 50% of the liver, the presence of four or more metastases, metastases greater than 5cm in diameter, the presence of satellite metastatic lesions, regional lymph node metastases, the presence of portal vein invasion, hepatic vein invasion, the presence of tumour in the resection margins indicating that residual tumour may be present and interestingly the absence of a fibrous pseudocapsule on histological examination (Ballantyne and Quin, 1993; Ambiru et al.,

1999; Nagashima et al., 1999; Yamamoto et al., 1999; Okano et al., 2000; Rodgers and McCall, 2000).

Liver resection in selected patients is associated with a 30-40%, five-year survival and a 20% long-term disease free survival, with an associated mortality of less than 5% in experienced centres. The majority of deaths arise from either, peri-operative haemorrhage, infection or liver failure (Fong et al., 1996; Harmon et al., 1999). Major complications include bile leak and biliary fistula (3-8%), peri-hepatic abscesses (2-10%), pneumonia (5-22%), significant haemorrhage (1-3%) and liver failure (3-8%) (Schlag et al., 1990; Doci et al., 1991; Scheele et al., 1991; Ballantyne and Quin 1993; Harmon et al., 1999).

In patients in whom liver resection surgery is not performed, the duration of survival is closely related to the extent of liver involvement, where the one-year survival is 60% in patients with a solitary liver metastasis but is less than 10% in patients with widespread liver disease (Wood et al., 1976). There are several retrospective case-controlled studies comparing hepatic resection with no resection, five-year survival in the resectional group was approximately 25% but was less than 5% in the non-resectional group (Wilson and Adson, 1976; Scheele et al., 1991).

Most patients surviving after liver resection surgery die of recurrent disease, indicating that microscopic disease is present at the time of the surgery, the most common sites for recurrence being the liver and lungs (Ekberg et al., 1987). At present the use of adjuvant chemotherapy after liver resection surgery for colorectal cancer metastases remains unproven, but since the liver is the prime site of recurrence, hepatic artery chemotherapy may provide a useful modality (Koea and Kemeny, 2000).

1.10 UVEAL MELANOMA

Melanoma of the uveal tract (iris, ciliary body and choroid) is the commonest malignancy of the eye, with 5-7 cases per million population each year in the USA. Uveal melanomas can be divided into two sub-types, anterior affecting the iris and posterior affecting the ciliary body and choroids, with the most common subtype being the posterior melanoma, which affects 80% of all patients (Char, 1997). Uveal melanomas are rare in childhood, with the median age at diagnosis for posterior melanomas being 55 years (Egan et al., 1988), whilst for iris melanomas the average age of presentation being in the mid forties (Rennie, 1991).

1.10.1 Aetiology

There are a number of risk factors associated with the development of uveal melanomas, which include pre-existing ocular naevi, ocular melanocytosis, primary acquired melanosis, eye colour, skin colour, cutaneous naevi, ultra-violet light and genetic factors, but as yet no definitive causative factor has been found (Egan et al., 1988; Seddon et al., 1990).

Familial occurrences of uveal melanoma appear to be rare, however in a survey of kindreds with a first degree relative also affected with uveal melanoma, it was shown that these cases were unlikely to be due to coincidence, suggesting that a genetic predisposition to uveal melanoma was present in these families (Singh et al., 1996). Some cases of uveal melanoma may indeed be related to systemic hereditary cancers, with some evidence to suggest a link between uveal melanoma and, breast and ovarian cancers (Sinilnikova et al., 1999). Certain pre-neoplastic syndromes have also been associated with the predisposition to uveal melanoma. Familial atypical multiple mole and melanoma syndrome (FAMM-M), also known as dysplastic naevus syndrome is an autosomal dominant inherited syndrome, characterised by the presence of large numbers of irregular and multi-coloured naevi and a hereditary susceptibility to cutaneous melanoma and possibly uveal melanoma (Singh et al., 1995). Congenital ocular melanocytosis is a disorder characterised by melanocytic hyper-pigmentation and which is associated with melanomas of the uveal tract, optic nerve, skin and the central nervous system (Kanski 1999).

1.10.2 Clinical Course

The majority of uveal melanomas occur in the choroid, followed by tumours in the ciliary body, with tumours occurring in the iris the least common. Melanomas of the iris are relatively slow growing and rarely metastasise, with a 5-year survival of approximately 95%. Iris melanomas are generally detected at an early stage and are usually small. Posterior melanomas tend to be more malignant, are usually detected later and metastasise more frequently than iris melanomas, presenting with either blurred vision in the case of ciliary body melanomas or with symptoms similar to that of retinal detachment (flashing lights or visual shadows), in the case of choroidal melanomas (Rennie, 1991).

Diagnostic investigations include slit lamp biomicroscopy, ultrasonography, indocyanine green angiography, CT and/or MRI and fine needle aspiration biopsy in selected cases. A general medical examination is essential in order to either exclude a metastasis to the choroids (most frequently from the lung or breast, but occasionally from the kidneys or gastrointestinal tract), and to detect any metastatic spread to either the lungs or the liver (Kanski, 1999).

1.10.3 Treatment

Iris melanomas can generally be managed conservatively, with careful observation and serial photography for small asymptomatic tumours, but surgical intervention may be required for large tumours or those that are growing rapidly and have become symptomatic (Geisse and Robertson, 1985). Surgical options include broad iridectomy for small tumours, iridocyclectomy for tumours invading the corneo-conjunctival angle or enucleation for large diffusely growing tumours (Kanski, 1999).

The management of posterior melanomas is more complex, with treatment being individualised for each patient taking into consideration the size and location of the tumour, together with the state of the unaffected eye and the general fitness of the patient. However, there is still no general consensus as to the management of these melanomas and a number of treatment options are available. A major clinical trial, The Collaborative Ocular Melanoma Study (COMS) is in progress in order to address this question. Treatment options include, careful and frequent observation of asymptomatic

small tumours (Barr et al., 1978; Thomas et al., 1979), local irradiation with either cobalt-60 or iodine-125 plaques (Shields et al., 1982; Packer et al., 1992), proton beam (Seddon et al., 1986; Gragoudas et al., 1988) or helium ions (Char et al., 1990) and enucleation with or without external-beam irradiation (Fine et al., 1989).

1.10.4 Prognosis

A number of factors influence prognosis. The most important are size, location, cell type, extra-ocular extension and cytogenetic abnormalities (Gragoudas et al., 1991; White et al., 1998). The selection of treatment modality depends on the location (whether iris, ciliary body or choroid), size, any co-morbid conditions that the patient suffers from and whether any extra-ocular invasion or metastases have occurred (Gragoudas et al., 1991).

The size of the tumour is determined by its elevation/height and basal diameter. Small tumours are 2-3mm or less in elevation, medium-sized tumours range from 2-3mm up to 10mm in elevation and have a basal diameter of up to 16mm and large tumours have a basal diameter of more than 16mm with an elevation greater than 10mm, where large tumours are associated with a poorer prognosis (McLean et al., 1982; Sato et al., 1997).

Uveal melanomas can be further classified according to the major cell type present within the tumour. These are the spindle cell variety (types A and B) and non-spindle cell variety (epithelioid melanomas, mixed cell type melanomas and necrotic melanoma). Patients with tumours composing of spindle cells have a better prognosis than those with non-spindle cells (Jensen, 1982).

The major determinant for prognosis as in the case of colorectal cancers is the extent of local spread and the development of metastases (Rennie, 1991).

1.11 GENETICS OF UVEAL MELANOMA

Uveal melanomas unlike most solid tumours are amenable to karyotypic studies and also seem to have relatively simple chromosomal abnormalities (Horsman and White, 1993; Wiltshire et al., 1993). Early reports suggested that abnormalities of chromosomes 1, 6, 3 and 8 (in particular isochromosome 8q) were associated with posterior melanomas (Horsman et al., 1990; Prescher et al., 1990, 1992; Sisley et al., 1990, 1992; Horsthemke et al., 1992; Dahlenfors et al 1993; Horsman and White, 1993; Gordon et al., 1994; Singh et al., 1994; Speicher et al., 1994; Ghazvini et al., 1996; Becher et al., 1997). Later studies confirmed these early findings and also associated monosomy 3 and increase in 8q with tumours of the ciliary body, which are also associated with a poorer prognosis (Prescher et al., 1996; Sisley et al., 1997; White et al., 1998). It was initially postulated that monosomy 3 was the primary event with gains of chromosome 8q as a secondary event in the development of uveal melanomas (Prescher et al., 1994). However, it has been shown that both monosomy 3 and gain of 8q are independent indicators of poor prognosis, with additional copies of 8q being directly related to reduced survival (Sisley et al., 1997).

Numerous tumour suppressor genes are thought to be located on chromosome 3, with deletions of chromosome 3 being a frequent finding in a number of malignant tumours, such as lung, renal and cervical cancer (Kok et al., 1997). Loss of heterozygosity of chromosome 3 in uveal melanomas can also occur through the loss of one copy of chromosome 3 followed by the duplication of the remaining copy, thus resulting in a functional monosomy, termed isodisomy, and so on cytogenetic analysis appears to be normal with two copies of chromosome 3, but by molecular analysis there appears to be only one copy (White et al., 1998). Similarly, gain of 8q also appears to be a consistent finding in a number of malignant tumours, notably breast, endometrial and ovarian cancers, with the amplification of the *c-myc* oncogene appearing to play a major role in tumourigenesis (Fejzo et al., 1998).

There are as yet no prospective studies analysing chromosomal abnormalities of 3 and 8 with long-term follow-up, but there have been several retrospective studies relating the presence of monosomy 3 and gains of 8q to a worse prognosis (Prescher et al., 1996; Sisley et al., 1997; White et al., 1998).

1.12 UVEAL MELANOMA METASTASIS

The major routes of spread for uveal melanomas are by local invasion and metastatic spread via the blood stream (haematogenous metastasis). The uveal tract is extremely vascular but has no lymphatic system and so when regional lymph nodes are involved (pre-auricular, sub-mandibular and cervical nodes), it is usually as a result of sub-conjunctival extension of the primary tumour. Uveal melanomas invariably metastasise to the liver, accounting for 70-90% of all metastatic cases, with other sites including the skin (24%), presenting as subcutaneous nodules, the lungs (7%) and the vertebrae (7%) (Kath et al., 1993; Luyten, 1996; Char, 1997).

The majority of patients show no evidence of metastases at the time of presentation of the primary tumour (Gragoudas et al., 1991), with the incidence of metastases peaking usually at 2-4 years after primary enucleation (Diener-West et al., 1992), but may present many years after the primary has been excised (Shields et al., 1985). Therefore, it is likely that metastases have already been established, as micro-metastases, prior to the detection of the primary tumour (Wang et al., 1993). As a consequence, by the time liver metastases present clinically, resectional surgery is not possible as the metastases tend to be multiple, and present within both lobes of the liver (Salmon et al., 1998; Pyrhonen, 1998), thus, liver metastatic tissue from uveal melanomas is rarely available for study. Consequently, prognosis is generally poor, with a 5-year tumour-related mortality of between 16-53% (McClean et al., 1982; Diener-West et al., 1992; Kath et al., 1993). Most studies have reported a recurrence rate of approximately 20-30% within 5 years following treatment (Pach et al., 1986; Gamel et al., 1992).

1.13 AIMS OF THE STUDY

Liver metastases pose significant morbidity and mortality to patients with colorectal cancer, occurring either synchronously or several years after the primary tumour has been resected, and as yet no specific histopathological or genetic factor is available to predict the development of metastases. The principle aim of the study was to identify either a single or set of genetic abnormalities which could predict the development of liver metastases.

At the commencement of the study no protocol was in place for the collection and storage of fresh-frozen specimens of primary colorectal cancers and their liver metastases, and as such multi-centre ethical approval was sought and obtained prior to establishing collection, storage and analysis of tumour specimens. Since this would take time to institute, and the prospective collection of uveal melanomas had already been established for several years, the decision was taken to commence analysis of archival formalin-fixed paraffin-embedded colorectal cancer and their liver metastases and simultaneously that of the fresh-frozen uveal melanoma samples, prior to the analysis of fresh specimens of colorectal cancers and their liver metastases.

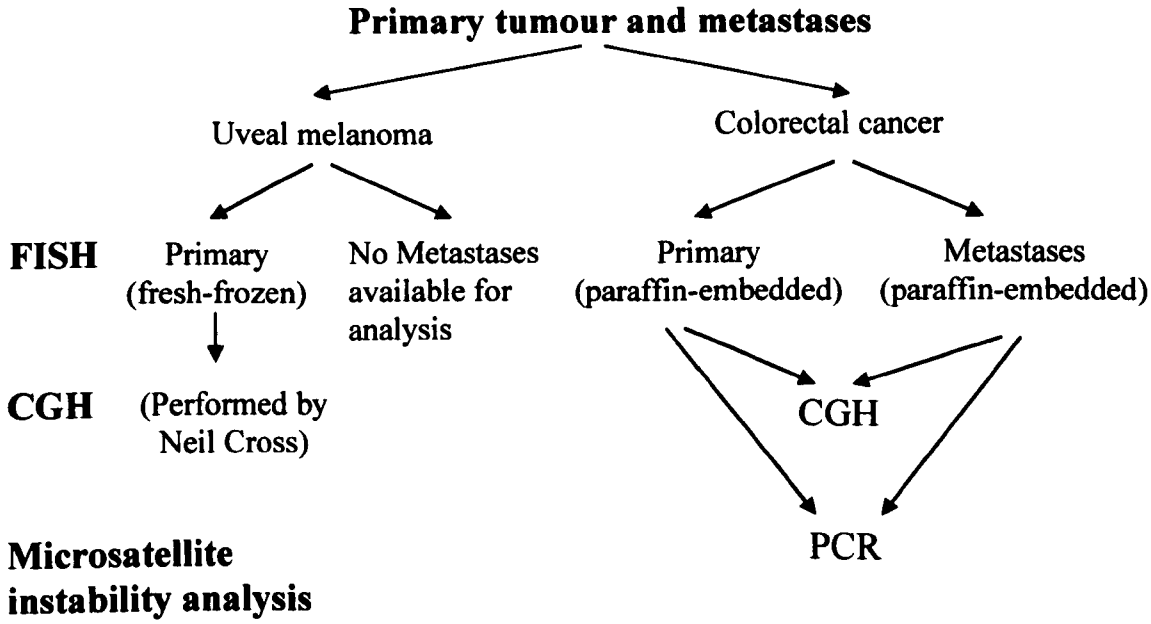
Abnormalities of chromosomes 3 and 8 are known to be associated with liver metastasis in the case of uveal melanomas and as such would provide a starting point for our study into the genetic changes involved in the development of liver metastases from primary colorectal cancers and uveal melanomas. Thus, the study was divided into three main phases, which were as follows:

1. The analysis of abnormalities in copy numbers of chromosomes 3 and 8 in primary colorectal cancers and uveal melanomas with their paired liver metastases, using fluorescent *in situ* hybridisation (FISH), in order to establish the clinical significance of changes in copy numbers of chromosomes 3 and 8 with liver metastasis and to assess the feasibility and efficacy of FISH to predict prognosis.
2. The analysis of gross genomic changes in primary colorectal cancers and uveal melanomas compared with their liver metastases using comparative genomic hybridisation (CGH), in an attempt to elucidate any specific chromosomal abnormalities associated with liver metastasis.

3. As microsatellite instability appears to play an important role in a group of colorectal cancers, the third study was to assess the presence of microsatellite instability in primary colorectal cancers and uveal melanomas to the presence or absence of liver metastases.

As the outlined investigations were too extensive for the duration of the study, some of the study was divided between a colleague who was investigating uveal melanomas (Mr Neil Cross) and myself; this is outlined more clearly in figure 1.7. Unfortunately, no liver metastases were available from uveal melanomas for analysis, as clinically patients with liver metastases from uveal melanomas tend to present late and with metastases present in both lobes of the liver, thus precluding curative resection. The Department of Ophthalmology and Orthoptics had already established the collection of fresh uveal melanoma tissue with storage at -80°C, whereas no such system of collection and storage had been established for colorectal cancers or their liver metastases, thus parts of the study was reliant on formalin-fixed paraffin-embedded tissue specimens.

Figure 1.7 Diagrammatic representation of the studies undertaken, with the techniques used and the archival tissue available for analysis.



CHAPTER 2 – MATERIALS AND METHODS

CONTENTS

CHAPTER 2

2.1 MATERIALS

2.1.1 General Laboratory Equipment and Reagents

Chemicals

All chemicals were purchased from BDH Laboratory Suppliers or Sigma-Aldrich Co Ltd. unless stated otherwise, and all were at least of analar grade.

Water

Deionised water for preparing aqueous solutions and rinsing equipment was obtained from either, a Purite Select Analyst HP system or Permulab HQ filter system.

Glassware

Disposable glass Pasteur pipettes were purchased from John Poulton Ltd. Low iron 'Superfrost' slides were purchased from BDH Laboratory Suppliers. Coplin jars were obtained from general glassware supplies.

Plastics and disposable laboratory equipment

Sterile serological pipettes (10ml), 12ml centrifuge tubes. 0.5ml and 2ml microcentrifuge tubes (eppendorf tubes) were purchased from Sarstedt Ltd. Abgene Thermo-Fast 96 low profile plates were purchased from Advanced Biotechnologies Ltd. Sterile disposable scalpels were purchased from Swann Morton Ltd. Disposable plastic syringes (1ml and 10ml) and sterile needles (Microlance 3) were purchased from Becton Dickinson Ltd., the accompanying filters (Acrodisc 0.8µl/0.2µl) were purchased from Pall Gelman Sciences. Petri dishes (100x15mm) and 20ml universals were purchased from Bibby Sterilin Ltd. Disposable gloves were purchased from Ansell Medical. Blotting paper for use in slide preparation was bought from Raymond Lamb Ltd.

Heating equipment

Grant water baths were purchased from Scientific Laboratory Supplies Ltd., while the heating block was a Dri-block DB.2A purchased from Techne Ltd.

Automatic pipettes and tips

Gilson Pipetmans (20µl, 200µl and 1000µl) were purchased from Anachem Ltd., the accompanying tips were provided by Sarstedt, and sterilised by autoclaving at 15p.s.i. and 120°C for 15 minutes.

Centrifugation

Cells obtained from tumour disaggregation were pelleted at 1000rpm for 10mins unless stated otherwise in a Megafuge 1.0R purchased from Deraeus Sepatech. For all other techniques a microcentaur MSE purchased from Scientific Laboratory Supplies Ltd. was used.

Storage

Reagents were either stored at 4°C in an Electrolux fridge or at –20°C in a LabCold 0020 freezer purchased from Scientific Laboratory Supplies Ltd. Fresh frozen tissue samples were stored in a BioStor liquid nitrogen container purchased from Statebourne Cryogenics.

General Equipment

A water-jacketed CO_2 incubator from Sanyo Gallenkamp was used. Sartorius Basic weighing scales were purchased from Sartorius Ltd. A Stuart Scientific mini orbital shaker and test-tube rotator was purchased from Scientific Laboratory Supplies Ltd. Adjustment of pH of prepared solutions was carried out on a 3020 pH meter from Jenway. The vortex rotamixer was from Hook and Tucker Instruments Ltd. Both the fume safety cabinet and microbiological safety cabinet were purchased from Walker Safety Cabinets Ltd. A Speed Vac Concentrator connected to a Vacuubrand diaphragm vacuum pump were purchased from Savant Instruments Inc. and Vacuubrand GMBH + Co. respectively. DNA spectrometry was conducted on a Lambda Bio UV/VIS spectrophotometer purchased from Perkin-Elmer Ltd. Finally, the microscope for analysis of slide preparations was an Olympus CK2.

2.1.2 Biological Samples

Fresh uveal melanoma tumour specimens were obtained following surgical procedures carried out at the Royal Hallamshire Hospital in Sheffield. Ethical approval was obtained for specimen collection. The majority of patients were treated by enucleation, with tumour material being removed immediately after the globe had been opened and then embedded in optimal cutting temperature (OCT) compound and subsequently stored at –20°C.

All liver metastatic tissue was obtained from the Royal Hallamshire Hospital, while primary colorectal cancer tissue was obtained either from the Royal Hallamshire Hospital, Barnsley District General Hospital, Chesterfield Royal Hospital, Doncaster Royal Infirmary or Rotherham District General Hospital. Ethical approval for specimen collection was obtained from each individual NHS Trust Local Research and Ethics Committee. Tissue specimens obtained were either paraffin-embedded or were collected from theatre and stored in liquid nitrogen until required.

All tissue samples were coded and stored in a secured area.

2.1.3 Reagents used in Cell Culture and Cell Harvesting

Cell culture medium

All short-term cell cultures were grown in modified RPMI 1640 medium with L-glutamine, purchased from GibcoBRL. Standard RPMI 1640 medium (500ml) was modified with the addition of 10% fetal calf serum purchased from Helena Biosciences, 50000 IU of penicillin-streptomycin purchased from GibcoBRL, 250UG of fungizone also purchased from GibcoBRL and 45% d-glucose.

Phosphate buffered saline solution with EDTA (PBSe)

PBS tablets were purchased from Oxoid. Prepare by dissolving 1 tablet in 100mls of deionised water followed by the addition of 0.2g EDTA. Sterilize in an autoclave.

Trypsin 1:20

Trypsin 1:20 is produced by the dilution of trypsin 1:250 with PBSe.

2.1.4 Reagents used for Chromosome Banding

Sorensen's buffer

Dissolve 9.47g of disodium hydrogen orthophosphate (Na_2HPO_4) with 9.08g of potassium dihydrogen orthophosphate (KH_2PO_4) in one litre of deionised water.

Gurr's buffer

Buffer tablets pH6.8 were purchased from BDH. Prepare by dissolving 1 tablet in 100mls of deionised water and store at room temperature.

Leishman's staining solution

Prepare the Leishman stain working solution by diluting the Leishman stain stock solution (purchased from BDH) with Gurr's buffer to a ratio of 1:4.

2.1.5 Reagents used in Fresh-Frozen Tumour Disaggregation

Hypotonic solution (0.075M KCl)

Dissolve 5.5875g of potassium chloride (KCl) into 1 litre of deionised water and sterilize by autoclaving. Store at 4°C and pre-warm in an incubator at 37°C prior to use.

Fixative

Fixative is freshly prepared, by adding methanol to glacial acetic acid in a ratio of 3:1.

2.1.6 Equipment and Reagents used in Fluorescent *In Situ* Hybridisation (FISH)

RNase

RNase A was purchased from Boehringer Mannheim. Stock solution was prepared by dissolving Rnase with 15mM of sodium chloride (NaCl) into 20mg/ml of 10mM Tris-HCl (pH 7.5) then stored at -20°C, in aliquots of 100µl until required.

Pepsin solution

Pepsin stock 10% (100µg/ml) was purchased from Sigma-Aldrich. Add 25µl of 10% pepsin stock solution (100µg/ml) to 100ml of pre-warmed 0.01M HCl solution. Pepsin stock solution was stored at –20°C until required.

Phosphate buffered saline (PBS) solution

PBS tablets were purchased from Oxoid. Dissolve 1 tablet per 100mls of deionised water and sterilise in an autoclave.

Magnesium chloride solution ($MgCl_2$)

A 1M solution of magnesium chloride is prepared by making up to 500ml, by adding 101.65g of $MgCl_2.6H_20$ to deionised water. The resulting solution is then sterilised by autoclaving.

PBS – magnesium chloride solution (PBS-$MgCl_2$)

Add 50ml of the 1M magnesium chloride solution to 950ml PBS, mix thoroughly and store at room temperature.

Alpha satellite centromeric probes

Initially, both probes were purchased from Cambio, the chromosome 8 probe was directly labelled with FITC, whereas the probe for chromosome 3 was conjugated with biotin and indirectly labelled with avidin-Texas Red. The probes purchased from Appligene-Oncor were indirectly labelled. The chromosome 8 probe being conjugated with biotin and the chromosome 3 probe with digoxigenin, thus indirectly labelled with avidin-FITC and anti-digoxigenin rhodamine respectively.

Denaturation solution

Add 35ml formamide, 5ml 20xSSC and 10ml deionised water into a Coplin jar, mix thoroughly and adjust the pH to 7.0.

FISH hybridisation buffer

A 10μl hybridisation buffer solution consists of 5μl of formamide, 2μl of 50% dextran sulphate (which had been prepared with deionised water and autoclaved), 2μl of deionised water and 1μl of 20xSSC.

Rubber solution

This is a polyurethane adhesive purchased from Phillips.

Tween 20

10% Tween 20 was purchased from Pierce.

20xSSC

Add 175.3g of sodium chloride (NaCl) to 88.2g of tri sodium citrate ($Na_3C_6H_5O_72H_2O$) and make up to 800ml with deionised water. Adjust to pH 7.0 and make up to 1 litre with deionised water before being autoclaved.

2xSSC

Add 50ml of 20xSSC to 450ml of deionised water and adjust to pH 7.0.

Stringency wash

Add 50ml formamide, 10ml 20xSSC and 40ml deionised water into a Coplin jar, mix thoroughly and adjust pH to 7.0.

SSCT

This consisted of 100ml of 20xSSC, 2.5ml of 10% Tween 20 and mixed with 397.5ml of deionised water.

SSCTM

Add 10ml of SSCT through a 0.45μm filter to 0.5g of dried semi-skimmed milk powder (Marvel™). The resulting solution is vortexed and then mixed on a test-tube rotator for 5 minutes.

Detection reagents

Texas red conjugated to avidin was obtained from Vector for use with the Cambio chromosome 3 probe. Indirect Labelling Solution containing avidin-FITC and anti-digoxigenin rhodamine was purchased from Appligene-Oncor for use with their chromosome 8 and 3 probes.

Slide mountant

Antifade (PBS/Glycerol) was purchased from Citifluor. Counterstain contained 2μl of DAPI stock solution added to 1ml of antifade.

FISH analysis

FISH preparations were analysed using a Cohu high performance CCD camera attached to a fluorescent microscope. Images of FISH results were captured and manipulated using a PSI Powergene programme running on a Power PC 8500/180 computer from Apple Macintosh.

2.1.7 Reagents used for DNA Extraction

Proteinase K

Proteinase K (fungal) 100mg (>20 Units/mg) was purchased from Life Technologies. Stock solution was prepared by dissolving proteinase K in 10mM Tris HCl (pH 7.5), 20mM Calcium chloride and 50% glycerol. Then stored in aliquots of 50μl at -20°C, until required.

Digestion buffer

Prepare by dissolving 60.57g Tris (pH 8.5), 0.372g EDTA and 5ml 10% Tween into 1000ml deionised water to prepare a 50mM Tris (pH 8.5), 1mM EDTA, 0.5% Tween 20 stock solution.

QIAamp® DNA Extraction Kit

The DNA extraction kit was purchased from Qiagen Ltd. The kit contained QIAamp® spin columns, 2ml collection tubes, AL buffer (lysis buffer), ATL buffer (wash buffer), AW1 buffer (wash buffer), AW2 buffer (wash buffer), AE buffer (elution buffer) and proteinase K enzyme solution.

TE buffer

Add 8g of Tris HCL and 2g EDTA into 500ml of deionised water and mix thoroughly, adjust pH to 7.4.

2.1.8 Reagents used in DNA Labelling

Two methods for DNA labelling were employed. One was nick translation, reagents for which were purchased from Vysis UK, Ltd., the other was Ulysis® labelling purchased from Kreatech diagnostics. The QIAquick™ PCR purification and QIAquick™ nucleotide removal kits, used in the purification of ULS® labelled DNA probes, were purchased from Qiagen Ltd. The kits were supplied with PB buffer (binding buffer for use in the QIAquick™ PCR purification kit), PN buffer (binding buffer for use in the QIAquick™ nucleotide removal kit), PE buffer (wash buffer containing ethanol) and EB buffer (elution buffer-10mM Tris-Cl, pH 8.5).

2.1.9 Equipment and Reagents used for Comparative Genomic Hybridisation (CGH)

All reagents, normal metaphase target slides and control DNA used for CGH were purchased from Vysis UK, Ltd.

20xSSC, pH5.3

Add 66g of 20xSSC to 200ml deionised water and mix thoroughly. Make up to a final volume of 250ml and adjust pH to 5.3.

Denaturation solution

Add 49ml formamide, 7ml 20xSSC (pH 5.3) and 14ml deionised water into a Coplin jar and mix thoroughly. Adjust the pH to 7.4.

0.4xSSC/0.3% NP-40 Stringency wash solution

Add 20ml of 20xSSC to 950ml of deionised water and mix thoroughly. Add 3ml of NP-40 and using a magnetic stirrer mix thoroughly until the NP-40 is completely dissolved. Adjust the ph to 7.4. Aliquot out 50ml into clean 50ml centrifuge tubes and store at ambient temperature. Discard these aliquots after six months or if the solution appears cloudy.

2xSSC/0.1% NP-40 Stringency wash solution

Add 100ml of 20xSSC to 850ml of deionised water and mix thoroughly. Add 1ml of NP-40 and using a magnetic stirrer mix thoroughly until the NP-40 is completely dissolved. Adjust the ph to 7.4. Aliquot out 50ml into clean 50ml centrifuge tubes and store at ambient temperature. Discard these aliquots after six months or if the solution appears cloudy.

Test and Control DNA

Test and control DNA were labelled using either nick translation or ULS direct labelling methods.

CGH analysis

CGH slides were analysed using a Cohu high performance CCD camera attached to a fluorescent microscope. Images of CGH results were captured and manipulated using a MacProbe programme running on a Power PC 8500/180 computer from Apple Macintosh.

2.1.10 Equipment and Reagents used for Polymerase Chain Reaction (PCR)

Primers

Infra-red dye (IRD) labelled and unlabelled primers were purchased from MWG-Biotech (UK) Ltd. (For details of primer sequence and annealing temperatures see appendix III).

***Taq* polymerase**

Taq polymerase together with the nuclease free water, ammonium buffer, magnesium and dNTP master mix were purchased from Bioline Ltd.

PCR thermo cyclers

PCR thermo cyclers used included the Primus 96 Plus thermo cycler purchased from MWG Biotech (UK) Ltd. and the Biometra UNO-Thermoblock purchased from Scientific Laboratory Supplies Ltd.

2.1.11 Equipment and Reagents used for Agarose and Polyacrylamide Gel Electrophoresis

All reagents were purchased from either BDH Laboratory Suppliers or Sigma-Aldrich Co Ltd. A Bio Rad 1000/500 power supply was used for all electrophoresis procedures. A Bio Rad Protean II xi Cell tank was used for polyacrylamide gel electrophoresis and a Bio Rad Mini Sub DNA Cell was used for agarose gel electrophoresis. All Bio Rad equipment was purchased from Life Science Research. Gels stained with ethidium bromide were visualised on a UV transilluminator purchased from UVP Inc. and images were captured using a Kodak DC290 Zoom digital camera connected to an Apple iMac computer running Kodak Scientific Imaging Systems 1D software purchased from Anachem Ltd.

PCR loading buffer

Add 0.25g of bromophenol blue to a 40% glycerol solution and mix thoroughly.

10xTBE buffer

Add 121g of Tris HCL, 61g of boric acid and 7.4g of EDTA into 800ml of deionised water, mix thoroughly, adjust pH to 8.0 and make up to a final volume of 1 litre.

20% Ammonium persulphate solution (20% APS)

Add 4g of ammonium persulphate to 20ml of deionised water, mix thoroughly and store at 4°C.

Polyacrylamide gel solution

A 20ml polyacrylamide gel solution was prepared according the concentration of gel required (table 2.1). The 20% APS and TEMED (N, N, N', N', -tetramethylethylenediamine) were added just prior to the gel being poured.

Table 2.1 Volumes of the various constituents used to prepare a specific concentration of polyacrylamide gel.

	6%	8%	10%
Deionised Water	30ml	28ml	26ml
40% 29:1 Polyacrylamide	6ml	8ml	10ml
10xTBE	4ml	4ml	4ml
20% APS	200µl	200µl	200µl
TEMED	20µl	20µl	20µl

2.1.12 Reagents used for Ethidium Bromide Staining and Silver Staining

All reagents were purchased from either BDH Laboratory Suppliers or Sigma-Aldrich Co Ltd. Ethidium bromide is a potent carcinogen and all precautions must be used in handling and disposing of this compound.

Solution 1 (Initial fix solution)

A solution of 50ml 100% ethanol, 5ml glacial acetic acid and 445ml deionised water.

Solution 2 (Silver nitrate solution)

Dissolve 0.5g silver nitrate into 500ml deionised water.

Solution 3 (Developer)

Dissolve 1.5ml formaldehyde and 2.25g sodium hydroxide into 500ml deionised water.

Solution 4 (Final fix/stop solution)

Dissolve 7.5g sodium carbonate into 500ml deionised water.

2.1.13 Equipment and Reagents used for LongRanger Gel Electrophoresis

All reagents were purchased from either BDH Laboratory Suppliers or Sigma-Aldrich Co Ltd., with the exception of 50% LongRanger Gel Solution which was purchased from BioWhittaker Molecular Applications Inc., urea from United States Biological and Chill-out 14 liquid wax from MJ Research Inc. Electrophoresis was performed on a LiCor 4200 LongRead IR automated sequencer (Li-Cor Technologies), with the products visualised using the LiCor ImagIR software on a PC computer, purchased from MWG-Biotech (UK) Ltd.

Stop solution

Prepare a solution of 95% (v/v) formamide with 10mM EDTA, 0.4% basic fuchsin and adjust pH to 9.0.

LongRanger gel solution

A 25cm LongRanger gel was prepared by mixing 4ml of 50% LongRanger Gel Solution with 10.5g urea, 2.5ml of 10xTBE buffer, 250µl DMSO and make up to 25ml with deionised water. Add 25µl TEMED and 175µl of fresh APS just prior to pouring the gel.

2.2 METHODS

2.2.1 Cell Culture Protocols

2.2.1.1 Producing a Short-Term Cell Culture

1. All the steps are performed in a sterile safety cabinet.
2. Transfer the tumour tissue into a Petri dish. Discard any fat, muscle, fibrous or necrotic tissue.
3. Add a small amount of PBS and fragment the tumour tissue manually using two scalpels. Try and squeeze cells loose from the clumps of tissue. Tilt the Petri dish to allow the larger fragments to settle and harvest the cell suspension into a sterile plastic centrifuge tube.
4. Centrifuge the harvested cell suspension at 1000rpm for 10 minutes.
5. Remove the supernatant and add 5ml of cell culture medium (RPMI 1640) gently and re-suspend the pelleted cells.
6. Divide this sample into three and aliquot out into sterile T25 culture flasks.

2.2.1.2 Cell Culture Passage

1. Assess the culture flasks to ensure that the cell culture layer is 60-80% confluent.
2. Remove the media. Note that some cells that are actively dividing will detach from the flask bottom. To assess whether these cells in suspension are viable or not, perform a tryphan blue test.
3. Add 5ml of trypsin 1:20 to the flask and gently agitate the flask and incubate for 15 minutes at 37°C.
4. Assess the flask to ensure that the cells have lifted off the bottom of the flask and are in suspension. The flask may need to be tapped to dislodge the cells completely. If the cells are still adhering to the flask bottom, incubate for a further 10 minutes and re-assess.
5. Once the majority of the cells are in suspension, aliquot a small volume into a sterile flask and add 10ml of fresh medium. Other aliquots may be cryopreserved in 20% DMSO/RPMI 1640 medium.

2.2.1.3 Tryphan Blue Test for Cell Viability

1. Remove the cell/medium suspension and transfer to a clean centrifuge tube.
2. Centrifuge the cell suspension at 1000rpm for 10 minutes.
3. Remove the supernatant except for 0.5-1ml remaining above the cell pellet and gently re-suspend the pelleted cells in the remaining medium.
4. Transfer 50µl of the suspended cells into a clean microcentrifuge tube and add 50µl of tryphan blue.
5. Pipette out 10µl of the cell/tryphan blue suspension onto a clean haemocytometer and examine under a microscope.
6. Dead cells will be stained dark blue, whilst living cells will not be stained.

2.2.1.4 Slide Washing

1. Place 20ml of Decon 90 detergent into a 2l beaker, and dilute with 1500ml of hot tap water.
2. Place as many slides as required into this solution and cover with aluminium foil. Leave the slides to soak overnight.
3. Rinse the slides thoroughly with hot tap water on each side for approximately 10 seconds. And then rinse with cold tap water and finally deionised water.
4. Store the washed slides in a covered beaker of deionised water at 4°C.

2.2.1.5 Preparation of Metaphase Chromosome Spreads

1. Assess the culture flasks to ensure that the cell culture layer is 60-80% confluent so that the cells will be still dividing and to ensure a good yield of cells.
2. Add 0.1ml of colcemid (final concentration 10µg/ml) to 10ml of media and incubate for 3-4 hours at 37°C.
3. Remove the media and any non-adherent cells and transfer to a clean plastic centrifuge tube.
4. Add 5ml of trypsin 1:20 to the flask and gently agitate the flask and incubate for 15 minutes at 37°C.

5. Assess the flask to ensure that the cells have lifted off the bottom of the flask and are in suspension. The flask may need to be tapped to dislodge the cells completely. If the cells are still adhering to the flask bottom, incubate for a further 10 minutes and re-assess. Once the majority of the cells are in suspension, transfer them into the clean tube containing the initial sample.
6. Centrifuge the cells at 1000rpm for 10 minutes.
7. Remove the supernatant, except for 0.5-1ml remaining above the cell pellet. Gently re-suspend the pelleted cells in the remaining medium and carefully add 5ml of pre-warmed (37°C) hypotonic solution, drop-by-drop, with gentle agitation. (Hypotonic treatment causes a swelling of the cells, thus causing the cell membrane to burst when the cells are dropped onto a microscope slide to prepare metaphase spread). The optimal time of hypotonic solution treatment varies for different cell types and must be determined empirically.
8. Incubate for 15 minutes at 37°C.
9. Centrifuge the cells at 1000rpm for 10 minutes.
10. Remove the supernatant, except for 0.5-1ml remaining above the cell pellet. Gently re-suspend the pelleted cells in the remaining medium and carefully add 5ml of freshly prepared fixative, drop-by-drop, with gentle agitation.
11. Repeat steps 9 and 10 two to three times.
12. After the last centrifugation, re-suspend the cells in a small volume of fixative and drop a drop of the suspension onto a cold, wet, clean microscope slide. The height from which the drop is dropped is determined empirically. Then add a drop of fixative onto the cell spot to facilitate further spreading. The quality of the metaphase spreading is dependent on a number of factors, including humidity, airflow and cell concentration.
13. After the required number of slides have been prepared, add 5ml of fixative to the cells gently and store at –20°C.

2.2.1.6 Leishman's Stain Banding

1. Age the prepared metaphase spread slides for one week at room temperature.
2. Place a slide on a rack over the sink and cover with trypsin solution 1:20 for 10-20 seconds.

3. Wash the slide with Sorenson's buffer for 10-20 seconds and then quickly drain.
4. Cover the slide with Leishman's stain working solution and leave for 30-90 seconds and then quickly drain and wash with Gurr's buffer. The optimal time for staining will need to be determined empirically.
5. Carefully blot-dry the slides and examine without mounting.

2.2.2 Tumour Cell Disaggregation Protocols

2.2.2.1 Disaggregation of Tumour Cells from Formalin-Fixed Paraffin-Embedded Tissue

1. Cut a 50μm section from each block of paraffin-embedded tumour specimen using a cryostat machine.
2. Remove excess paraffin wax using a scalpel. Then transfer the tumour sample to a polyurethane test tube and place in a water bath at 70°C for ~20 minutes, in order to melt the wax.
3. Once the wax has completely melted, add 5ml of xylene into the tube, and leave in a water bath at 70°C for 10mins. Gently vortex the tube midway through the wash to aid removal of the wax. Centrifuge the tube at 1000rpm for 10 minutes and carefully pipette off the supernatant.
4. Add 5ml of 100% ethanol into the tube and gently vortex. The tumour sample is then left in suspension for 10 minutes at room temperature. Centrifuge the tube at 1000rpm for 10 minutes and carefully pipette off the supernatant, so as not to disturb the pelleted tissue. This procedure is repeated at ethanol concentrations of 95%, 70% and 50%.
5. Add 10ml of deionised water into the tube and gently vortex. The tumour material is then left in suspension for 10 minutes at room temperature. Centrifuge the tube at 1000rpm for 10 minutes and carefully pipette off the supernatant, so as not to disturb the pelleted tissue.
6. The tumour sample is then digested with 1ml of protease at 37°C. The aim of which was to release single tumour cells into suspension, allowing these cells to be more accessible for FISH. The tumour sample is digested until a fine suspension is

produced. During the digestion process gently vortex the tumour sample every five minutes. (The length of time required for digestion is partially dependent on the individual tumour; however, most tumours were digested for approximately 30mins). Following the digestion process, remove any large clumps of undigested tumour material centrifuge at 1000rpm for 10 minutes. Then carefully pipette off the supernatant except for 0.5-1ml remaining above the cell pellet.

7. Gently re-suspend the pelleted cells in the remaining supernatant and carefully add 5ml of freshly prepared fixative, drop-by-drop, with gentle agitation. Then centrifuge the tube at 1000rpm for 10 minutes and carefully pipette off the supernatant except for 0.5-1ml remaining above the cell pellet. Repeat this fixation step twice.
8. Finally, re-suspend the disaggregated tumour cells in 5ml of fixative and store at -20°C until required.

2.2.2.2 Disaggregation of Tumour Cells from Fresh-Frozen Tissue

1. Retrieve fresh-frozen tumour samples out of frozen storage and place in a Petri dish.
2. If the sample has been stored in optimal cutting temperature (OCT) compound, remove excess OCT from around the sample and add a small amount of deionised water, in order to dissolve out the remaining OCT.
3. Using a scalpel blade, mince the tumour as finely as possible in order to release individual intact tumour cells. Use two scalpels in a sweeping action rather than a cutting action, so as to reduce the amount of damage to the individual cells.
4. Transfer the suspension containing the released tumour cells into a clean centrifuge tube using a pipette. Rinse the Petri dish with deionised water, to ensure that the maximum numbers of disaggregated cells are collected. Then centrifuge at 1000rpm for 10 minutes and carefully pipette off the supernatant.
5. Re-suspend the pelleted tumour cells with 3ml of hypotonic solution with gentle agitation, so as to cause the cells to swell and incubate at 37°C for 15mins. Following the incubation, centrifuge the tube at 1000rpm for 10 minutes and carefully pipette off the supernatant except for 0.5-1ml remaining above the cell pellet. (Initially, the cells were digested with protease at this stage; however, this was found to destroy the cells. Consequently this step was abandoned).

6. Gently re-suspend the pelleted cells in the remaining supernatant and add 5ml of PBS. Then carefully add 5ml of freshly prepared fixative, drop-by-drop, with gentle agitation. Centrifuge the tube at 1000rpm for 10 minutes and carefully pipette off the supernatant except for 0.5-1ml remaining above the cell pellet. Repeat this fixation step twice.
7. Finally, re-suspend the disaggregated tumour cells in 5ml of fixative and store at -20°C until required.

2.2.3 Slide Preparation for FISH

1. Centrifuge the tumour cell suspension at 1000rpm for 10 minutes. Remove the supernatant, except for 0.5-1ml remaining above the cell pellet.
2. Gently re-suspend the pelleted cells in the remaining supernatant and carefully add 5ml of freshly prepared fixative, drop-by-drop, with gentle agitation.
3. Allow any large particles of tumour to settle to the bottom of the tube, before 3-4 drops of the re-suspended cells are dropped onto the centre of a cooled washed slide, from a height of about 1cm. The ends of the slide are then blotted dry with blotting paper.
4. Air-dry the slide slightly until the surface appears frosted. At this point, a further 1-2 drops of the re-suspended cell suspension is dropped onto the cell spot, if required.
5. Finally, a drop of fixative is dropped onto the cell spot, causing the cells to spread out into a single layer.
6. Air-dry the slides completely, and store in slide holders for 1 week prior to Fluorescent *In Situ* Hybridisation.

2.2.4 Fluorescent *In Situ* Hybridisation

Day 1

Preparation of Solutions

1. Freshly prepare denaturation solution (consisting of 35ml formamide, 5ml 20xSSC and 10ml deionised water and adjusted to pH7) and pre-warm to 70°C in a water bath placed in a fume cupboard.

2. Prepare the working solution for pepsin digestion (consisting of 0.5ml of 1NHCl in 49.5ml of deionised H_2O) and pre-warm to 37°C in a water bath.

Pre treatment with RNase

1. The RNase working solution is made by adding 50µl of RNase stock to 10ml of 2xSSC in a universal container and mixing.
2. Pipette 120µl of the RNase solution onto each 22x50mm cover slip. Each slide is the carefully touched on top of each cover slip and incubated in a humid chamber at 37°C for 1 hour.
3. Remove the cover slips by tapping the slides vertically. Then immerse the slides into a Coplin jar containing 2xSSC at room temperature, and agitate on a shaker for 5 minutes. Repeat this wash twice.

Pepsin Digestion

1. During the third wash in 2xSSC, add 25µl of pepsin stock into the working solution prepared at the beginning of the procedure.
2. Immerse the slides into the pepsin solution for between 10-15 minutes depending on the cells under investigation. Tumour cells are generally digested for 10-15 minutes; whereas the blood spreads (positive controls) require only 10minutes.
3. Then wash the slides in PBS at room temperature on the shaker for 5 minutes. The wash is repeated once. Following this, the slides undergo a wash in PBS-$MgCl_2$ (50ml $MgCl_2$ in 950ml PBS) at room temperature on the shaker for 5 minutes.

Fixation

1. Freshly prepare the fixative solution by adding 1.35ml of formaldehyde to 50ml of PBS-$MgCl_2$.
2. Immerse the slides into the fixative solution at room temperature for 10 minutes and place the Coplin jar in a fume cupboard.
3. Immediately following fixation, wash the slides in PBS at room temperature on a shaker for 5 minutes.
4. Then dehydrate the slides through an ethanol series (70%, 95% and 100%) at room temperature for 3 minutes each.
5. Allow the slides to air-dry at room temperature.

Probe Preparation

1. The probes are prepared while the slides are in the fixative solution.
2. Prepare a master mix in a microcentrifuge tube. For each slide add 3μl of salmon sperm, 6μl of 100% ethanol and 0.5μl and 1.0μl of chromosome 3 and 8 centromeric probes. Microfuge the microcentrifuge tube at 13000rpm for 3 minutes.
3. Then place the microcentrifuge tube onto a heating block at 80°C, in order to remove all traces of ethanol, which could seriously affect hybridisation. This usually takes approximately 12-15 minutes.
4. Once dry, add 10μl of hybridisation mix for each slide into microcentrifuge tube containing the master mix. Denature the probes by placing the microcentrifuge tube onto the heating block at 80°C for 8 minutes.
5. Then place the microcentrifuge tube immediately onto ice so as to prevent re-annealing of the DNA probes.

Slide Denaturation

1. Place the slides momentarily onto a heating block at 80°C to warm.
2. Then rapidly immerse the slides into the denaturation solution, which had been prepared at the beginning of the procedure. Leave the slides to stand for exactly 2 minutes.
3. Having been denatured, the slides are then swiftly transferred into an ice-cold (-20°C) ethanol series (70%, 95% and 100%) for 3 minutes each, before being left to air-dry at room temperature.

Loading the Probes

1. Pulse-microfuge the probe mixture immediately prior to use.
2. Pipette 10μl of the probe mixture onto a 22x22mm cover slip. Touch each slide on top of each cover slip and seal with rubber solution. It is important to ensure that the position of the probe mix on the slides coincide with that of the greatest concentration of cells.
3. Incubate in a humid chamber at 37°C for 24 hours.

Day 2

Post-Hybridisation Stringency Washes

1. Freshly prepare a stringency solution (containing 50ml formamide, 10ml 20xSSC and 40ml deionised water and adjusted to pH 7.0) and warm to 42°C in a water bath, in a fume cupboard.
2. Remove the slides from the humid chamber and remove the cover slips by removing the rubber sealant and tapping the slides vertically. Then place the slides into the stringency solution for 5 minutes. Repeat this wash once.
3. Then immerse the slides into 2xSSC (adjusted to pH 7.0), also at 42°C, for 5 minutes. Repeat this wash once.

Detection

1. Wash the slides in SSCT for 3mins.
2. Prepare SSCTM and transfer 1ml to a microcentrifuge tube. Microfuge at 13000rpm for 2 minutes and pipette 120µl of the supernatant onto the centre of a 22x50mm cover slip. Touch each slide on top of each cover slip and incubate in a humid chamber at room temperature for 10 minutes.
3. Remove the cover slips by tapping the slides vertically, and then immerse the slides in SSCT for 3 minutes.
4. Pipette 30µl of the detection solution onto the centre of a 22x50mm cover slip. Touch each slide on top of each cover slip and incubate in a humid chamber at 37°C for 20 minutes.
5. Remove the cover slips by tapping the slides vertically and immerse the slides in SSCT at room temperature for 3 minutes, in the dark. Repeat this wash twice.
6. Then immerse the slides in PBS for 5 minutes, in the dark. Repeat this wash once.
7. Dehydrate the slides through an ethanol series (70%, 95% and 100%) at room temperature for 3 minutes each, in the dark. The slides are then left to air-dry, in the dark.
8. The counterstain is produced by adding 2µl of DAPI to 1ml of antifade in a microcentrifuge, which is then vortexed and pulsed in a microfuge.
9. Pipette 25µl of the counterstain onto the centre of a 22x50mm cover slip. Touch each slide on top of each cover slip, care being taken to avoid the introduction of air

bubbles, and allow the counterstain to spread right across the area of hybridisation. Use nail varnish to seal the edges of the cover slip.

10. Use lightproof slide boxes to hold the completed slides and store at 4°C until required for viewing.

2.2.5 DNA Extraction Protocols

2.2.5.1 DNA Extraction using Phenol:Chloroform for Formalin-Fixed Paraffin-Embedded Tissue for use in CGH

De-waxing of Paraffin-Embedded Tissue

Slide preparation

Prepare twelve 5μm sections from the paraffin-embedded tissue. The first and last slides are stained with haematoxylin and eosin (H&E), to ensure that there is no abrupt change in the tumour cell population throughout the sectioned tumour specimen. The remaining slides are left unstained and the tumour cell population microdissected using the H&E stained slide as a guide.

The various protocols utilised for the de-waxing of the microdissected paraffin-embedded tissue are discussed more fully in chapter 4.2.1 Optimisation of DNA Extraction from Formalin-Fixed Paraffin-Embedded Tumour Samples.

Tissue Digestion

1. Place the microdissected tissue sample into a 2ml microcentrifuge tube and add 600μl of digestion buffer and 30μl of proteinase K and incubate on a rotator at 55°C for 24 hours.
2. Centrifuge at 13000rpm for 10 minutes at room temperature.
3. Place on a heating block and boil for 10 minutes in order to de-activate the proteinase K.
4. Centrifuge again at 13000rpm for 10 minutes at room temperature.

DNA Purification using Phenol:Chloroform

1. Add an equal volume of phenol:chloroform to the de-waxed and digested DNA solution sample in a polypropylene tube with a plastic cap. (Ensure that the phenol has been equilibrated to ph 7.8-8.0, otherwise the DNA will partition into the organic phase).
2. Mix the contents of the tube until an emulsion forms.
3. Centrifuge at 13000rpm for 10 minutes at room temperature.
4. Remove the aqueous layer (top clear layer, which contains the DNA) avoiding the white interface layer (which contains protein) and place into a fresh tube.
5. Repeat steps 1 through to 4 until no white interface layer is present.
6. Add an equal volume of cold (-20°C) chloroform (to remove any phenol) and repeat steps 2 through 4.
7. Add an equal volume of cold (-20°C) 100% ethanol and precipitate the DNA by centrifuging at 13000rpm for 30 minutes.
8. Carefully remove the supernatant, ensuring not to disturb the pellet and then vacuum dry at ambient temperature, so as to remove all traces of ethanol.
9. Finally, resuspend the DNA pellet in either 10-20µl of deionised water (if the DNA is to be used immediately) or 10-20µl of TE buffer (pH 7.4) and store at -20°C.

2.2.5.2 DNA Extraction using the QIAamp® DNA Mini Kit for Formalin-Fixed Paraffin-Embedded Tissue for use in CGH

1. Microdissect 8-10 unstained 5µm sections and place in a 2ml microcentrifuge tube.
2. Add 1200µl xylene and vortex vigorously for at least 30 seconds.
3. Centrifuge at 13000rpm for 5 minutes at room temperature.
4. Carefully remove the supernatant, without disturbing the tissue pellet.
5. Add 1200µl of 100% ethanol and gently vortex.
6. Centrifuge at 13000rpm for 5 minutes at room temperature.
7. Carefully remove the supernatant, without disturbing the tissue pellet.
8. Repeat steps 5-7 once more.
9. Place the microcentrifuge tube onto a heating block set at 37°C, with the lid off for between 10-20 minutes, until all the ethanol has evaporated.
10. Re-suspend the tissue pellet in 180µl of Buffer ATL.

11. Add 20µl of proteinase K, gently vortex and incubate at 56°C until the tissue has been completely digested. Vortex occasionally during incubation to disperse the sample. (The tissue digestion usually takes 24 hours but the addition of a further 20µl of proteinase K after 24 hours and incubation for a further 24 hours will improve DNA yields).
12. Briefly centrifuge and add 200µl of Buffer AL. Gently vortex the microcentrifuge tube and place on a heating block set at 70°C for 10 minutes. (A white precipitate may form during this step, but it will dissolve during the incubation).
13. Add 200µl of 100% ethanol and gently vortex for 15 seconds. Briefly centrifuge to remove drops from the inside of the lid.
14. Transfer the contents of the microcentrifuge tube into a QIAamp spin column without wetting the rim. Close the cap and centrifuge at 8000rpm for 2 minutes. Place the spin column in a clean 2ml collection tube and discard the tube containing the filtrate. (Ensure that all of the solution has passed through the membrane of the spin column, if it has not, then centrifuge at 13000rpm for a further 2 minutes).
15. Carefully open the lid and add 500µl of Buffer AW1 without wetting the rim. Close the cap and centrifuge at 8000rpm for 2 minutes. Place the spin column in a clean 2ml collection tube and discard the tube containing the filtrate.
16. Carefully open the lid and add 500µl of Buffer AW2 without wetting the rim. Close the cap and centrifuge at 13000rpm for 5 minutes. Place the spin column in a clean 2ml collection tube and discard the tube containing the filtrate.
17. Place the spin column in a clean 2ml collection tube and centrifuge again at 13000rpm for 5 minutes, to ensure that there is no carry over of any Buffer AW2.
18. Place the spin column in a clean 2ml collection tube and discard the tube containing the filtrate. Carefully open the lid and add 50µl of Buffer AE. Close the lid and incubate at room temperature for at least 30 minutes. Centrifuge at 13000rpm for 5 minutes and transfer the filtrate to a clean 0.5 ml centrifuge tube.
19. Store the eluted DNA at –20°C until required. Determine the DNA yield using a spectrophotometer.

2.2.5.3 DNA Extraction using the QIAamp® DNA Mini Kit for Fresh Frozen Tissue for use in CGH

1. Retrieve the tissue sample from storage and place in a Petri dish.
2. Finely mince approximately 25mg of the tissue into small pieces and place in a 2ml microcentrifuge tube.
3. Add 180µl of Buffer ATL and gently vortex.
4. Follow steps 11-19 as for the DNA Extraction Protocol for Paraffin-Embedded Tissue (QIAamp® DNA Mini Kit).

2.2.5.4 DNA Extraction of Formalin-Fixed Paraffin-Embedded Tissue for use in PCR

Slide Preparation

Two 5µm sections are required. One is stained with H&E the other with 0.01% aqueous toludine blue. The H&E stained slide is used as a guide when dissecting the toludine blue stained slide. Wet the toludine blue slide before microdissection with digestion buffer, so that the microdissected cells are not blown away.

Tissue Digestion

1. Pipette 200µl of digestion buffer into a microcentrifuge tube (100µl for small samples).
2. Place the microdissected material into the microcentrifuge tube.
3. Add 10µl (5µl for small samples) proteinase K.
4. Incubate at 55°C overnight (after 1.5 to 3 hours, give the bottom of the tube a "flick" to re-suspend any settled material; **do not** vortex.
5. Centrifuge at 13000rpm for 10 minutes at room temperature.
6. Place on a heating block and boil for 10 minutes in order to de-activate the proteinase K.
7. Centrifuge again at 13000rpm for 10 minutes at room temperature.
8. Store at -20°C.

2.2.6 DNA Labelling Protocols for Comparative Genomic Hybridisation

2.2.6.1 Nick Translation Labelling of DNA

Nick translation is a process of incorporating a fluorochrome label to the test DNA. The process fragments large pieces of DNA into smaller pieces tagged at the 3' – end with either Spectrum Green or Spectrum Red dUTP, depending on which fluorochrome is used to label the control DNA. Test DNA would be labelled with Spectrum Green dUTP, when the control DNA is labelled with Spectrum Red dUTP, and vice-versa.

1. Place a clean 0.5ml microcentrifuge tube on ice and allow to cool.
2. The following constituents are added into the microcentrifuge tube in this order:

   (17.5 - χ)µl nuclease free water

   χµl for 1µg extracted genomic test DNA

   2.5µl 0.2mM Spectrum Green or Spectrum Red dUTP

   5µl 0.1mM dTTP

   10µl dNTP mix

   5µl 10 x nick translation buffer

   10µl nick translation enzyme

   ——

   50µl total volume

   χ = volume of extracted test DNA in solution which is equal to 1µg of test DNA

3. Vortex the microcentrifuge tube briefly and incubate at 15°C for 2-4hrs.
4. The reaction is stopped by heating the microcentrifuge tube on a heating block to 70°C for 10minutes.
5. Then either place the microcentrifuge tube on ice or store at –20°C until required.

Determining Nick Translated DNA Probe Size

Determining the probe size is essential, in order to ensure that the genomic test DNA has been effectively nick translated to the desired size of ~ 600-2000 bp. If the probe

size is either too large or too small then the hybridisation will either appear too dim or appear granular. The probe size is determined using 1% agarose gel electrophoresis with ethidium bromide staining.

2.2.6.2 Ulysis® ULS Labelling of DNA

1. Add 2µl of either rhodamine-ULS® or dGreen- ULS® to 1µg of the test DNA to label it with rhodamine or dGreen respectively into a 0.5ml microcentrifuge tube.
2. Adjust the volume with labelling solution to 20µl and vortex.
3. Incubate at 65°C on a heating block for 15 minutes.
4. Briefly centrifuge the tube to remove droplets from the lid.
5. Purify the labelled DNA using QIAquick™ PCR purification Kit spin columns.

2.2.6.2.1 QIAquick PCR Purification/Nucleotide Removal Protocol

1. Add 100µl of PB buffer to 20µl of the Ulysis labelled DNA and vortex briefly and pulse microcentrifuge. (Originally, the QIAquick nucleotide removal protocol was employed to purify the labelled DNA, which involved the addition of either 100µl or 200µl of PN buffer to 20µl of the Ulysis labelled DNA. However, it was found that more efficient CGH hybridisations were obtained using the PCR purification protocol).
2. Transfer the solution into a QIAquick spin column and centrifuge at 13000rpm for 1 minute.
3. Discard the filtrate and add 750µl of PE buffer into the spin column. Centrifuge at 13000rpm for 1 minute and discard the filtrate. Repeat the centrifugation at 13000rpm for a further 1 minute and ensure that the membrane in the spin column appears dry.
4. Place the spin column into a clean 2ml microcentrifuge tube and place 30µl of EB buffer onto the membrane of the spin column.
5. Leave at room temperature for at least 30 minutes and the centrifuge at 13000rpm for 5 minutes. Transfer the eluted filtrate into a clean 0.5ml microcentrifuge tube.
6. Store the eluted labelled DNA at –20°C until required.

2.2.7 Comparative Genomic Hybridisation

Day 1

Preparation of Denaturation solution

1. Freshly prepare the denaturation solution (containing 49ml formamide, 7ml 20xSSC and 14ml deionised water and adjusted to pH 7.0) and heat in a water bath to 73±1°C, in a fume cupboard.

Preparing the Probe Mix

1. Combine the following in a 1.5ml microcentrifuge tube:
   10µl (200ng) labelled test DNA
   1µl (100ng) labelled total genomic reference DNA
   10µl (10µg) Human Cot-1 DNA
2. Add 2.1µl (0.1 volume) of 3M sodium acetate to the mixture followed by 52.5µl (2.5 volumes) of 100% ethanol, in order to precipitate the DNA. The sodium acetate acts as a carrier. Then place the microcentrifuge tube in a -70°C freezer for 15-30 minutes.
3. Microfuge the microcentrifuge tube at 12,000rpm for 30 minutes in order to pellet the DNA.
4. Carefully remove the supernatant so as not to disturb the pellet and then vacuum dry in a vacuum centrifuge at room temperature so as to remove all traces of ethanol.
5. Re-suspend the dried DNA pellet in 3µl of purified water and 7µl of CGH hybridisation buffer.
6. Then denature the DNA mixture by heating in a water bath at 73°C for exactly 5 minutes.

Hybridising the Probe DNA to the Target Metaphase

1. Mark the hybridisation areas on the target metaphase slides using a diamond tipped scribe.
2. Then immerse the slides in the denaturation solution for exactly 5 minutes.

3. Having been denatured, dehydrate the slides at room temperature in an ethanol series (70%, 85% and 100%) for 1 minute each, before being left to air-dry at room temperature.
4. Apply 10µl of the denatured DNA mixture to the marked hybridisation area.
5. Then apply a cover slip immediately to each hybridisation area and seal with rubber solution.
6. Incubate the slides in a humid chamber at 37°C for between 48-72 hours.

Day 3

Preparation of Solutions

1. Prepare a fresh stringency wash solution (containing 0.4xSSC/0.3% NP-40 and adjusted to pH 7.0-7.5) was and heat to 74±1°C in a water bath placed in a fume cupboard.
2. Freshly prepare a second stringency wash solution (containing 2xSSC/0.1% NP-40 and adjusted to pH 7.0-7.5) and place at room temperature in a fume cupboard.

Post-Hybridisation Stringency Washes

1. Remove the slides from the humid chamber and remove the cover slips by removing the rubber sealant and tapping vertically. Immerse the slides into the 0.4xSSC/0.3% NP-40 stringency solution, agitate gently for 1-3 seconds and then allow to stand in the stringency solution for 2 minutes.
2. Remove the slides and immediately immerse them into the 2xSSC/0.1% NP-40 stringency solution, agitate gently for 1-3 seconds and allow to stand for 1 minute.
3. Then allow the slides were to air-dry in darkness.

Detection and Visualising the Hybridisation

1. Once the slides are dry apply 10µl of DAPI II counterstain to each hybridisation area and apply a cover slip. Use nail varnish to seal edges of the cover slip. The slides can then be stored in lightproof slide boxes at 4°C, until required for viewing.
2. Visualise the hybridisation using a CCD camera and image enhancement software, with appropriate filters for the fluorochromes and DAPI used.

2.2.8 Polymerase Chain Reaction

2.2.8.1 PCR using 0.5ml Microcentrifuge tubes and the Biometra UNO-Thermoblock for use on Agarose or Polyacrylamide Gels

1. Place all 0.5ml microcentrifuge tubes to be used for the PCR reaction on ice and allow to cool.
2. Add the following constituents into a 2ml microcentrifuge tube, also placed on ice, to produce a master mix in this exact order:

19.1µl nuclease free water	Each constituent was multiplied by (n+2) before being added into the master mix. Where n = number of tumour DNA samples and 2 extra volumes to allow for one negative control and any mis-pipetting
2.5µl 10xNH_4 buffer	
0.75µl $MgCl_2$	
0.5µl dNTP mix	
0.5µl forward primer (100pmol/µl)	
0.5µl reverse primer (100pmol/µl)	
0.15µl *Taq* polymerase	
24µl total volume per reaction	

3. Place an aliquot of 24µl from the master mix into each 0.5ml microcentrifuge tube.
4. Pipette 1µl of the tumour DNA to be amplified into the microcentrifuge tube.
5. Add a drop of mineral oil to each microcentrifuge tube to prevent evaporation of the PCR reaction mixture during thermo-cycling.
6. Finally, pulse each microcentrifuge tube briefly in the microfuge and place on the PCR block for thermo cycling. All samples underwent an initial denaturation step at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, an annealing step at the optimal temperature as shown in appendix III for 30 seconds and extension at 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes.

2.2.8.2 PCR using the Abgene Thermo-Fast 96 Low Profile Plate and the Primus 96 Plus thermo cycler for use on LongRanger Sequencing Gels

1. Place a 2ml microcentrifuge tube on ice and allow to cool.
2. Add the following constituents into the microcentrifuge tube to produce a master mix in this exact order:

7.05µl nuclease free water 1µl 10xNH_4 buffer 0.3µl $MgCl_2$ 0.2µl dNTP mix 0.15µl unlabelled forward primer (100pmol/µl) 0.15µl unlabelled reverse primer (100pmol/µl) 0.15µl *Taq* polymerase	Each constituent was multiplied by (n+2) before being added into the master mix. Where n = number of tumour DNA samples and 2 extra volumes to allow for one negative control and any mis-pipetting
9µl total volume per reaction	

3. To the final master mix add 3µl of the IRD labelled forward primer.
4. Place an aliquot of 9µl from the master mix into each well of the 96 well plate.
5. Pipette 1µl of the tumour DNA to be amplified into the well.
6. Add a drop of Chill-out 14 liquid wax (purchased from MJ Research Inc.) into each well to prevent evaporation of the PCR reaction mixture during thermo-cycling.
7. Place the 96-well plate immediately onto the PCR block for thermo cycling. All samples underwent an initial denaturation step at 94°C for 3 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, an annealing step at the optimal temperature as shown in appendix III for 30 seconds and extension at 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes.

2.2.9 Agarose Gel Electrophoresis

1. The agarose gel is prepared by mixing 1g agarose to 100ml 1xTBE buffer, and heating the solution in a microwave until the agarose is in solution. Take care not to super heat the solution.
2. Allow the agarose solution to cool to approximately 45°C and add 2.5µl of ethidium bromide.
3. Pour the agarose solution into a mini-gel fitted with combs and allow to set.
4. Pour TE buffer over the gel so as to cover it completely by approximately 1mm.
5. In a 0.5ml microcentrifuge tube, mix 9µl of the reaction mixture with 1µl of PCR loading buffer and pipette the resultant solution into one of the wells.
6. Electrophorese the reaction mixture with a 1kb DNA marker placed in another well, in order to determine the size of the DNA product.
7. Electrophorese the gel at 10 V/cm until the PCR loading buffer is approximately 2-3cm from the edge of the gel. Visualise the gel with UV light, using all appropriate precautions.

2.2.9.1 Ethidium Bromide Staining

Ethidium bromide is a potent carcinogen and all precautions must be used in handling and disposing of this compound. Ethidium bromide may either be incorporated directly into a gel or may be added to a volume of electrophoresis buffer placed in a plastic tray and the gel immersed into the buffer for a variable period of time so as to stain the DNA. This immersion technique may be used to stain either agarose or polyacrylamide gels.

2.2.10 Polyacrylamide Gel Electrophoresis

1. Prior to pouring the gel, ensure that the glass plates are perfectly clean by cleaning with methanol and wiping dry with tissue paper.

2. Place the spacers between the glass plates and clamp the plates onto a casting tray, ensuring that there is a tight seal between the glass plates and the casting tray, in order to prevent leakage of the polyacrylamide gel solution.
3. Pour the gel solution quickly in between the glass plate and carefully insert the comb, ensuring that no air bubbles are trapped between the comb and gel.
4. Allow the gel to set for approximately 30 minutes.
5. Once the gel is set, the comb is carefully removed and the wells washed with 1xTBE buffer using a syringe, in order to remove any polyacrylamide gel that has not yet set.
6. The gel and glass plates are removed from the casting tray and clamped into the gel running tank and the tank filled with 1xTBE buffer.
7. Pre-electrophorese the gel at 175 volts for 30 minutes, so as to warm the gel, so that the PCR products electrophorese uniformly along the gel.
8. 5µl of PCR loading buffer is added to each sample.
9. Using a Hamilton syringe, load 20µl of each PCR sample into each well, ensuring that the syringe is washed out prior to loading the next sample.
10. The gel is then electrophoresed at 175 volts for approximately 16 hours.
11. Once the loading dye has reached the end of the gel, the power is switched off and the glass plates and gel removed from the tank. Carefully separate the glass plates and slide the gel into a clean plastic tray containing 1xTBE buffer. Cut off the top right corner of the gel for orientation, prior to silver staining.

2.2.10.1 Silver Staining

1. Remove the polyacrylamide gel from the tray containing the 1xTBE buffer and carefully place into a clean plastic tray containing solution 1 (initial fix solution) and leave at room temperature for 10 minutes.
2. Remove the gel and place in a clean tray containing solution 2 (silver nitrate solution) for approximately 15 minutes.
3. Remove the gel and place in a clean tray containing deionised water and rinse once.
4. Remove the gel and place in a clean tray containing solution 3 (developer) and gently shake the tray until the bands appear on the gel and have reached the desired

intensity. (Over-development will result in a dark brown background with low contrast bands rather than a grey background with sharp bands).

5. Remove the gel and place in a clean tray containing solution 4 (final fix/stop solution) for 5 minutes.
6. After the final gel fix the gel is removed and sealed in a plastic sleeve.

2.2.11 LongRanger Gel Electrophoresis

1. Prior to pouring the gel, ensure that the glass plates are perfectly clean by cleaning with methanol and wiping dry with tissue paper.
2. Place the spacers between the glass plates and clamp the plates onto a casting tray, ensuring that there is a tight seal between the glass plates and the casting tray, in order to prevent leakage of the polyacrylamide gel solution.
3. Pour the gel solution quickly in between the glass plate and carefully insert the comb, ensuring that no air bubbles are trapped between the comb and gel.
4. Allow the gel to set for approximately 30 minutes.
5. Once the gel is set, the comb is carefully removed and the wells washed with 1xTBE buffer using a syringe, in order to remove any gel that has not yet set.
6. The gel and glass plates are removed from the casting tray and clamped into the LiCor 4200 LongRead automated sequencer and the tank filled with 1xTBE buffer.
7. Pre-electrophorese the gel at 800 volts for 20 minutes, so as to warm the gel to 50°C.
8. Prior to loading the gel, add 4µl of stop solution to each well and denature at 75°C for 5 minutes.
9. Using a Hamilton syringe, load 1µl of each PCR sample into each well, ensuring that the syringe is washed out prior to loading the next sample.
10. The gel is then electrophoresed at 800 volts.
11. The PCR products will be visualised automatically with the LiCor Base ImagIR software on a PC computer.

2.3 LIST OF SUPPLIERS

Advanced Biotechnologies Ltd.

Longmead Business Centre, Blenheim Road, Epsom, KT19 9QQ.

Ansell Medical

Ansell House, 119 Ewell Road, Surbiton, KT6 6AY.

Anachem Ltd.

Anachem House, Charles Street, Luton, LU2 OEB.

Appligene-Oncor

Parc d'Innovation, B.P.72, F-67402, Illkirch, France.

Becton Dickinson Ltd.

Between Towns Road, Cowley, Oxford, OX4 3LY.

BDH Laboratory Suppliers

Merck Ltd, Hunter Boulevard, Lutterworth, LE17 4XN.

Bibby Sterilin Ltd.

Tilling Drive, Stone, Staffordshire, ST15 0SA.

Bioline Ltd.

16 The Edge Business Centre, Humber Road, London, NW2 6EW.

Bio Whittaker Molecular Applications Inc.

191 Thomston St, Rockland, Maine, USA.

Boehringer Mannheim

Boehringer Mannheim House, Bell Lane, Lewes, BNY 1LG.

Cambio

34 Millington Road, Cambridge, CB3 9HP.

Citifluor

UKC, Chemical Laboratories, Canterbury, CT2 7NH.

Corning Costar UK Ltd.

1 The Valley Centre, Gordon Rd, High Wycombe, HB13 6EQ.

Flowgen

Novara House, Excelsior Road, Ashby de la Zouch, LE65 1NG.

Gibco Life Technologies Ltd.

PO Box 35, Trident House, Renfrew Rd, Paisley PA3 4EF.

Helena Biosciences

Sunderland Enterprise Park, Sunderland SR5 3XB.

John Poulton Ltd.

75-93 Tanner Street, Barking, IG11 8QD.

Kreatech Diagnostics

Vlierweg 20, 1032 LG Amsterdam, The Netherlands.

LI-COR

4308 Progressive Avenue, Lincoln, Nebraska, USA.

Life Science Research

Bio-Rad House, Mayland Avenue, Hemel Hempstead, HP2 7TD.

MJ Research Inc.

590 Lincoln Street, Waltham, Massachusetts, USA.

MWG-Biotech (UK) Ltd.

Mill Court, Featherstone Road, Milton Keynes, MK12 5RD.

Oxoid Ltd.

Wade Rd, Basingstoke, RG24 OPW.

Pall Gelman Sciences

Blackmills Business Park, Caswell Rd, Northampton, NN4 7EZ.

Pearce Laboratories

Lotherton Way, Garforth, Leeds, LS25 2JY.

Perkin-Elmer Ltd.

Post Office Lane, Beaconsfield, HP9 1QA.

Phillips

Phillips Rubber, Dantzic St, Manchester, M4 4JH.

Pierce

Rockford, Illinois 61105, USA.

Purite Ltd.

Bandet Way, Oxon, OX9 3SJ.

Qiagen Ltd.

Boundary Court, Gatwick Road, Crawley, RH10 2AX.

Raymond Lamb

6 Sunbeam Rd, London, NW10 6JL.

Sanyo Gallenkamp Plc.

Monarch Way, Becton Park, Loughborough, LE11 5XG.

Sartorius Ltd.

Longmead, Blenheim Rd, Epsom, KT19 9QN.

Sarstedt Ltd.

68 Boston Road, Beumont Leys, Leicester, LE4 IAW.

Savant Instruments Inc.

Farmingdale, New York, USA.

Scientific Laboratory Supplies Ltd.

Wilford Industrial Estate, Nottingham, NG11 7EP.

Sigma-Aldrich Co Ltd.

The Old Brickyard, New Road, Gillingham, SP8 WXT.

Statebourne Cryogenics

18 Parsons Road, Washington, NE37 1EZ.

Swann Morton Ltd.

Owlerton Green, Sheffield, S6 2BJ.

Techne Ltd.

Duxford, Cambridge, CB2 4PZ.

United States Biological

Swampscott, Massacheusetts, USA

UVP Inc.

San Gabriel, California, USA.

Vacuubrand GMBH + Co.

Alfred-Zippe Strasse 4, Wertheim, Germany.

Vector Laboratories

30 Ingold Rd, Burlingame, CA 94010, USA.

Vysis UK, Ltd.

Rosedale House, Rosedale Road, Richmond, TW9 2SZ.

Walker Safety Cabinets Ltd.

3 Pyegrove, Glossop, SK13 8RA.

CHAPTER 3 – FLUORESCENCE *IN SITU* HYBRIDISATION ANALYSIS OF CHROMOSOME 3 AND 8 USING ALPHA SATELLITE PROBES, ON PRIMARY UVEAL MELANOMAS AND PRIMARY COLORECTAL CANCERS AND THEIR CORRESPONDING LIVER METASTASES

CONTENTS

CHAPTER 3

3.1 INTRODUCTION

Uveal melanomas are the commonest malignancy of the eye with an annual incidence of 5-7 cases per million population (Egan et al., 1988; Jensen, 1982). They account for approximately 80% of all non-cutaneous melanomas (Scotto et al., 1976), and are classified as either posterior when located in the ciliary body or choroid or anterior when located in the iris (Char, 1997). Uveal melanomas invariably metastasise to the liver, with the incidence of metastases peaking usually at 2 years after primary enucleation (Diener-West et al., 1992). Thus prognosis is generally poor, with a 5-year tumour-related mortality of between 16-53% (Diener-West et al., 1992).

A number of prognostic indicators have been established for uveal melanomas, which include: a maximum tumour diameter of greater than 15mm; the presence of epithelioid cells; extra-scleral extension; tumour location in the ciliary body (Coleman et al., 1993; McLean et al., 1982) and possibly vascular patterns (Folberg et al., 1993; Rummelt et al., 1995). More recently, cytogenetic studies have shown that a loss of chromosome 3 is a significant prognostic indicator of early relapse and poor survival (Prescher et al., 1996; Sisley et al., 1997). Several groups have also shown that a gain of chromosome 8, principally in the form of an isochromosome 8q, is also associated with a worse prognosis (Dahlenfors et al., 1993; Horsman and White, 1993; Horsthemke et al., 1992; Prescher et al., 1992, 1996; Singh et al., 1994; Sisley et al., 1990, 1992). Other groups have found that the presence of chromosome 6 abnormalities is predictive of a good prognosis even when associated with abnormalities of chromosomes 3 and 8 (White et al., 1998).

However, cytogenetic analysis requires the short-term culture of tumours followed by producing good quality metaphase spreads. This process is not only time consuming but could also induce in-vitro genetic changes (Fox et al., 1995). This presence of aneuploidy has also been studied using flow cytometry and static image analysis on both fresh and archival material, with conflicting results (Mooy, 1998), which may in part be as a result of limitations of the various techniques (Koss et al., 1989; Shankey et al., 1993). The development of fluorescence *in situ* hybridisation (FISH) allows the interphase cytogenetic analysis of either fresh or archival tumour tissue not only quickly,

but also accurately. It also allows for the examination of multiple genetic changes, by the use of probes labelled with spectrally distinct fluorescent dyes (Pinkel et al., 1988; Matsumura et al., 1992; Kallioniemi et al., 1996).

3.1.1 Fluorescence *In Situ* Hybridisation (FISH)

In situ hybridisation is a method of localising either mRNA within the cytoplasm or DNA within the nucleus, by hybridising a strand of nucleotide probe to the complimentary target sequence of interest. *In situ* hybridisation techniques were developed in the late 60's based mainly on work undertaken by Gall and Pardue (1969). However, it was only in the 1980's that scientists started using the procedure to localise single copy base sequences cloned from individual genes (Lichter and Cremer, 1992). Formerly radioactive isotopes (^{32}P, ^{35}S or ^{3}H) were used as markers, but the recent development of fluorescent dyes (fluorochromes) has not only improved the safety aspect, but also the resolution of the procedure (McNeil et al, 1991).

FISH not only allows the examination of cells in the interphase stage - so called interphase cytogenetics (Cremer et al., 1986), it also allows for the examination of multiple genetic changes, by the use of probes labelled with spectrally distinct fluorescent dyes. The resulting examination of the cells can be performed by a charge-coupled device camera, each image being recorded with a specific filter for each fluorochrome (Morrison, 1993).

In situ hybridisation is based on the site specific annealing of single stranded DNA molecules (probes) to denatured complementary DNA sequences (targets) in the cells of the tissue under study. Probes can be as small as 20-40 base pairs (bp), and up to 1000 bp. There are essentially three types of DNA probes used; oligonucleotide probes (Lengauer et al., 1994a; Meyne and Moyzis, 1994), single-stranded DNA probes (Meyne and Goodwin, 1994) and double stranded DNA probes (Lengauer et al., 1994b). The DNA probe can consist of base sequences specific for the whole chromosome, chromosomal region or specific loci. Chromosome specific repetitive probes target the tandemly repeated chromosome specific alpha satellite DNA, which is present at or near the centromeres of chromosomes (Hopman et al., 1997). These probes are useful in determining the copy number of various chromosomes and can easily be applied to interphase nuclei. The centromeric regions tend to be highly condensed and so a probe

hybridised to these regions, appears as a small fluorescent dot. By using a number of probes each labelled with a spectrally distinct fluorochrome, it is possible to simultaneously detect a number of chromosomal aberrations within each hybridisation. However, the use of these alpha satellite centromeric probes is limited to the assessment of copy number changes (Fox et al., 1995).

Target cells for FISH analysis can be obtained from a number of sources, including fine needle aspirates, touch or imprint preparations or histological sections from either fresh or paraffin-embedded. Cells may also be disaggregated from either archival fresh frozen or formalin-fixed tissue samples (Matsumura et al., 1992; Schofield and Fletcher, 1992). The disadvantage of using isolated cells from disaggregated tissues is that it can produce a bias in the sample of cells, as examination may only be undertaken of those cells that have been easily dislodged. Another disadvantage is the loss of morphological correlation from the tissue sample. Morphological correlation can be achieved by using tissue sections. However, thin sections (4-6 μm) result in the cutting of cells and thus producing partial nuclei, which can lead to an underestimation of polysomy (Hopman et al., 1991; Weremowickz et al., 1994; Brothman et al., 1994). The use of thick sections (40-60 μm) overcomes this, however this can lead to difficulties in the interpretation of cell superimposition (Southern and Herrington, 1996). A suitable compromise is to use thin sections together with the analysis of inflammatory and stromal cells, acting as internal controls (Kim et al., 1993). A recent technique of using laser-scanning confocal microscopy on thick (20μm) sections may solve this problem. Confocal microscopy allows different planes within the section to be observed, thus allowing the presence of hybridisation signals to be related to the histological architecture (Kozubek et al., 2001).

The use of a control specimen with normal nuclei, preferably from the same tissue of origin is essential in order to recognise hybridisation efficiency problems and possible signal cross-reactions between different probes (Kallioniemi et al., 1996). The interpretation of FISH relies on the degree to which the apparent hybridisation signals accurately correspond to the specific target site for the probes. The number of apparent signals will be smaller than the true number of target sites if target DNA is lost during preparation or, remains inaccessible to the probe detection reagents or, if signals overlap. Extra signals can arise from the fragmentation of target DNA or from the non specific binding of the detecting reagents (Devilee et al., 1988; Matsumura et al., 1992).

There are shortcomings to FISH technology. Firstly, there needs to be the development of a specific probe for each genetic defect, by contrast, conventional karyotypic analysis looks at the entire genome. Secondly, FISH has a minimum size of visualisation, requiring a probe size of around 40-200 kb of genomic DNA, and so can miss very small genetic alterations (Kallioniemi et al., 1996). For aberrations that are too small to be detected by conventional FISH (~ 10 kb or less), other techniques may be used, such as high-resolution FISH mapping (Raap et al., 1996), primed *in situ* hybridization (Koch et al., 1996) and ligase-mediated chain reaction (Landegren et al., 1996).

FISH has already proved useful in clinical practice and is routinely used in the diagnosis of trisomy 21 (Klever et al., 1992; Klinger et al., 1992; Ward et al., 1993). FISH may also prove useful in the diagnosis of certain constitutional microdeletion syndromes (Ledbetter, 1992), the diagnosis of carrier status in X-chromosomal recessively inherited diseases associated with deletions, such as Duchenne muscular dystrophy (Ried et al., 1990), and the identification of deleted tumour suppressor genes in certain types of cancers (Stilgenbauer et al., 1993)

3.1.2 Aims of this Study

The first part of this study was to perform a retrospective analysis of uveal melanoma tumours using centromeric probes for chromosomes 3 and 8 and to correlate the analysis with various clinicopathological characteristics and patient survival, in an attempt to define any prognostic factors.

The study was to be repeated using the same centromeric probes to analyse primary colorectal cancers and their corresponding liver metastases, in order to compare the findings between colorectal cancers and uveal melanomas, and to assess whether abnormalities in the copy numbers of chromosomes 3 and 8 are associated with the development of liver metastases, in the case of colorectal cancers.

3.2 RESULTS

A series of 33 tumour samples from 33 patients treated between 1987 and 1995 were analysed using FISH. All tumours were categorized histopathologically according to the AFIP system of classification for uveal melanomas (Spencer, 1986). Tumour diameter was assessed pre-operatively by B scan ultrasonography and subsequent clinical follow-up was conducted at the Department of Ophthalmology and Orthoptics, University of Sheffield.

Six normal blood controls were analysed using FISH to assess the sensitivity of the centromeric probes, 300 cells being counted for each sample. For each uveal melanoma tumour sample, again 300 cells were counted (table 3.3). Cells were excluded from analysis if they were clumped together or if they appeared to have been cut.

Patient outcomes were divided into two groups: 1) absence of genetic abnormalities (no genetic imbalance) and 2) presence of genetic abnormalities (genetic imbalance). No genetic imbalance was defined as a cell having the normal complement of two target hybridisation signals for each chromosome (figures 3.4 and 3.5), and the presence of genetic imbalance was defined as any combination of target hybridisation signals other than two for chromosome 3 and two for chromosome 8 (figures 3.6 and 3.7). Genetic imbalance was said to be present if the number of abnormal cells exceeded 70% of the total population observed. A value of 70% was taken, following the manufacturers recommendations for probe sensitivity, which indicated that approximately 70% of normal cells analysed would show constitutional hybridisation signals for either centromeric probe. Therefore if the total number of cells analysed showed a normal complement in more than 70% of cells analysed, the tumour was said to show no genetic imbalance. FISH was performed on six normal blood controls to confirm this sensitivity value.

Statistical Analysis

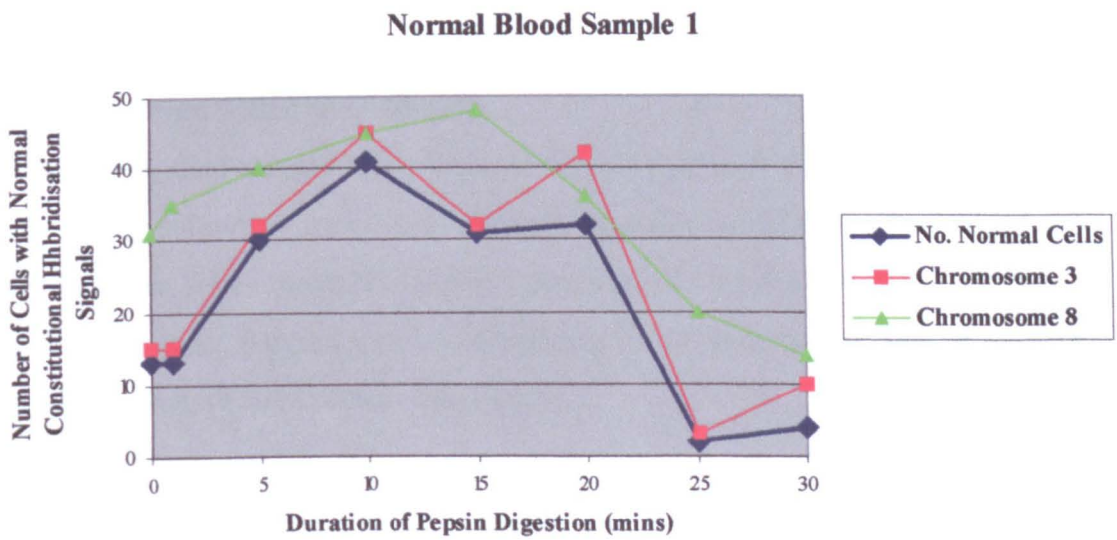
Independent samples t-test, chi-square test or Fisher's exact test were used to assess any differences in sex, age, tumour cell types, location and mean tumour diameter between the two groups. The log-rank test was used to compare survival, which was represented by Kaplan-Meier survival curves. Statistical analysis was performed using

SPSS statistical software package on a PC computer, with a p value for significance set at <0.05.

3.2.1 Optimising Conditions for FISH on Normal Blood Controls

A standard FISH protocol was initially used (see Materials and Methods section 2.2.4 Fluorescent *In Situ* Hybridisation), and optimum pepsin digestion times were evaluated on several normal blood samples. Figure 3.1 show the results of pepsin digestion of a normal blood sample (Normal Blood Sample 1), ranging from 0 to 30 minutes. Optimum hybridisation for both probes i.e. two constitutional hybridisation signals for either probe was with 10 minutes pepsin digestion, where the number of normal cells with constitutional hybridisation signals was 82%. Two further blood samples were also analysed to confirm this with the duration of pepsin digestion ranging from 5 to 15 minutes and both samples confirmed optimal hybridisation efficiency at 10 minutes pepsin digestion. Normal constitutional hybridisation signals were observed in 91% and 90% of cells in normal blood samples 2 and 3 respectively.

Figure 3.1 FISH results for Normal Blood Sample 1 with pepsin digestion for various times. Fifty cells were counted at each pepsin digestion time.



A total number of six normal blood sample controls were analysed using FISH with chromosomes 3 and 8 centromeric probes. Three hundred cells were counted for each

sample with the average number of cells with a normal number of hybridisation signals being 96.72% (range 94.33% - 97.67%), and a median of 97%. Thus, the sensitivity of the two probes in combination concurred with the value supplied by Appligene Oncor, which stated that a normal sample of cells would show two hits for each chromosome in 70% of cells analysed.

3.2.2 Optimising Conditions for FISH on Formalin-Fixed Paraffin-Embedded Tumours

Preservation of tumour material initially fixed in formalin and then embedded in paraffin wax, allows for the convenient storage of tumour tissue over many years. Tissue architecture and morphology are both conserved in these paraffin blocks, allowing retrospective analysis to be undertaken. This allows a collection of tumour specimens to be built up which can be analysed a number of years later. These retrospective studies have the advantage of facilitating the correlation of data acquired to clinical progression and outcome determined by follow-up undertaken over many years.

Tissue preservation in paraffin wax is not without its problems when attempting to use novel techniques to analyse the tissue. A number of protocols exist to allow the analysis of paraffin-embedded tissue material. However, difficulties arise when chemicals and procedures for tissue fixation are not standardised either between Institutions or even within an Institution.

In this study, dual colour FISH analysis was attempted on eight tissue samples of paired primary colorectal cancer and metastatic formalin-fixed paraffin-embedded tissue. However, following hybridisation a great deal of auto-fluorescence was observed, thus concealing the number of true specific hybridisation signals. A number of factors could have been responsible for this:

1. Operator error.
2. Problems with the reagents and solutions.
3. Inefficient removal of paraffin from the tumour sample.
4. Insufficient permeabilization of tumour cells by pepsin.

Operator error and substandard solutions could be discounted as all hybridisations were performed with a normal blood sample to act as a control. Normal blood samples consistently showed bright, clear constitutional hybridisation signals for both chromosomes 3 and 8.

To improve the removal of paraffin from the tumour samples, extra washes of xylene and ethanol were introduced. However, this had very little effect on reducing auto-fluorescence and improving hybridisation signals. It was concluded that auto-fluorescence was an inherent problem with using paraffin-embedded material. An attempt was made to improve the intensity of the hybridisation signal by increasing the permeablization of the nuclei to the centromeric probes. This was initially performed by increasing the duration of pepsin digestion. Care would need to be taken during this procedure, as over-digestion by pepsin would destroy cellular and nuclear morphology, resulting in a loss of true target hybridisation signals (Köpf et al., 1996). Several slides were prepared from two formalin-fixed paraffin-embedded tissue samples of normal colon tissue and digested in pepsin for various durations, ranging from 10-60 minutes (figures 3.2 and 3.3).

Figure 3.2 FISH results for Normal Colon Tissue Sample 1 with pepsin digestion for various times. Fifty cells were counted at each pepsin digestion time.

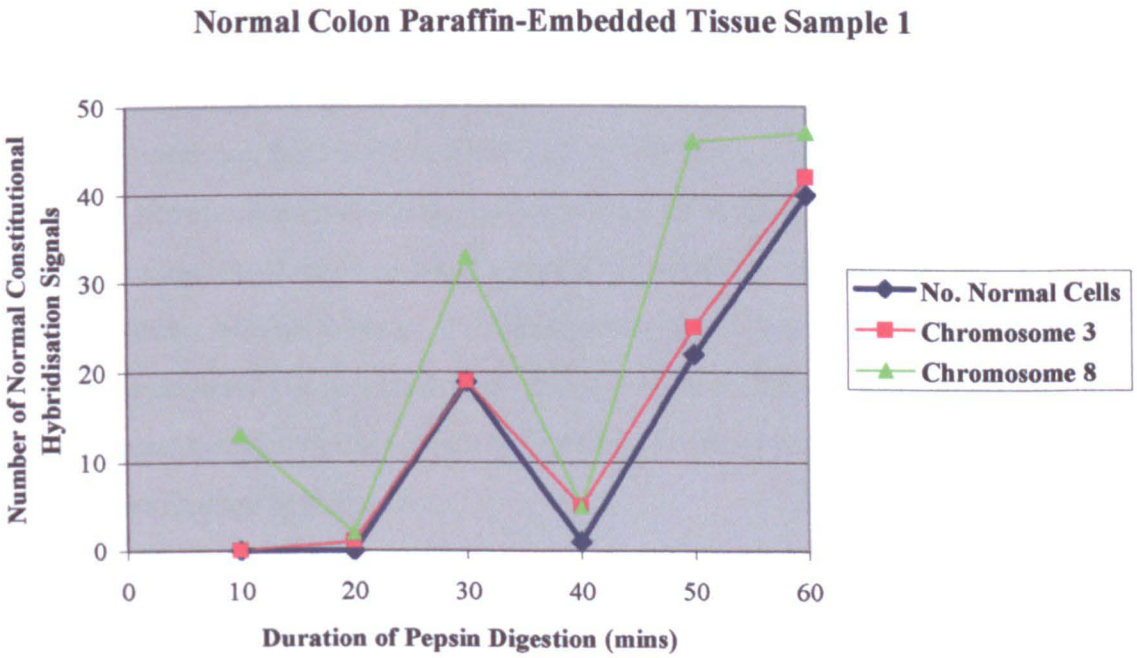
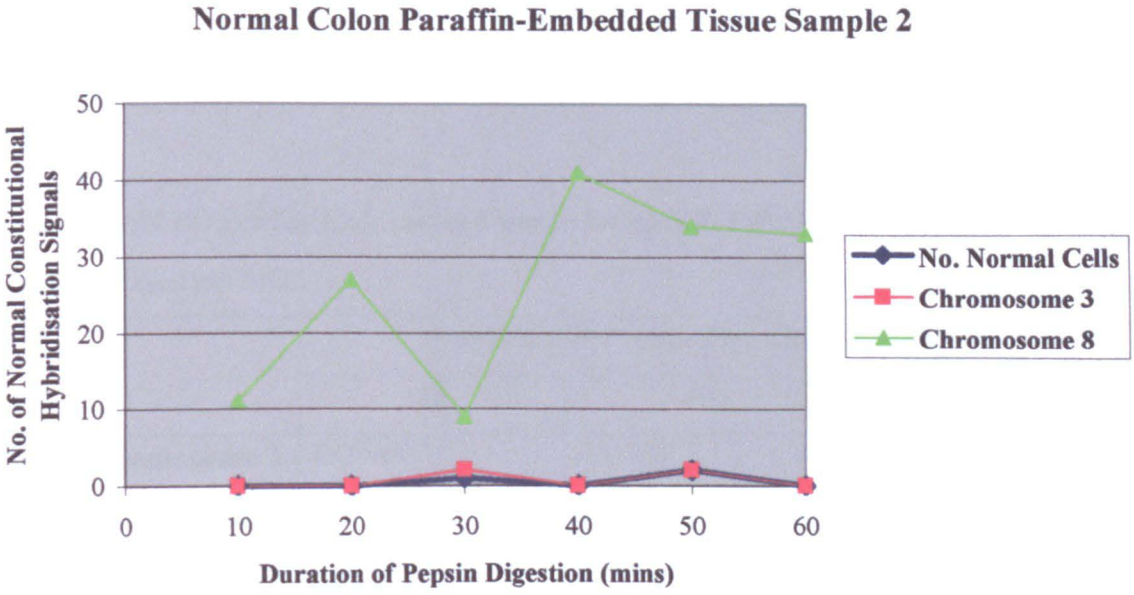


Figure 3.3 FISH results for Normal Colon Tissue Sample 2 with pepsin digestion for various times. Fifty cells were counted at each pepsin digestion time.



One would expect to observe two constitutional hybridisation signals for both the chromosome 3 and chromosome 8 centromeric probes, however, the number of normal hybridisation signals recorded were maximal at 60 minutes for sample 1 (80% of cells analysed) but could not be evaluated for sample 2 as there appeared to be poor hybridisation of the chromosome 3 centromeric probe, even though optimum hybridisation of the chromosome 8 centromeric probe appeared to be at 40 minutes. Although not investigated, the better hybridisation efficiency of the chromosome 8 centromeric probe may have been as a result of its smaller size, thus being able to penetrate the permeablised nucleus more readily than its larger counterpart. Another interesting finding was the two peaks of optimal hybridisation with the chromosome 8 centromeric probe. Similar findings are sometimes seen with chromosome banding, although the reason for this has never been fully elucidated. Thus, it was concluded that different tumour samples would require different durations of pepsin digestion to facilitate hybridisation by the probes.

Two paraffin-embedded colorectal cancer tissue samples were analysed using FISH for abnormalities in copy numbers of chromosomes 3 and 8. However, results were inconsistent at the various pepsin digestion times, ranging from 30-50 minutes. For colorectal cancer sample 1, the majority of cells showed no hybridisation signals for

either probe at any duration of pepsin digestion (table 3.1). While for colorectal cancer sample 2 the majority of cells at 30 and 40 minutes pepsin digestion showed 3 hybridisation signal for chromosome 8 and none for chromosome 3, while at 50 minutes pepsin digestion the majority of cells showed no hybridisation signal for either probe (table 3.2).

Table 3.1 FISH results for Colorectal Cancer Sample 1. Fifty cells were counted at each pepsin digestion time.

	Duration of Pepsin digestion (minutes)		
	30	**40**	**50**
Results (Chromosome 3 hybridisation signals: Chromosome 8 hybridisation signals)	0:0=49 0:1=1	0:0=31 2:0=11 1:1=10 0:1=7 0:2=3 1:2=2 2:2=2	0:0=25 1:1=10 2:2=6 0:2=3 1:2=2 0:1=2 2:1=2

Table 3.2 FISH results for Colorectal Cancer Sample 2. Fifty cells were counted at each pepsin digestion time.

	Duration of Pepsin digestion (minutes)		
	30	**40**	**50**
Results (Chromosome 3 hybridisation signals: Chromosome 8 hybridisation signals)	0:3=19 1:3=8 0:2=8 2:3=4 1:2=3 3:3=2 0:4=2 2:4=2 2:2=2	0:3=18 1:3=15 2:2=5 1:2=4 0:2=3 2:3=2 1:4=2 0:4=1	0:0=26 0:3=7 0:2=7 1:2=4 1:3=2 0:1=2 1:1=1 3:2=1

Therefore, in order to ascertain the true number of hybridisation signals for either probe for each paraffin-embedded primary and metastatic tumour analysed, a normal tissue control would need to be disaggregated from an adjacent block of paraffin-embedded tissue, so as to control for tissue fixation and processing differences.

A further attempt to improve permeablization of the tumour sample was performed by increasing the duration of protease digestion in the tumour disaggregation procedure. Tumour sample were digested with protease for between 10-60 minutes. The optimal duration of digestion was determined by visualising the disaggregated tumour samples under a microscope, to ensure that cellular morphology remained intact. However, even this procedure failed to improve the intensity of the hybridisation signals.

It was concluded that, as the duration of digestion with either pepsin or protease could not be standardised for all samples and that hybridisation would have to be repeated with normal tissue controls for each tumour analysed, the total number of hybridisations required for all tumour samples would not be feasible in terms of time or reagents.

3.2.3 Optimising Conditions for FISH on Fresh-Frozen Tumour Samples

FISH analysis was successfully completed on 33 fresh-frozen uveal melanoma tumour samples that had been embedded in optimal cutting temperature (OCT) compound and subsequently stored at –20°C. FISH performed on the original disaggregated tissue samples was extremely poor, with very few intact cells and abundant cellular debris. This was thought to be attributable to poor disaggregation techniques and attempts were employed to rectify this. In these early samples, tissue disaggregation employed a combination of coarse tumour mincing using two scalpel blades followed by protease digestion. Omitting the protease digestion step and instead relying solely on mechanical disaggregation improved disaggregated tumour slide preparations. To prevent large clumps of disaggregated tissue from being dropped onto slides during slide preparation, tumour samples were freshly fixed using methanol: glacial acetic acid fixative and then left to stand for several minutes in order for the large clumps of tissue to settle and the upper clearer solution used to prepare slides. Thus, with slight adjustments in the tissue disaggregation protocol and in the preparation of slides, high quality hybridisations were obtained.

3.2.4 FISH Analysis of Fresh-frozen Uveal Melanoma Tumours Using Centromeric Probes for Chromosomes 3 and 8

Of the 33 tumours analysed, 16 showed evidence of genetic imbalances. Of these 16 tumours, 14 patients had died by the end of the study, with 10 having died of liver metastases. The cause of death was unknown in two patients, with the rest having died of unrelated causes. The median duration of survival for these patients was 37months (14-52 months). Of the tumours without evidence of genetic imbalances, 5 patients had died by the end of the study, although none had died as a result of either liver metastases or from the primary uveal melanoma. The median duration of survival for these patients was 114 months (44-204 months), (table 3.5). Two patients were lost to follow up, one from each group and were thus excluded from the survival analyses (tables 3.4 and 3.5).

Of the 16 tumours that showed genetic imbalance, imbalance was due to a loss of chromosome 3 in 15 of these cases, with associated additional copies of chromosome 8 in 5 tumours only. Only one tumour had a gain of chromosome 8 not associated with a loss of chromosome 3 (Mel 50) where the patient died due to liver metastases and survived only 52 months (table 3.4). In several tumours the results obtained using FISH were in accord with previous cytogenetic analysis (Sisley et al., 1990).

If only those deaths due to liver metastases are considered, the difference between the two groups is statistically significant ($p<0.0001$). If it is assumed that all patients who died may have had occult metastases, the difference between the two groups is again statistically significant ($p<0.0001$). Both scenarios are represented by the Kaplan-Meier survival curves (figures 3.8 and 3.9).

Specific analysis of the predictive value of monosomy 3 with survival showed it to be significantly associated with reduced survival in all patients ($p<0.0001$) and in those with liver metastases ($p<0.0001$). Both scenarios are represented by the Kaplan-Meier survival curves (figures 3.10 and 3.11). However, the gain of chromosome 8 and reduced survival did not reach statistical significance ($p=0.133$), (figure 3.12).

No significant differences were observed in sex ($p=0.732$), age ($p=0.889$), tumour cell type ($p=0.73$), location ($p=0.498$), or mean tumour diameter ($p=0.574$) between the two groups (table 3.5).

Table 3.3 Results of dual colour FISH analysis.

Patient	Results (Chromosome 3 hybridisation signals:Chromosome 8 hybridisation signals)	GI/No GI
Mel 1	2:2=285 1:2=8 2:1=5 1:1=1 4:4=1	**No GI**
Mel 5	2:2=272 1:3=14 1:2=9 3:3=2 2:1=2 4:4=1	**No GI**
Mel 7	2:2=282 1:3=4 1:2=3 3:3=3 1:1=2 2:1=2 2:3=2 1:4=1 2:4=1	**No GI**
Mel 8	2:2=244 2:1=27 1:1=14 1:2=15	**No GI**
Mel 13	2:2=275 1:2=25	**No GI**
Mel 14	1:2=166 1:3=89 2:2=42 1:1=2 2:1=1	**GI**
Mel 15	1:3=164 1:2=116 2:2=14 1:4=6	**GI**
Mel 16	1:2=207 2:2=55 1:3=29 1:1=9	**GI**
Mel 21	2:2=290 2:1=3 1:3=3 1:2=3 1:1=1	**No GI**
Mel 22	2:2=283 2:1=4 1:2=4 2:3=3 3:3=2 4:2=1 4:4=1	**No GI**
Mel 24	2:2=236 1:2=26	**No GI (8%GI)**
Mel 26	2:2=200 1:2=97 1:3=3	**GI**
Mel 28	2:2=240 1:2=57 1:3=1 3:1=1 4:2=1	**No GI (19%GI)**
Mel 31	2:2=289 1:2=11	**No GI**
Mel 36	2:2=180 1:2=113 1:3=6 2:1=1	**GI**
Mel 37	2:2=169 1:2=65 1:3=60 2:4=2 3:3=2 1:4=1 2:1=1	**GI**
Mel 40	2:2=128 1:2=87 1:3=78 1:4=3 1:1=2 3:3=1 2:3=1	**GI**
Mel 44	2:2=186 1:2=69 1:3=12	**No GI (27%GI)**
Mel 45	1:2=167 2:2=98 1:3=28 2:4=2 1:4=1 2:1=1 2:3=1 1:1=2	**GI**
Mel 46	1:2=240 2:2=45 1:3=13	**GI**
Mel 47	2:2=261 2:1=19 1:2=9 4:4=3	**No GI**
Mel 48	2:2=269 1:2=20 2:1=6 1:3=2 1:1=3	**No GI**
Mel 50	2:4=195 2:2=37 1:4=28 2:6=15 1:5=7 1:3=5 2:5=3 1:6=2	**GI**
Mel 51	1:2=233 1:3=46 2:2=13 1:4=4 1:1=3 2:1=1	**GI**
Mel 52	1:2=129 2:2=35 1:3=26 1:1=7 2:4=2 2:1=1	**GI**
Mel 53	1:3=173 1:2=116 2:2=11	**GI**
Mel 57	1:2=213 1:3=62 2:2=16 1:1=8 1:4=1	**GI**
Mel 68	1:2=112 2:2=105 1:3=57 2:4=10 3:3=5 2:1=4	**GI**
Mel 69	2:2=203 1:2=86 1:3=3 2:1=3 1:1=5	**No GI (29%GI)**
Mel 71	2:2=296 1:3=2 4:4=1 3:3=1	**No GI**
Mel 73	2:2=278 4:4=12 1:2=3 2:4=3 2:1=4	**No GI**
Mel 75	2:2=281 1:2=12 2:1=7	**No GI**
Mel 94	1:2=216 1:3=51 1:1=10 1:4=2	**GI**

GI = Genetic imbalance present; No GI = No Genetic imbalance present.

Table 3.4 Results of GI status and tumour characteristics.

Patient	Sex	Age (years)	Cell Type	Location	Mean tumour diameter (mm)	Results	Status (months alive since diagnosis)
Mel 1	M	52	S	C	12	**No GI**	A (153)
Mel 5	F	48	Mix	CB	4.75	**No GI**	LFU
Mel 7	M	82	E	C	16.5	**No GI**	DUR (111)
Mel 8	M	40	Mix	C	12.2	**No GI**	A (146)
Mel 13	F	55	Mix	CB/C	12.7	**No GI**	DUR (88)
Mel 14	M	52	Mix	C	15.25	**GI**	DLM (32)
Mel 15	M	56	Mix	C	11.75	**GI**	DUK (70)
Mel 16	F	61	Mix	CB/C	19.75	**GI**	DUR (14)
Mel 21	M	54	Mix	CB/C	16	**No GI**	A (134)
Mel 22	M	79	Mix	CB/C	12.7	**No GI**	DUR (44)
Mel 24	F	69	Mix	C	10.05	**No GI**	A (134)
Mel 26	M	72	Mix	C	8.5	**GI**	DLM (55)
Mel 28	F	59	E	C	25	**No GI**	DUR (128)
Mel 31	F	38	Unknown	C	Unknown	**No GI**	A (204)
Mel 36	M	55	E	Conj.	9	**GI**	DLM (40)
Mel 37	F	70	Mix	CB/C	15	**GI**	DLM (8)
Mel 40	F	48	Mix	C	11	**GI**	A (120)
Mel 44	F	76	Mix	CB/C	15.3	**No GI**	A (120)
Mel 45	F	57	Mix	C	13.4	**GI**	LFU
Mel 46	F	64	E	CB/C	14.35	**GI**	DLM (69)
Mel 47	M	67	Mix	C	13.25	**No GI**	A (118)
Mel 48	M	35	Mix	C	15.25	**No GI**	A (117)
Mel 50	F	48	Mix	CB/C	10.35	**GI**	DLM (52)
Mel 51	F	61	E	CB	Unknown	**GI**	DLM (26)
Mel 52	F	72	Mix	C	17	**GI**	DUR (46)
Mel 53	M	46	Mix	CB/C	15.6	**GI**	DLM (54)
Mel 57	F	63	Mix	CB/C	14.75	**GI**	DLM (38)
Mel 68	M	71	S	Conj.	30	**GI**	DUK (19)
Mel 69	F	53	Mix	C	9	**No GI**	A (102)
Mel 71	M	35	S	C	13.45	**No GI**	A (100)
Mel 73	M	64	Mix	CB/C	20.6	**No GI**	DUK (63)
Mel 75	F	70	S	C	12.3	**No GI**	A (98)
Mel 94	M	33	Mix	C	16.6	**GI**	DLM (17)

M = Male; F = Female; E = Epithelioid; Mix = Mixed; S = Spindle; C = Choroid; CB = Ciliary Body; Conj. = Conjunctiva; A = Alive; DLM = Died with liver metastases; DUR = Died of unrelated causes; DUK = Died of unknown causes; LFU = Lost to follow-up. GI = Genetic imbalance present; No GI = No Genetic imbalance present.

Table 3.5 Results of statistical analysis.

	Genetic Imbalance Absent (No GI)	**Genetic Imbalance Present (GI)**	**p value for difference**
Number of patients	17	16	
Sex M/F	9/8	7/9	0.732
Age (years)			
Mean (SD)	57.41 (15.18)	58.06 (10.96)	0.889
Median	55	59	
Tumour diameter (mm)	Incomplete data for 1 tumour	Incomplete data for 1 tumour	
Mean (SD)	13.82 (4.60)	14.82 (5.23)	0.574
Median	12.975	14.75	
Cell type	Incomplete data for 1 tumour		
Spindle	3	1	0.730
Mixed	11	12	
Epithelioid	2	3	
Location			
Choroid	7	11	0.498
Ciliary body	1	1	
Ciliary body/choroid	6	5	
Conjunctiva	2	0	

Figure 3.4 A Captured FISH image of Normal Blood Sample 1. A captured image showing the normal complement of two target hybridisation signals for each chromosome pair, thus signifying no genetic imbalance.

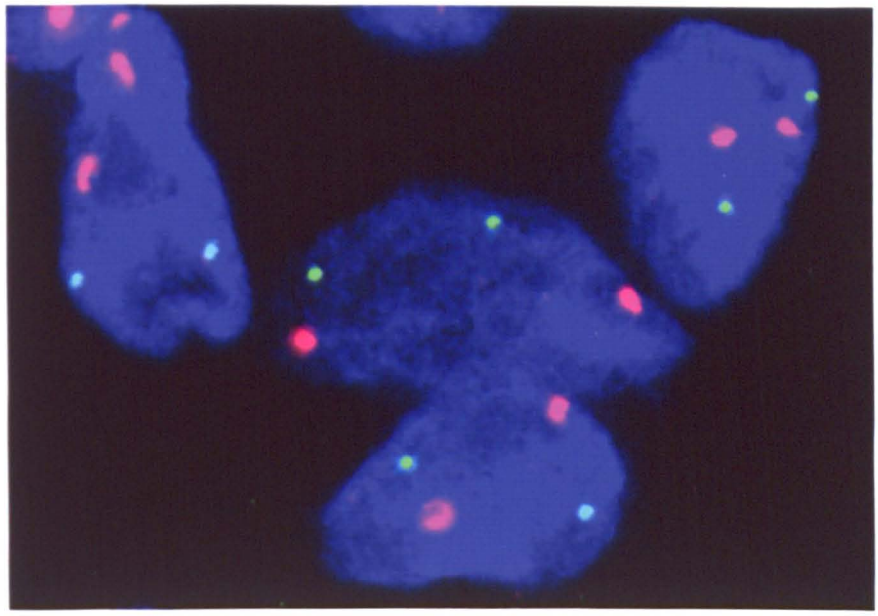


Figure 3.5 A Captured FISH image of Normal Blood Sample 5. A captured image showing a normal complement of split-target hybridisation signals. The split signals are distributed equally and are of similar intensity for each chromosome pair.

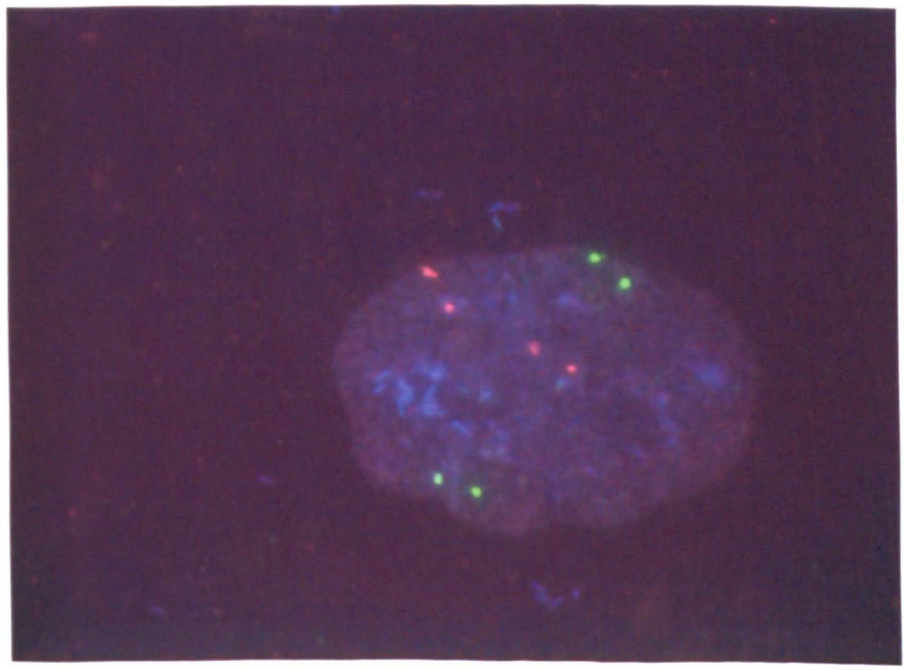


Figure 3.6 A Captured FISH image of Uveal Melanoma Tumour Mel 53. A captured image showing cells with monosomy 3 (red hybridisation signals - rhodamine) and trisomy 8 (green hybridisation signals - FITC), thus signifying genetic imbalance.

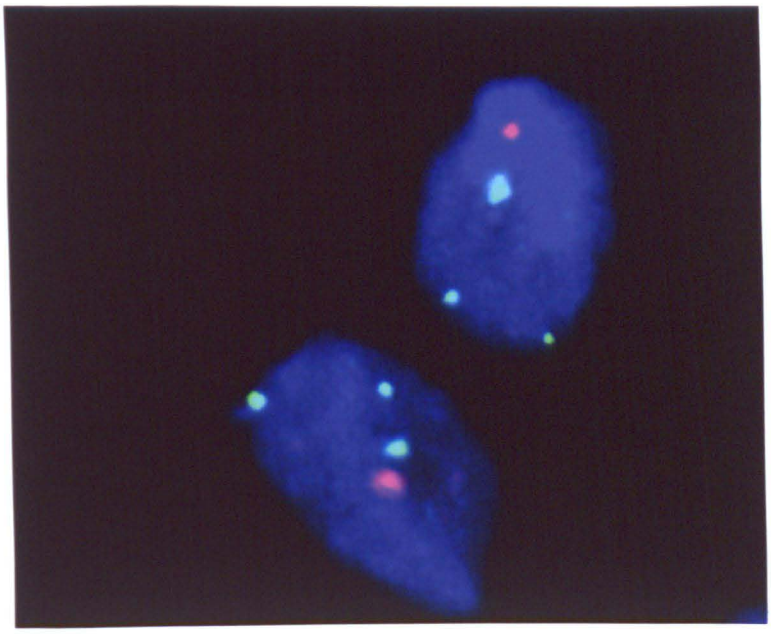


Figure 3.7 A Captured FISH image of Uveal Melanoma Tumour Mel 50. A captured image also showing genetic imbalance with four hits for chromosome 8 (green hybridisation signals - FITC) but with a normal complement of two hybridisation signals for chromosome 3 (red hybridisation signals - rhodamine).

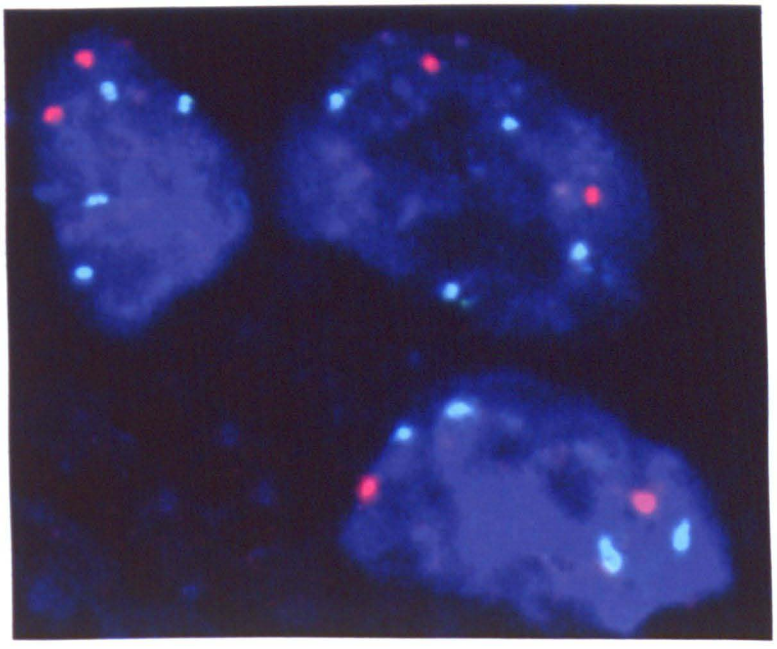


Figure 3.8 Kaplan-Meier survival curves for all deaths due to liver metastases – Analysis of chromosomes 3 and 8.

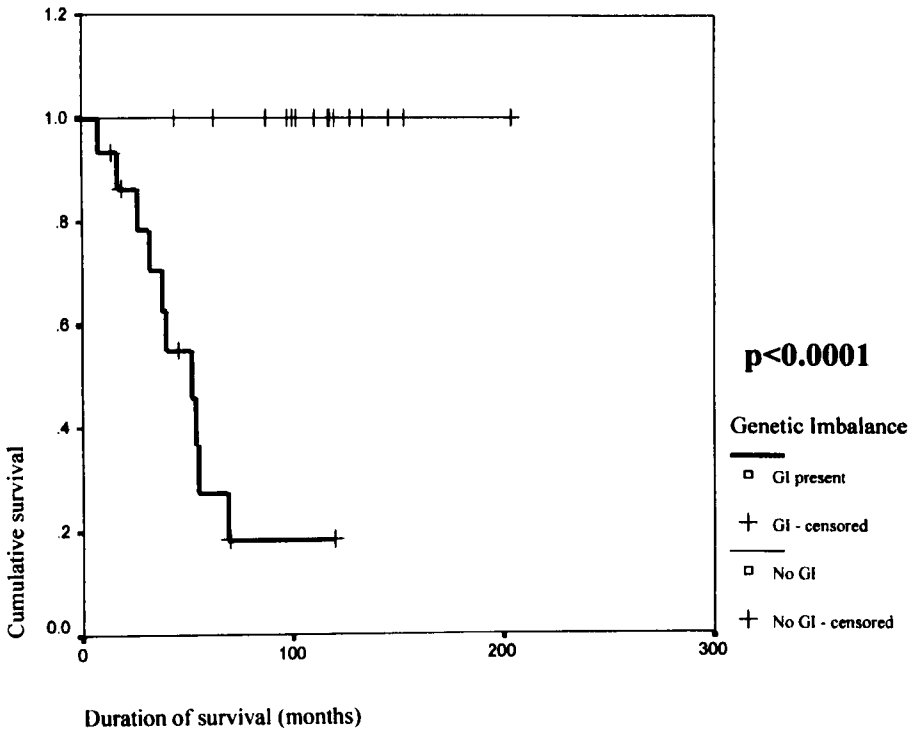


Figure 3.9 Kaplan-Meier survival curves for all deaths – Analysis of chromosomes 3 and 8.

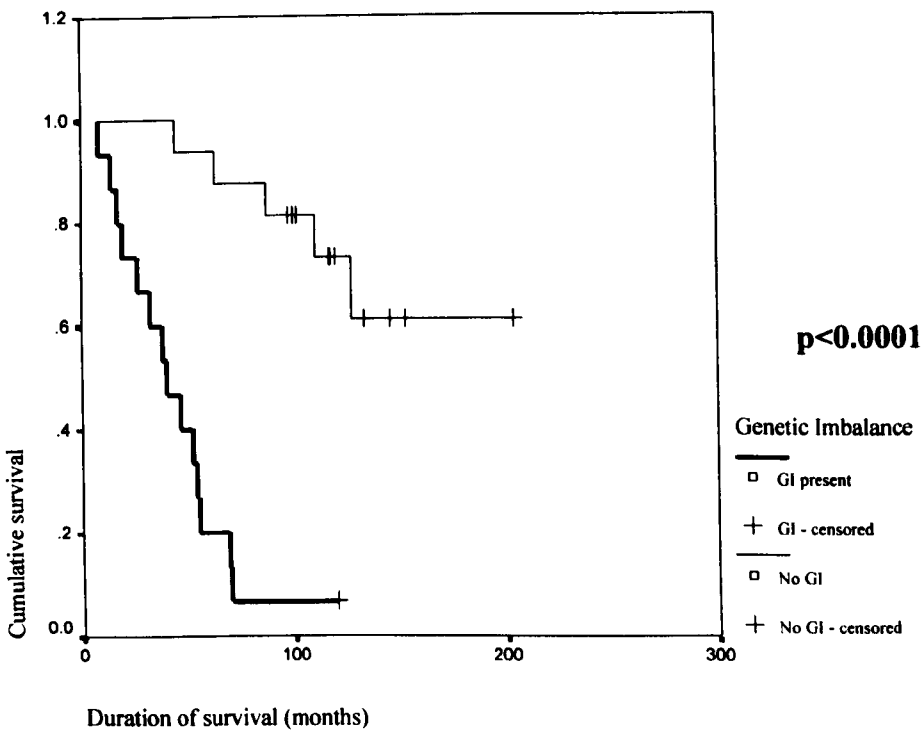


Figure 3.10 Kaplan-Meier survival curves for all deaths - Monosomy 3.

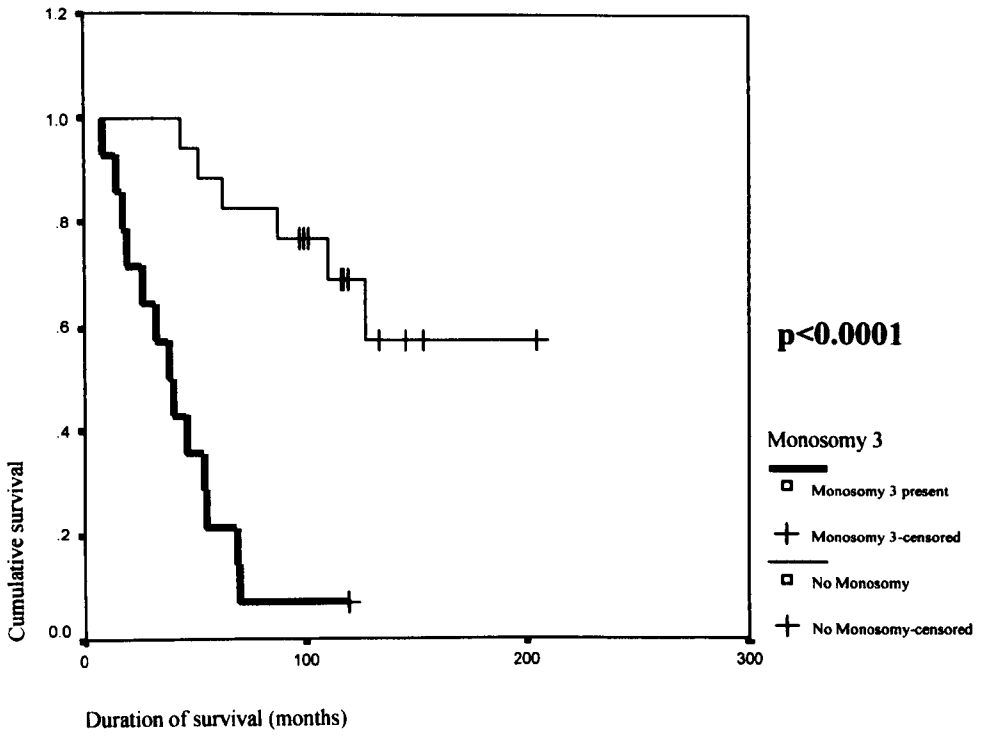


Figure 3.11 Kaplan-Meier survival curves for all deaths due to liver metastases – Monosomy 3.

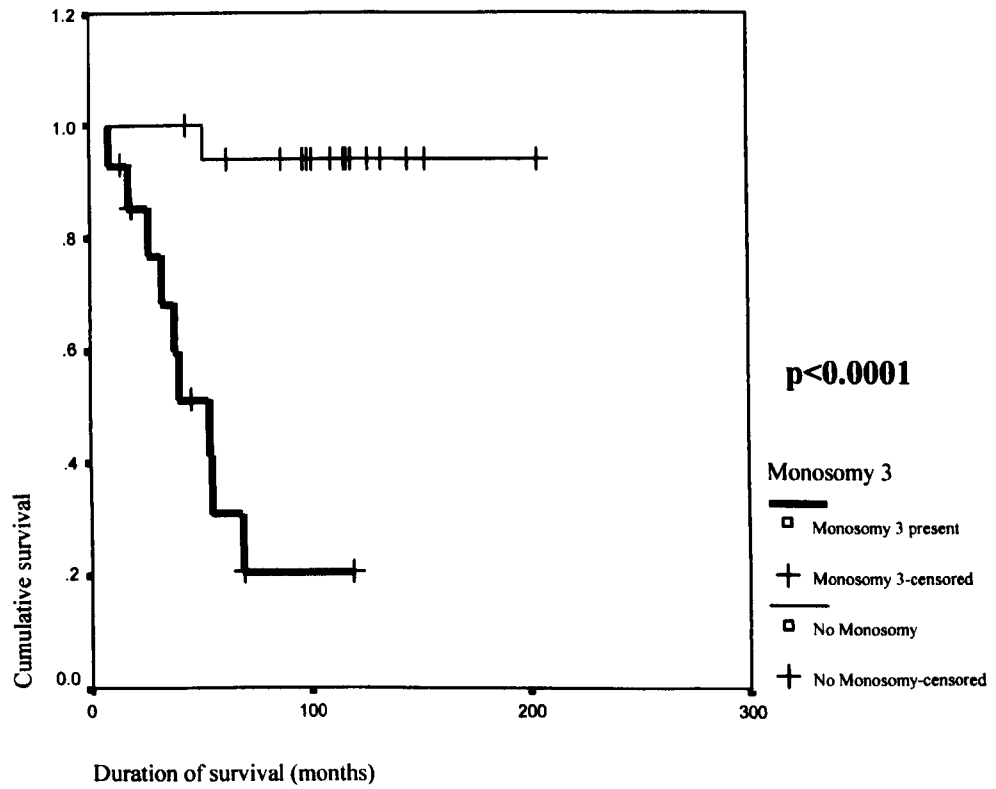
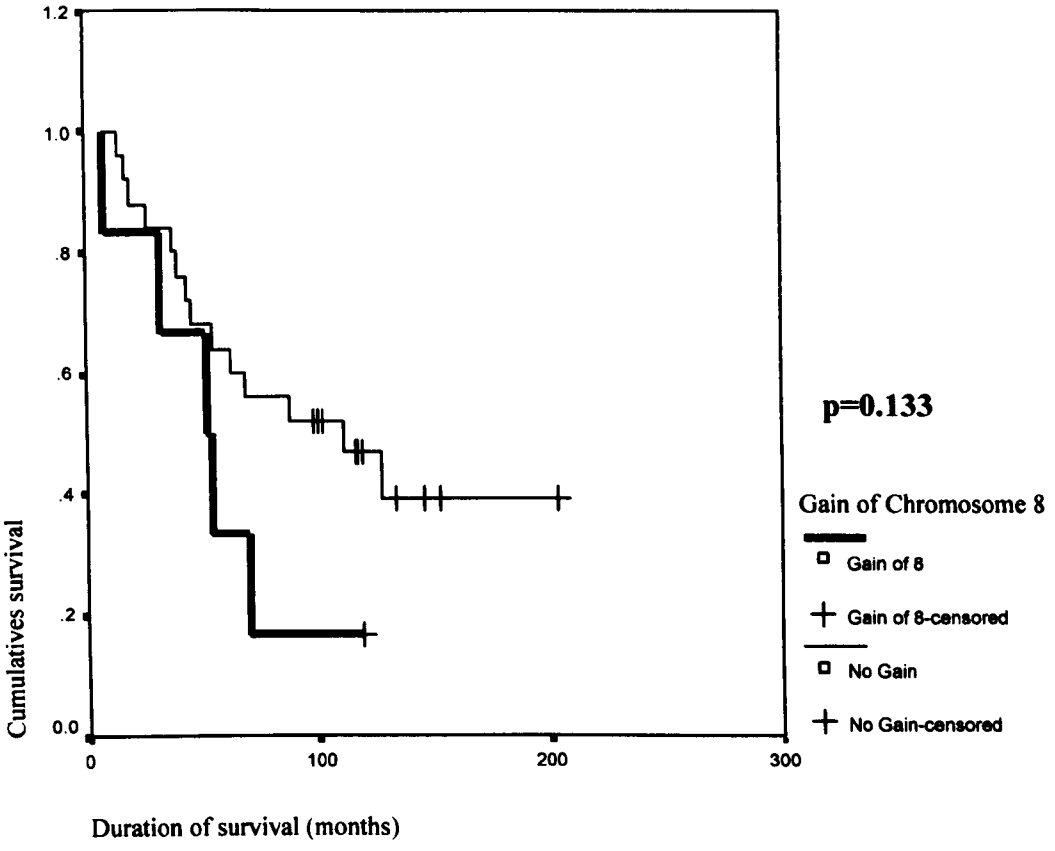


Figure 3.12 Kaplan-Meier survival curves for all deaths – Gain of Chromosome 8.



3.3 DISCUSSION

3.3.1 Effect of Tissue Fixation on FISH

FISH is an effective technique for analysing genetic changes in interphase nuclei and a number of studies on archival paraffin-embedded tissues have been published (Deviliee et al., 1988; Hopman et al., 1991; Matsumura et al., 1992; Qian et al., 1996). However, results are dependent on the method of tumour fixation with a number of protocols available for tumour fixation prior to being embedded in paraffin wax (Baker, 1960). Tumour material may not necessarily be fixed in formalin but a solution of various chemicals, some of which will hinder analysis by FISH (Kapranos et al., 1997). The purpose of fixation of fresh tissue samples is two-fold. Firstly, the process of autolysis and bacterial degradation should be prevented. Secondly, the tissue should be preserved in its original state without the loss of small molecules. Ideally, the tissue must be left in a condition following fixation, to allow subsequent processing. The aim of tissue processing is to progressively dehydrate the tissue of fixative and tissue water and then embed the tissue in a solid medium, firm enough to support the tissue, yet soft enough to allow a knife to cut sections without damaging the tissue. It is essential that the embedding medium thoroughly penetrates the tissue in its liquid form and that it solidifies with as little damage to the tissue. At the Royal Hallamshire Hospital, neutral buffered formaldehyde is used for fixation, which consists of formalin (which is a solution containing 35-40% gas by weight of formaldehyde), sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate anhydrous, with paraffin wax being used as the embedding medium. Tissue samples are left in this solution for between 4-6 hours generally before being processed. However, if specimens are large, as in the case of colon cancer resections, the tissue may be left in the solution overnight to allow penetration of the fixative into the tissue, occasionally this time period may extend to 48 hours if a cancer is resected over the weekend.

Fixatives form cross-links between proteins thereby producing a gel in which molecules are fixed relative to each other. In the case of formaldehyde these cross-links are formed between the lysine residues on the exterior surface of protein molecules, thus denaturing the proteins in the process (Baker, 1960). Fixation of tissues also brings about changes in the chemical and physical state of RNA and DNA. Although in their

native states, RNA and DNA do not react to any extent with formaldehyde at room temperature, when the temperature is increased to 45-65°C, as during tissue processing, then cross-links begin to form between proteins, namely histones and DNA. The length of time in formalin prior to being embedded in paraffin wax not only increases the extent of these DNA:protein complexes (Xiao et al., 1995), but can also result in the loss of up to 30% of the DNA (Díaz-Cano and Brady, 1997). Gomyo et al., (1995) found that with increasing durations of formalin fixation, hybridisation efficiencies when performing FISH reduced exponentially with results being unobtainable after 5 days of fixation and concluding that FISH analysis could easily be performed on formalin-fixed paraffin-embedded material, as long as tissue fixation was kept to as short a time as possible. Possible changes to the protocol to improve hybridisation include acid pre-treatment prior to pepsin digestion; heating the slide to 80°C for 10minutes in 1M sodium thiocyanate which dissociates proteins (notably histones) from DNA, and so allowing the DNA probes to access the target DNA more efficiently; suspending the disaggregated tumour cells in PBS or glycerol solution at 75-90°C which is thought to induce nuclei swelling and chromatin decondensation (Arnoldus et al., 1991; Hopman et al., 1991; Hyytinen et al., 1994) and microwave heating (Henke and Ayhan, 1994).

Thus, the effect of variable and prolonged fixation of the colorectal cancer specimens meant that FISH analysis was extremely difficult and although various pre-treatment methods could have been utilised, limitations in time precluded further investigation.

3.3.2 Clinical Parameters and FISH Analysis of Uveal Melanoma

Sex and Age

No significant difference was observed in either sex ($p=0.732$) or age ($p=0.889$) when using the t-test and Chi-Square test respectively, which is in concordance with other published series (McClean et al., 1982; Coleman et al., 1993).

Tumour Cell Type

No significant difference was observed in tumour cell type ($p=0.73$) when using Fisher's Exact test, which is in contrast to the studies by McClean et al. (1982) and Coleman et al. (1993) who found that tumours with a mixed or epithelioid cell type had

a poorer prognosis as compared to those with a spindle cell type. In our study there were a total of 4 tumours (12.1%) with a pure spindle cell type, of which 3 showed no genetic imbalance, whilst one tumour (Mel 68) did and in which the majority of the cells were observed to have monosomy 3, although the cause of death in this patient was unknown. There were a total of 23 tumours (69.7%) with a mixed cell type with almost equal numbers in each group (11 with no evidence of genetic imbalance and 12 with genetic imbalance). Of the tumours with an epithelioid cell type (15.2%), two tumours showed no genetic imbalance (Mel 7 and Mel 28) and three tumours showed genetic imbalance (Mel 36, Mel 46 and Mel 51), all with monosomy 3 and all of these patients dying as a result of liver metastases. There was one tumour in which the histological cell type was not recorded in the clinical notes.

Location

Uveal melanomas with a ciliary body location appear to be correlated with reduced survival (McClean et al., 1982; Prescher et al., 1996). However, in this study no significant association was shown between a specific location and a reduced survival, when using Fisher's Exact test ($p=0.498$), which concurs with other studies (Gordon et al., 1994; Parrella et al., 1999). Only two tumours (6%), Mel 5 and Mel 51 were situated entirely within the ciliary body, where Mel 5 showed no evidence of genetic imbalance, whilst Mel 51 showed monosomy 3, with the patient dying as a result of liver metastases. Eighteen tumours (54.5%) were situated entirely within the choroid, 11 of which showed no evidence of any genetic imbalance. Of the choroid tumours with genetic imbalance the majority had monosomy 3 and three tumours (Mel 14, Mel 15 and Mel 40) also had additional copies of chromosome 8. In a third group of tumours (33.3%), a definite site of origin could not be determined, as the tumours tended to be large and were thus classified as ciliary body/choroid. In this group there were near equal numbers of tumours with genetic imbalance ($n=6$) and showing no genetic imbalance ($n=5$). Of the two tumours situated in the conjunctiva (6%), Mel 36 and Mel 68, both showed evidence of genetic imbalance, both having monosomy 3. The patient, Mel 36, died as a result of liver metastases and patient Mel 68, the cause of death was unknown. However, patient Mel 68 did have a large tumour (mean tumour diameter of 30mm) and survived for only 19 months post-operatively.

Size

There was no significant difference shown between mean tumour diameter and the presence or absence of genetic imbalance, when using the t-test ($p=0.574$). The mean tumour diameter of those with no genetic imbalance was 13.82mm and in those with evidence of genetic imbalance was 14.82mm. There were two tumours in which the mean tumour diameter could not be ascertained from the clinical notes.

Tumours with and without Genetic Imbalance

Of the 33 tumours analysed16 tumours showed genetic imbalance, imbalance was due to monosomy 3 in 15 of these cases, with concurrent gains of chromosome 8 present in 5 tumours only (Mel 14, Mel 15, Mel 37, Mel 40 and Mel 53). Of these tumours, 4 patients had died by the end of the study, one where the cause was unknown and the rest having died as a result of liver metastases. The other patient (Mel 40), with a choroidal tumour is still alive 5 years post-operatively. Only one tumour had a gain of chromosome 8 (two extra copies) not associated with monosomy 3 (Mel 50). This tumour (Mel 50) was of a mixed cell type and located in the ciliary body/choroid with a mean tumour diameter of 10.35mm, with the patient dying as a result of liver metastases, surviving only 52 months post-operatively (table 2).

In several tumours the results obtained using FISH were in accord with previous cytogenetic analysis (Sisley et al., 1990, 1997), thus confirming the reliability of the technique in the assessment of abnormalities of chromosomes 3 and 8.

Survival Analyses

As stated previously, survival analyses were performed using the log rank test and represented by Kaplan-Meier survival curves. Two patients were lost to follow up, one from each group, and were excluded from survival analyses.

If only those deaths due to liver metastases were considered, the difference in duration of survival between those tumours with genetic imbalances and those with no evidence of genetic imbalance, was statistically significant ($p<0.0001$), suggesting that patients with tumours that had genetic imbalances were more likely to die sooner than patients in whom the tumours appeared to show no evidence of genetic imbalance (figure 3.8). If however, even if it was assumed that all patients who died may have had

occult liver metastases then the difference between the two groups was still statistically significant ($p<0.0001$), (figure 3.9).

A third analysis considered all tumours with monosomy 3 (irrespective of whether there was an associated gain of chromosome 8 and again assuming that all patients who died may have had liver metastases), and whether there was any difference in survival between tumours with monosomy 3 and those with a normal complement of chromosome 3 (figure 3.10). This analysis was also statistically significant ($p<0.0001$), thus confirming that patients with tumours having monosomy 3 have a worse prognosis than those patients in whom the tumours have a normal complement of chromosome 3. If the analysis is repeated, considering only those patients who died as a result of liver metastases again comparing tumours with and without monosomy 3, the difference is still statistically significant ($p<0.0001$) (figure 3.11). Note the step in the upper survival curve, which represents the patients with tumours having a normal complement of chromosome 3, this is due to the death of patient Mel 50, who had a tumour with two extra copies of chromosome 8 but without monosomy 3.

The final survival analysis considered all patients with tumours showing additional copies of chromosome 8 (irrespective of the presence of monosomy 3) and compared them with all those patients with tumours having a normal complement of chromosome 8. There appeared to be no difference in survival between the two groups ($p=0.133$), even though the survival curve for the patients with tumours showing additional copies of chromosome 8 is steeper than the survival curve for patients with a normal complement of chromosome 8 (figure 3.12). A survival analysis comparing tumours with additional copies of chromosome 8 and tumours with a normal complement of chromosome 3 could not be performed, as there was only one tumour (Mel 50), which would fit this criterion. These findings would at first appear to be in contrast to those of Horsman et al., (1990) and Sisley et al., (1997), who found a dose related correlation between additional copies of 8q and reduced survival. However, as only six tumours appeared to have additional copies of chromosome 8 and only one tumour with gains of chromosome 8 not associated with monosomy 3, the numbers were too small to reach statistical significance. These findings also concur with previous studies, which suggested that the gain of chromosome 8 (especially i8q) is a late event and tends to occur after either monosomy 3 or alterations of 6p (White et al., 1998; Parrella et al., 1999).

3.3.3 Monosomy 3 and Gain of Chromosome 8 as Prognostic Indicators of Liver Metastasis and Poor Survival in Uveal Melanoma

Several retrospective studies relating the presence of monosomy 3 and gains of 8q to a reduced survival and hence a poor prognosis have been undertaken previously (Prescher et al., 1996; Sisley et al., 1997; White et al., 1998). However, all of these studies were performed using the conventional cytogenetic analysis, which requires the short-term culture of tumours followed by the production of good quality metaphase spreads. The possibility also exists for missing genetic changes due to small number of cells studied. The development of fluorescence *in situ* hybridisation (FISH) allows the interphase cytogenetic analysis of a large number of cells accurately.

Prescher et al. (1996) examined uveal melanomas from 54 patients, either by karyotype analysis or comparative genomic hybridisation, in relation to clinical status, having been followed-up for a median of 40 months. Of the 54 tumours examined 30 had monosomy 3, of which 17 (54%) of these patients had relapsed with metastatic disease. In contrast, of the 24 patients in whom the tumours retained both copies of chromosome 3, none had developed metastatic disease. Thus concluding that monosomy 3 was the most significant predictor of poor prognosis, followed by ciliary body involvement and a tumour diameter greater than 10mm. Interestingly, histopathological subtype and the presence of extra-scleral growth had no additional predictive value in their study.

McNamara et al. (1997) assessed chromosome 3 copy numbers using fluorescent *in situ* hybridisation (FISH) on fresh touch preparations. Of the 17 uveal melanomas that they studied (all of which were located in the choroid) only two revealed monosomy 3, although the clinical significance of this was not stated.

Sisley et al. (1997) examined 42 patients using a combination of karyotypic analysis and FISH to confirm cytogenetic abnormalities. Twenty-three tumours (54%) had one or more additional copies of the long arm of chromosome 8, either as a result of translocation or in the form of an isochromosome i(8q) and of these 14 had two or more copies. Monosomy of chromosome 3 was found in 21 (50%) of the tumours examined. Other abnormalities detected, included deletion of chromosome 1p in 12 patients (29%), deletion of 6q in 13 patients (31%) and trisomy 6p in 8 patients (19%). FISH was used

to confirm cytogenetic abnormalities and was performed using chromosome paints for chromosomes 3 and 8 on prepared short-term cultures. Statistical analysis confirmed a significant association between chromosome 3 and 8 abnormalities with a ciliary body location and a reduced survival. The results also showed a significant correlation between increasing copies of chromosome 8q and a reduced survival.

Interestingly, White et al. (1998), performed cytogenetic analysis on 54 patients who underwent enucleation for uveal melanoma and divided the patients into two groups; those that had liver metastases (either alive or dead) and those who had no evidence of metastatic disease (again, alive or dead). Patients were followed-up for a median of 38 months and they found that abnormalities of chromosomes 3 and 8 (in the form of monosomy 3 and additional copies of chromosome 8q) were associated with a poor prognosis (presence of metastatic disease) but only when the two abnormalities were present together. When a chromosome 3 or 8 abnormality occurred alone, which was infrequent, the risk of a poor outcome was the same as if neither abnormality was present. However, they also found that additional copies of chromosome 6p correlated with a good prognosis even in the presence of chromosome 3 and 8 abnormalities. It appeared that gain of chromosome 6p material, either in the form of an isochromosome 6p or deletion of 6q was protective. And patients with the best outcomes were those with a lone abnormality of chromosome 6. Extra-scleral extension was also predictive of a poor outcome although there appeared to be no association between tumour location, size or histological cell type with clinical outcome. Although they concurred with the findings of Sisley et al. (1997), that abnormalities of chromosomes 3 and 8 were associated with tumours in a ciliary body location.

There have been a number of studies utilizing comparative genomic hybridisation (CGH) to assess genome wide changes in uveal melanoma (Speicher et al., 1994; Gordon et al., 1994; Ghazvini et al., 1996; Becher et al., 1997; Aalto et al., 2001; Naus et al., 2001), where the most common genetic changes were loss of chromosome 3, over representation of 6p, loss of 6q and additional copies of 8q. CGH allows analysis of the entire genome, highlighting regions of chromosomal gains and losses (Kallioniemi et al., 1992). Speicher et al. (1994), repeatedly observed the loss of chromosome arm 9p concluding that 9p loss may not entirely be restricted to the development of cutaneous melanomas. A similar conclusion was also drawn by Gordon et al. (1994), who also observed recurrent abnormalities with gains of chromosomes 7q and 13q. However,

both studies conceded small numbers within the study. Naus et al. (2001), used a combination of FISH, CGH and spectral karyotyping (SKY), to complement each technique to allow a more intensive exploration of chromosomal abnormalities in uveal melanomas. Examining two uveal melanoma cell lines and five primary uveal melanomas, they revealed a new abnormality using SKY, a der(17)t(7;17)(?;q?), that had not previously been recognised by conventional cytogenetics. CGH proved particularly useful in assigning abnormalities identified by SKY to specific chromosomal regions, in particular a small deletion of chromosome region 3q13~21. However, they did not observe monosomy 3 in any of their cases, which is not entirely unexpected as the incidence of monosomy 3 in our study was 45% (15/33) and clinicopathological data was unavailable for comparison in their study. Aalto et al. (2001), examined 14 non-metastasising uveal melanomas with 15 metastasising uveal melanomas and 6 of their metastases. They reported significantly more abnormalities in the metastases and metastasising uveal melanomas compared to the non-metastasising group, commenting that loss of chromosome 1p was only evident in the metastases and metastasising group suggesting a site of a putative tumour suppressor gene. They also reported that the frequency of chromosome 6p gains was higher in the non-metastasising group, although this was not statistically significant. However, loss of chromosome 6q in the metastases and metastasising group was statistically and clinically significant with all patients with 6q loss, dying from their disease.

There are thought to be several putative tumour suppressor genes on chromosome 3 and oncogenes on chromosome 8, which may be involved in uveal melanoma tumourigenesis and metastasis (Kok et al., 1997; Singh et al., 1996). A possible candidate tumour suppressor gene on chromosome 3 is the fragile histidine triad (FHIT) gene lying at 3p 14.2, which is targeted by tobacco smoke carcinogens and where allelic deletions has been linked to cancers of the lung, breast, colon, pancreas and head and neck (Burke et al., 1998). The other areas of deletions on chromosome 3p associated with cancers is at 3p 21.2-21.3 and at 3p 25-26, which contains the von Hippel-Lindau (*VHL*) syndrome tumour suppressor gene. Von Hippel-Lindau syndrome is a dominantly inherited familial cancer syndrome predisposing to a variety of malignant and benign neoplasms, most frequently retinal, cerebellar and spinal haemangioblastoma, renal cell carcinoma, phaeochromocytoma and pancreatic tumours. The gene is thought to function as a cellular gatekeeper, regulating cell cycle exit into the G0 phase, with loss

of function (as a result of mutation or deletion of both alleles) resulting in cells continuing to proliferate (Pause et al., 1998). Clifford et al. (1998) reported that hypermethylation could also lead to the silencing of the *VHL* tumour suppressor gene in renal cell carcinomas from patients with VHL disease. Another putative tumour suppressor gene located on chromosome 3p is the thyroid hormone receptor B (*THRB*) gene, which is also found to be deleted in uveal melanomas, predominantly those located in the ciliary body, although the significance of this finding is as yet unknown (Sisley et al., 1993).

In the case of chromosome 8 there not only appears to be a proto-oncogene on 8q but also a tumour suppressor gene on 8p. Studies using CGH indicate that a region from 8q 12 to 8qter are present at an increased relative copy number in a broad range of solid tumours (Fejzo et al., 1998). A region from 8p 22-p21.3 is commonly deleted in hepatocellular, colorectal and non-small cell lung cancers (Emi et al, 1992; Chinen et al., 1996). A region lying between 8p 11-p21 is also thought to be a region for one or more tumour suppressor genes involved in breast cancer (Adelaide et al., 1998). The *c-myc* oncogene (*c-myc* being the human homologue of an oncogene carried by an acutely transforming retrovirus known as Avian **myelocytomatosis** virus), which has been mapped to 8q 24 plays an important role in a variety of malignancies. However, in uveal melanomas there is contradictory evidence that over-expression of *c-myc* may be associated with a better prognosis (Chana et al., 1999). A number of genes also exist on chromosome 6p, including *WAF1/CIP1*, which encodes for a cyclin-dependant kinase inhibitor (p21) which is thought to bind to *p53* to control cellular progression through the cell cycle (El-Deiry et al., 1993; Waldman et al., 1995). The genes encoding MHC class 1 molecules are also located on 6p, expressions of which have been found to be altered in uveal melanoma using immunohistochemistry (Blom et al., 1997).

3.3.4 Summary

The results of this study are in accordance with previously published studies analysing uveal melanoma, correlating monosomy 3 with a poor prognosis (Prescher et al., 1992; Sisley et al., 1997; White et al., 1998). But this study also shows that analysing both chromosome 3 and 8 imbalances is a better prognostic indicator of liver metastasis and poor survival than analysing either one alone. Limitations of this study

include the small number of tumours analysed and the fact that centromeric probes were used, which only allow for the analysis of copy numbers of either chromosome. Therefore, subtle sequence changes such as base substitutions; deletions or insertions would be missed, as would chromosome translocations (Kallioniemi et al., 1996). Also the significance of chromosome 6 and 1p changes were not analysed, which in future studies could be addressed by the use of a hydroxycoumarin (blue) labelled probes. With the emergence of specific oncogenes and tumour suppressor genes in the tumourigenesis of uveal melanoma, these genes could also be specifically targeted using FISH.

This study has shown that FISH analysis for chromosome 3 and 8 is a reliable and efficient technique in the analysis of fresh-frozen tumour specimens and is valuable in the prediction of prognosis in individuals with uveal melanomas.

CHAPTER 4 – COMPARATIVE GENOMIC HYBRIDISATION ANALYSIS OF PRIMARY AND LIVER METASTATIC COLORECTAL CANCER

CONTENTS

CHAPTER 4

4.1 INTRODUCTION

The progression of colorectal adenoma to carcinoma is accompanied by the sequential accumulation of many genetic changes either as a result of chromosomal instability or microsatellite instability, with or without the influence of methylation (Fearon and Vogelstein, 1990; Frayling, 1999; Robertson and Wolffe, 2000).

Nanashima et al. (1997) conducted the FISH analysis of 18 paired samples of fresh-frozen primary colorectal cancers with their liver metastases using alpha satellite DNA probes for chromosomes 8, 18, 14/22 (combined) and 20. The authors reported that the gain of chromosome 20 was a frequently observed aberration in primary and liver metastatic tumours compared to 15 primary tumours which had not known to have metastasised. They concluded that the observation of a gain of chromosome 20 in the primary tumour could be used to predict liver metastasis.

One of the limitations of FISH is that it only provides information on one or a few loci at a time. This maybe adequate for single genetic abnormality disorders, such as Down's syndrome (trisomy 21) or Edward's syndrome (trisomy 13), but many malignancies have multiple genetic defects. So a technique that can examine the entire genome is more appropriate. One such technique is comparative genomic hybridisation (Kallioniemi et al., 1992; du Manoir et al., 1993; Kallioniemi et al., 1994).

The aim of this study was to look for any gross genomic changes in paired primary colorectal cancers and their liver metastases using comparative genomic hybridisation (CGH), in an attempt to delineate common regions of loss or gain, which may be prognostic for the development of liver metastases.

4.1.1 Comparative Genomic Hybridisation (CGH)

Comparative genomic hybridisation relies on the competitive hybridisation between fluorescently labelled tumour DNA and normal reference DNA onto a normal chromosome metaphase spread. Regions of gain or loss of DNA sequences, such as amplifications or deletions, are seen as changes in the differential ratio between the two

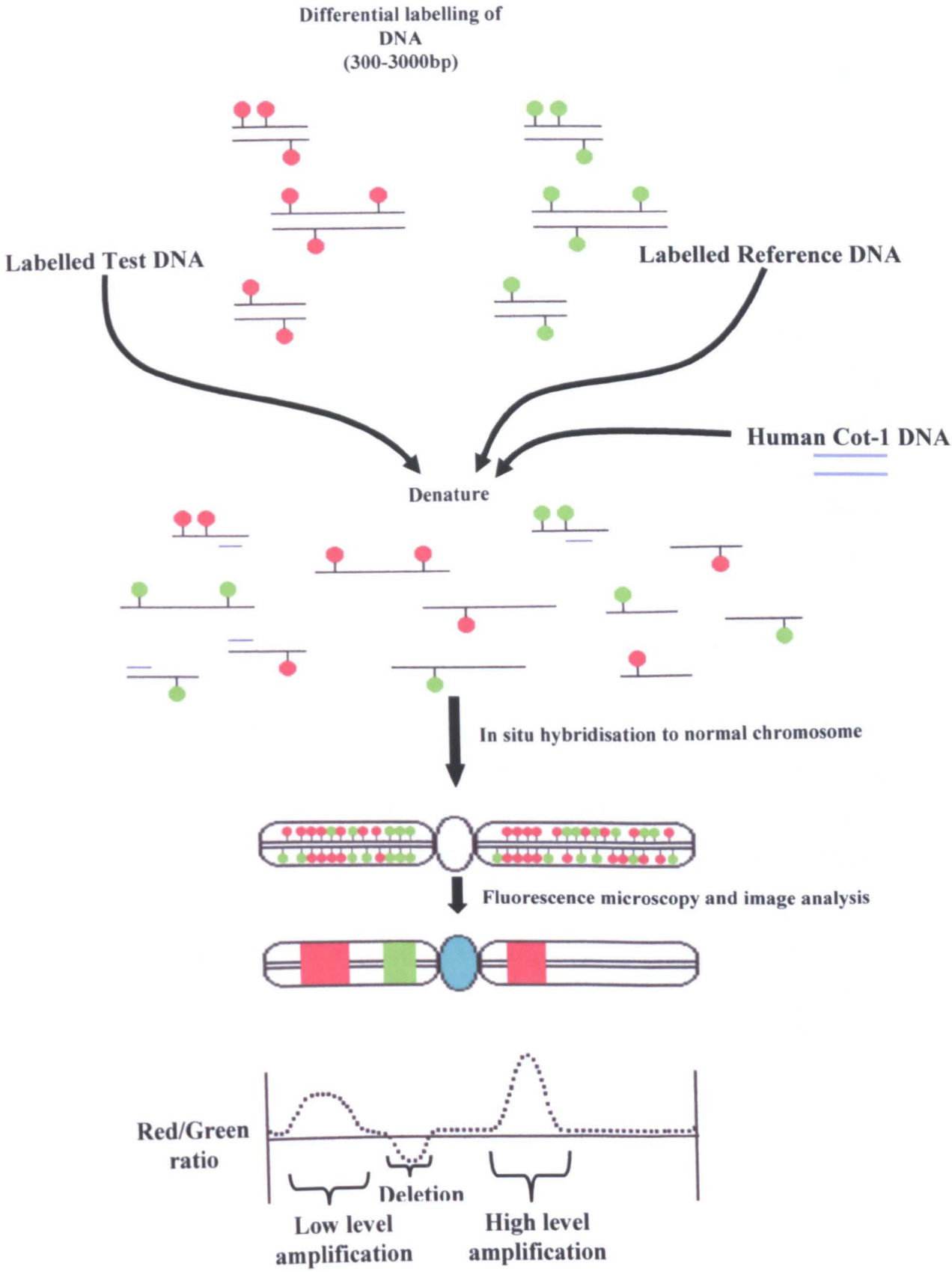
fluorochromes along the chromosomes. The analysis is undertaken using a charge coupled device camera and a digital image analysis system.

The simplest method of CGH relies on hybridisation of the labelled tumour DNA onto a normal metaphase spreads to elucidate areas of amplification, however this is not sensitive enough to detect low-level amplification or losses of DNA sequences (Kallioniemi et al., 1992; Joos et al., 1993). The inclusion of normal reference DNA, labelled in a different colour allows for compensation for differences in hybridisation efficiency found in one chromosomal region to another (Kallioniemi et al., 1996).

It is essential that DNA from tumour specimens contain at least 60% of the malignant cells under investigation (Kallioniemi et al., 1994). DNA extracted from fresh and frozen tissue and cell cultures are the most suitable, but recent developments in DNA extraction from formalin-fixed archival material have allowed this valuable source of DNA to be utilised (Speicher et al., 1993; Isola et al., 1994). The incorporation of directly labelled nucleotides FITC-dUTP (for tumour DNA) and Texas Red-dUTP (for reference DNA) has now replaced the previously used indirect labelling methods (Kallioniemi et al., 1992; Visakorpi et al., 1995), although directly labelled DNA tend to appear dimmer on visualisation, and so the use of digital image analysis is essential if low-level amplification and losses are to be detected (Piper et al., 1995), (figure 4.1).

The red-green ratios from several metaphase spreads are combined to account for any hybridisation inefficiencies to produce a mean profile with standard deviations for each chromosome (Kallioniemi et al., 1996). The degree of this variation is the best measure of the consistency and reliability of the CGH results (Kallioniemi et al., 1996). However, caution should be taken when interpreting ratio changes at the centromeres, heterochromatic regions and telomeres, and also at chromosome 1p32-pter and chromosomes 19, 22 and Y (Kallioniemi et al., 1994). It should also be noted that the differential intensities of the two fluorochromes do not always correlate with the level of copy numbers of each area of amplification, with another shortfall being that balanced translocations and ploidy shifts are undetectable (Persson et al., 1999). Small genetic changes, such as intragenic rearrangements and point mutations can also be missed and genetic changes affecting pericentromeric and heterochromatic regions are inconclusive (Kallioniemi et al., 1996).

Figure 4.1 A diagrammatic representation of comparative genomic hybridisation adapted from Houldsworth and Chaganti, (1994).



As CGH analyses the DNA from a population of cells, it cannot provide information on genetic changes present in a small subpopulation of cells or individual cells, important features of clonal heterogeneity (Kallioniemi et al., 1996). The development of spectral karyotyping and multicolour-FISH, allows the simultaneous visualisation of all chromosomes in specific colours (Schröck et al., 1996; Speicher et al., 1996). Thus, allowing the identification of subtle metaphase chromosomal aberrations, such as translocations and for the identification of small chromosomal structures, such as double-minutes, which may reflect oncogene amplification (Sawyer et al., 1998).

Comparative genomic hybridisation as a technique has been validated using independent methods, such as Southern blotting, loss of heterozygosity (LOH) analyses and fluorescence *in situ* hybridisation (FISH) (Kallioniemi et al., 1994). The results of CGH have also shown good correlation with classical cytogenetic analyses (Speicher et al., 1995). However, CGH tends to pick up more imbalances as compared to karyotype analyses, which require the growth of tumour cells in short-term cultures. A possible explanation for this could be that tumour cells with simple chromosomal abnormalities have a growth advantage over the complex/aneuploid tumour cells *in vitro* (Persson et al., 1999).

Conventional CGH analyses use normal metaphase chromosomes as targets for the mapping of gains and losses. However, this limits mapping of such imbalances to the resolution limit of the metaphase chromosomes (i.e. ~5-10Mb). A further development, is replacing the chromosomal target by cloned DNA, immobilised onto glass slides. The resolution then being dependant on the size of the immobilised DNA fragments. The technique is known as "matrix-CGH" (Solinas-Toldo et al., 1997; Pinkel et al., 1998).

4.1.2 Aims of this Study

The aim of the study was to investigate genomic changes in paired samples of primary colorectal cancers and their liver metastases, using CGH, to delineate potential chromosomal regions suggestive of putative oncogenes or tumour suppressor genes, which may be involved in the metastatic process.

4.2 RESULTS

A series of 25 paired tumour samples were obtained for the study. However, as an extensive amount of time and reagents had been utilised in optimising the CGH for formalin-fixed paraffin-embedded tissue, only two paired samples pCRC8/pLM8 and pCRC18/pLM18 were adequately hybridised and processed; although only partial results were obtained for these samples.

For each sample 10 to 15 target metaphase spreads were analysed, chromosomes being identified by DAPI banding.

4.2.1 Optimisation of DNA Extraction and Purification from Formalin-Fixed Paraffin-Embedded Tumour Samples

A number of DNA extraction protocols were attempted in order to obtain the optimum quantity and quality of DNA for use in CGH. DNA of poor quality (as a result of contamination from RNA or protein) would result in non-specific hybridisation and hence increased background fluorescence, whilst a poor yield of DNA would result in dim fluorescent signals.

Tissue for DNA extraction was obtained from microdissected formalin-fixed paraffin-embedded tissue material. Several different DNA extraction protocols were attempted in order to achieve the most efficient method of DNA extraction which would yield DNA of sufficient quantity and quality for use in CGH.

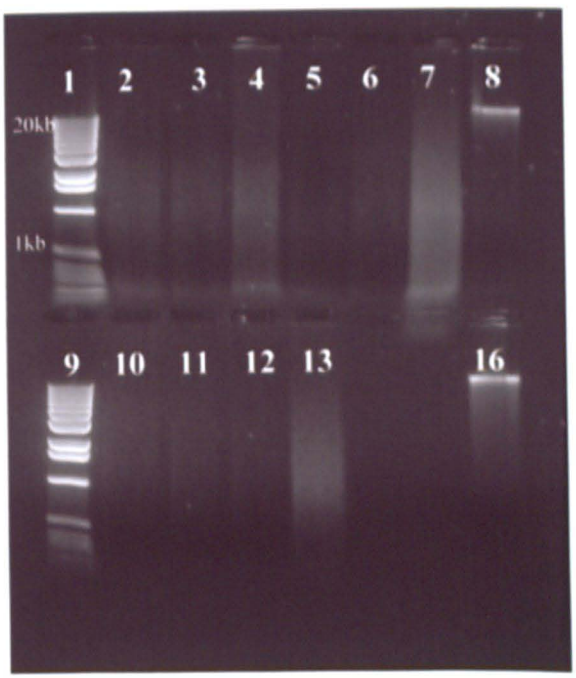
Tissue samples were de-waxed and digested with proteinase K as outlined in the Materials and Methods section 2.2.5.1 DNA Extraction using Phenol:Chloroform for Formalin-Fixed Paraffin-Embedded Tissue for use in CGH. DNA purification was initially performed using both cold (-20°C) chloroform and ethanol at both room temperature and in the cold room (~4°C) to assess whether extraction at 4°C would improve the yield of DNA. DNA yield was improved at 4°C, although only slightly when assessed quantitatively using the spectrophotometer and qualitatively by electrophoresis on an ethidium bromide stained 1% agarose gel. The DNA was not of sufficient quantity or quality for use in CGH, and as the amount of tissue available for extraction was limited, a more effective technique was required. A number of organic solvents were utilised in combination to improve both the quality and yield of DNA

extracted (table 4.1). Again the yield of DNA was assessed qualitatively on an ethidium bromide stained 1% agarose gel (figure 4.2). Method B1 proved to have yielded the largest quantity of DNA and analysis using the spectrophotometer showed no difference in the quality between methods B1 and C1. Method A1 produced the least yield and quality of extracted DNA.

Table 4.1 Combinations of the various organic solvents used in DNA purification. (All procedures were performed in polypropylene test tubes, with the aqueous layer being transferred into fresh test tubes between washes. All washes were performed at room temperature).

Organic solvent	Method A1	Method B1	Method C1
1st wash	Phenol	Phenol:Chloroform	Phenol:Chloroform
2nd wash	Phenol:Chloroform	Chloroform	Phenol:Chloroform
3rd wash	Isoprpanol	Ethanol	Chloroform
4th wash	-	-	Ethanol

Figure 4.2 Captured image of a 1% agarose gel showing the results of the various DNA purification methods utilised. (In lane 1 is a 1kb ladder and in lanes 8 and 16 a sample of DNA extracted from a whole blood sample, with the DNA smears produced using method A1 in lane 4, method B1 in lane 7 and method C1 in lane 13).



In a further attempt to improve the yield of DNA extracted a variety of methods were used to de-wax the tissue more effectively. The various methods used to de-wax the paraffin-embedded tissue are outlined in table 4.2.

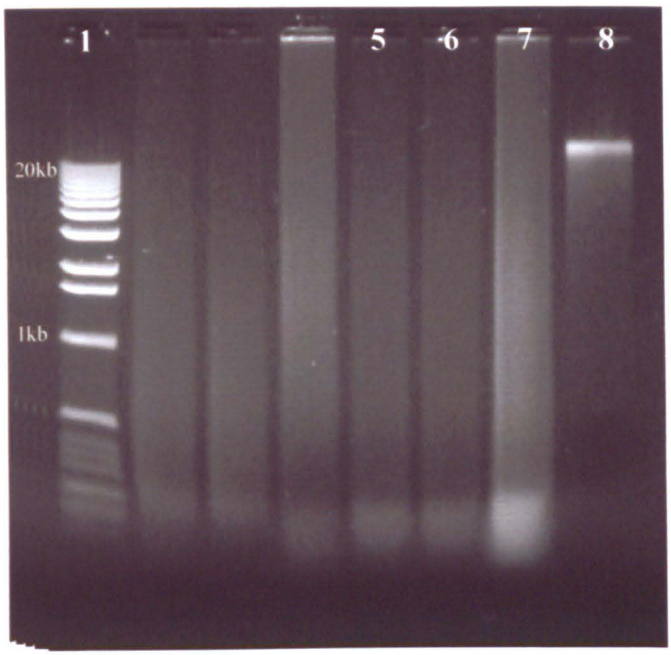
Table 4.2 The various organic solvents used in the de-waxing of formalin-fixed paraffin-embedded tissue. (In all the methods the microdissected tissue samples were transferred to a polypropylene test tube before undergoing de-waxing with the various organic solvents. Between each wash, the samples were centrifuged at 15000rpm for 2 minutes and the supernatant carefully removed).

Organic solvent	Method A2	Method B2	Method C2
1st wash	3ml xylene for 10mins at room temperature	3ml xylene for 10mins at 70°C (water bath in fume cupboard)	Slides warmed to 60°C before microdissection. Then 3ml xylene for 3 mins at room temperature
2nd wash	3ml xylene for 10 mins at room temperature	3ml xylene for 30 mins at 70°C (water bath in fume cupboard)	3ml xylene for 10mins at room temperature
3rd wash	3ml 100% ethanol for 10 mins at room temperature	3ml xylene for 30 mins at 70°C (water bath in fume cupboard)	3ml 100% ethanol for 3 mins at room temperature
4th wash	3ml 95% ethanol for 10 mins at room temperature	3ml 100% ethanol for 30 mins at room temperature	3ml 100% ethanol for 3 mins at room temperature
5th wash	3ml 70% ethanol for 10 mins at room temperature	3ml 100% ethanol for 30 mins at room temperature	3ml 95% ethanol for 10 mins at room temperature
6th wash	3ml 50% ethanol for 10 mins at room temperature	-	3ml 50% ethanol for 3 mins at room temperature
7th wash	3ml distilled water for 10 mins at room temperature	-	3ml distilled water for 3 mins at room temperature
Digestion	After the final wash the sample, centrifuge at 1000rpm for 10 minutes followed by the addition of 20µl proteinase K and 400µl digestion buffer and place in rotator at 55°C for 24 hours		

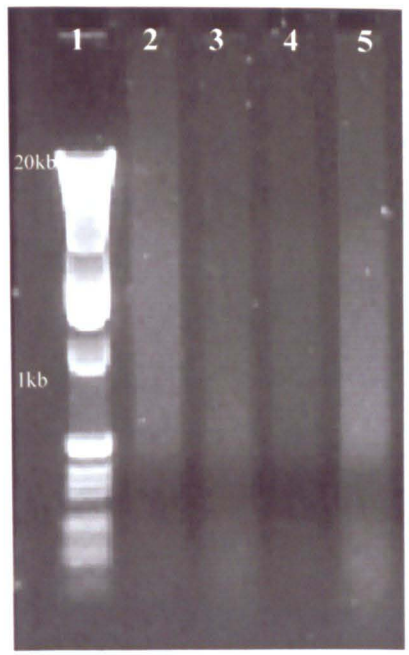
The de-waxed and digested tissue samples then underwent DNA extraction as per method B1, outlined previously and extracted DNA was assessed both qualitatively and quantitatively (figure 4.3a).

Figure 4.3 Two captured images of a 1% agarose gel showing the various DNA extraction methods used. Figure 4.3a shows a 1kb ladder in lanes 1 and a sample of whole blood DNA in lane 8, with the DNA smears produced using method A2 in lane 5, method B2 in lane 6 and method C2 in lane 7. **Figure 4.3b** shows a 1kb ladder in lane 1 and DNA smears of two samples of extracted tumour DNA using the Qiagen QIAamp® DNA Mini kit in lanes 2 and 5, and the DNA smears of two samples of extracted tumour DNA using the method C2 protocol in lanes 3 and 4.

4.3a



4.3b



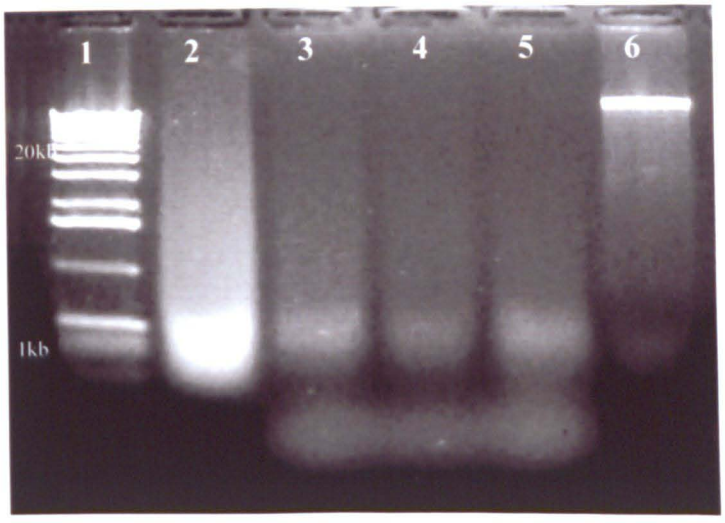
Although method C2 produced the greatest yield of DNA the ratio suggested contamination, most probably by protein. A relatively pure DNA sample would be essential for CGH, in order to minimise background fluorescence. Our DNA de-waxing protocols were compared to a commercially available DNA extraction kit (QIAamp® DNA Mini kit purchased from Qiagen Ltd). Tissue de-waxing and extraction was easier and cleaner using the kit, and the extracted DNA was of a slightly greater yield and purity. Thus, it was decided to use the QIAamp® DNA Mini kit for subsequent DNA extractions (figure 4.3b).

4.2.2 Optimisation of CGH Analysis of Formalin-Fixed Paraffin-Embedded Tumour Samples

DNA was extracted from 25 pairs of formalin-fixed paraffin-embedded primary colorectal cancers and their corresponding liver metastases, using the Qiagen QIAamp® DNA Mini kit.

CGH was performed as outlined in the Materials and Methods section 2.2.7 Comparative Genomic Hybridisation. Initially, nick translation was used to label the tumour DNA samples with Spectrum Green dUTP. The optimum time required to nick translate the DNA was assessed by taking 5µl aliquots from the 50µl reaction mix at 1 hour, 1½ hours, 2 hours and 2½ hours. The aliquots were run on a 1% agarose gel (figure 4.4). On inspection, there appeared to be little difference in the smears seen between the various times. However with longer durations of reaction, there appeared to be a greater intensity of smaller fragments of DNA visible on the gel, which would result in a more granular CGH result. In order to conserve the larger fragments, aliquots from the reaction mix were taken after 1 hour and run on a 1% agarose mini-gel, before the reaction was stopped.

Figure 4.4 Captured image of a 1% agarose gel showing DNA smears of aliquots of a nick translated DNA sample. In lane 1 is a 1kb ladder and in lane 6 a sample of whole blood DNA. A DNA smear of the original extracted DNA sample using the QIAamp® DNA Mini kit is seen in lane 2,with aliquots of the nick translated DNA samples taken out at 1½ hours (lane 3), 2 hours (lane 4) and 2½ hours (lane 5).

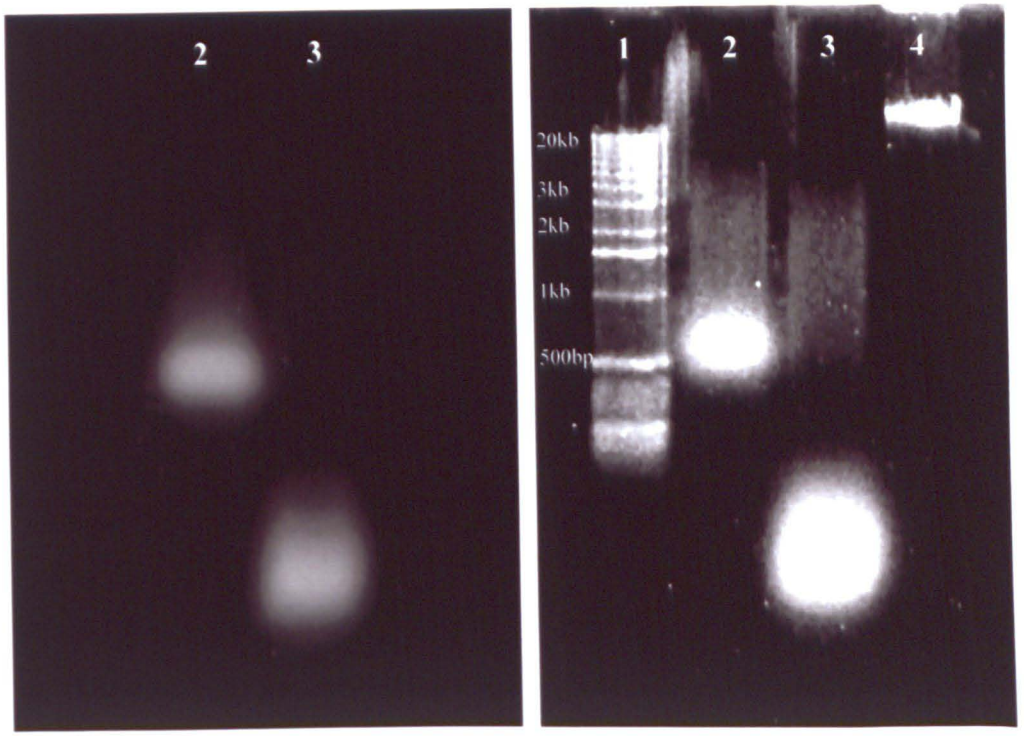


CGH results using extracted DNA nick translated with Spectrum Green dUTP, were poor, with very low intensities of labelled DNA. The metaphase images also appeared to be very granular, in keeping with very small, labelled DNA fragments. In order to determine whether tumour labelling with Spectrum Green dUTP was affecting the quality of hybridisations, tumour DNA was labelled with Spectrum Red dUTP. However, even labelling with Spectrum Red did not improve the intensity of the hybridised tumour DNA.

As both these fluorescent labels fluoresced in ultra-violet light, it was possible to visualise the intensity of labelling on a 1% agarose gel before the gel was stained with ethidium bromide to assess the DNA smear. Figure 4.5a shows that unlabelled Spectrum Red runs at a slower rate than Spectrum Green. One can also see that these fluorescent labels are associated with a smear of labelled DNA, which is stained more intensely with ethidium bromide (figure 4.5b).

Figure 4.5 Captured images of Spectrum Red and Spectrum Green labelled DNA run on a 1% agarose gel viewed under UV light without (figure 4.5a) and with (figure 4.5b) ethidium bromide staining. In lane 1 is a 1kb ladder and in lane 4a sample of whole blood DNA. Nick translated DNA smears using Spectrum Red labelling and Spectrum Green labelling are seen in lanes 2 and 3 respectively.

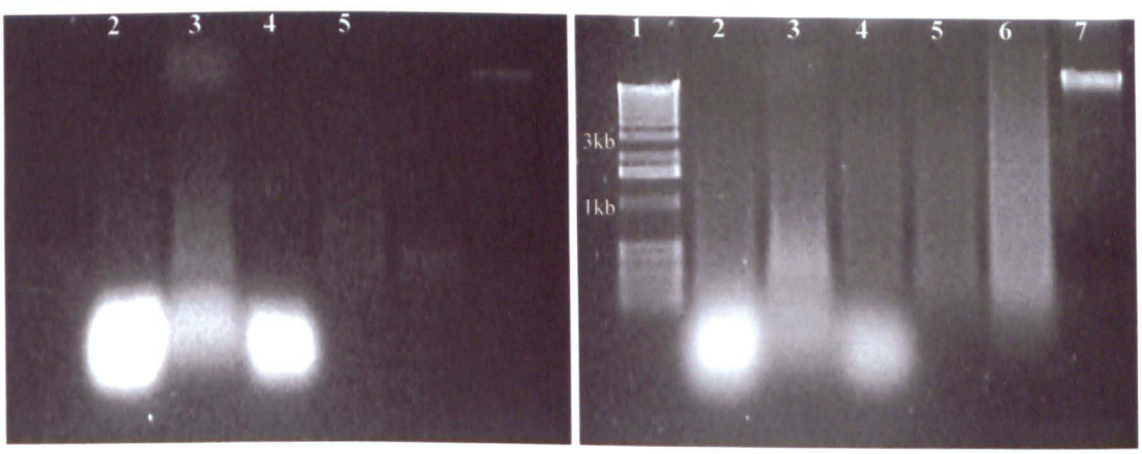
4.5a **4.5b**



At this stage a different method of labelling the extracted DNA was utilised. As the process of nick translation digests DNA into smaller fragments, a labelling system that avoids degradation would have a distinct advantage. The Ulysis ULS (Universal Linkage System) method labels DNA directly with a fluorescent label and thus does not degrade DNA. Both the Ulysis and nick translation techniques were compared side-by-side. Again images were taken of a 1% agarose gel before and after staining with ethidium bromide. Both showed similar levels of intensity, however as expected the DNA smear of DNA labelled using the Ulysis system was longer than using the nick translation method, as the larger fragments of DNA were not digested. Interestingly, a sample nick translated first and then having undergone the Ulysis method of labelling produced the greatest intensity of labelling (figure 4.6a).

Figure 4.6 Captured images of the various DNA labelling techniques used, run on the same 1% agarose gel viewed under UV light without (figure 4.6a) and with (figure 4.6b) ethidium bromide staining. In lane 1 is a 1kb ladder, with a sample of whole blood DNA in lane 7 and an aliquot of extracted DNA sample which has been used for all experiments in lane 6. Together with nick translated labelled DNA smears using Spectrum Green (lane 2 and 4), a dGreen Ulysis labelled DNA smear (lane 5) and a combined nick translated Spectrum Green and Ulysis dGreen labelled DNA (lane 3).

4.6a **4.6b**



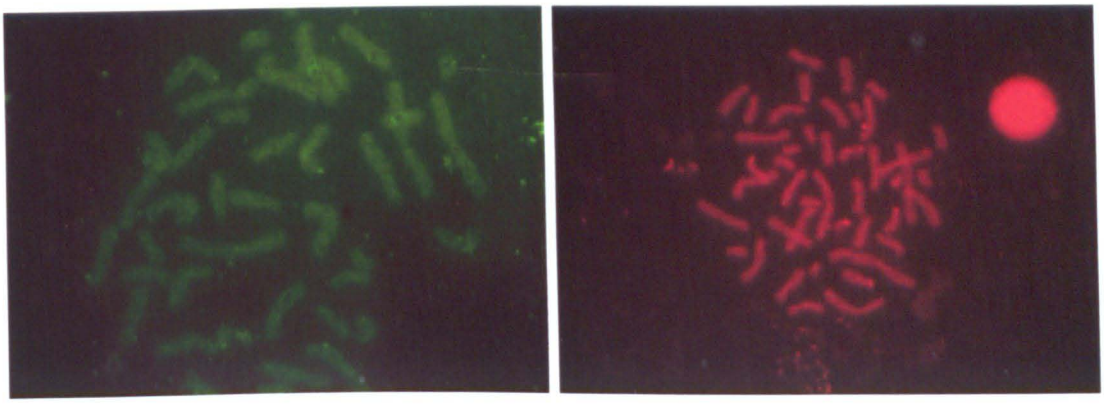
CGH was initially attempted using extracted DNA labelled with dGreen using the Ulysis system and hybridised against normal female nick translated DNA labelled with Spectrum Red. Images taken using the Ulysis dGreen labelling system, showed a 'halo-

effect' around each of the metaphase spreads (figure 4.7a). Although the software could analyse these images, it was possible that this 'halo-effect' could affect analysis.

CGH was repeated using extracted tumour DNA labelled with rhodamine using the Ulysis system, together with normal female nick translated DNA labelled with Spectrum Green. A 'halo-effect' around each metaphase was again observed. Therefore, CGH experiments were repeated using normal total genomic female blood DNA, which was digested with DNAse to produce fragmented DNA. The DNA was then labelled with rhodamine using the Ulysis system and hybridised against normal female nick translated DNA labelled with Spectrum Green. On this occasion, no 'halo-effect' could be seen, suggesting that this effect may be as a result of formalin-fixation or paraffin-embedding (figure 4.7b).

Figure 4.7 Captured images showing the green 'halo-effect' around a metaphase spread when CGH was performed using Ulysis dGreen labelled tumour DNA (figure 4.7a) and no 'halo-effect' when CGH was performed using Ulysis rhodamine labelled fragmented normal female blood DNA (figure 4.7b).

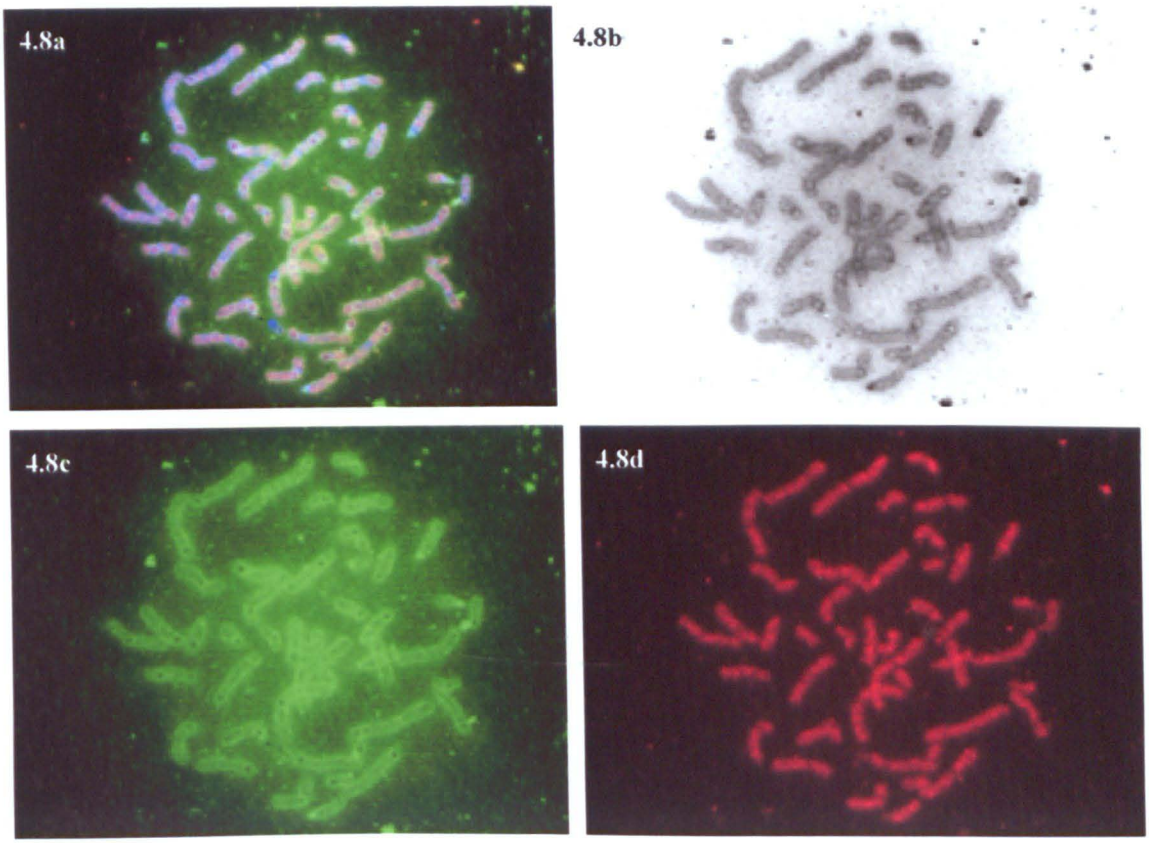
4.7a **4.7b**



The hybridised metaphases were analysed to test the validity of this labelling method and all CGH profiles were found to be within the normal range, as would be expected for a normal sample. To test the hypothesis that formalin-fixation or paraffin-embedding produced this 'halo-effect', a sample of normal formalin-fixed paraffin-embedded colon tissue was taken and its DNA extracted and labelled using Ulysis dGreen and hybridised against a normal female nick translated DNA labelled with Spectrum Red. The CGH image confirmed that the 'halo-effect' was most likely due to the effects of formalin-fixation or paraffin-embedding. The CGH results also showed that this 'halo-effect' did

not influence the analysis performed by the computer software, as the results showed normal CGH profiles (figures 4.8a-d).

Figure 4.8. A typical captured image of a Ulysis dGreen labelled DNA from formalin-fixed paraffin-embedded normal colon tissue (figure 4.8a), with its DAPI stained image (figure 4.8b). Figure 4.8c shows the Ulysis dGreen filtered image whilst figure 4.8d shows the Ulysis rhodamine filtered image.



The 'halo-effect' may also have resulted from non-specific hybridisation of very small, labelled fragments of DNA to the edges of chromosomes. We contacted Kreatech, who suggested we use Qiagen Clean-Up columns, to remove the very small fragments of labelled DNA and any unincorporated fluorochromes. Two kits are available from Qiagen for this task, a nucleotide removal kit (which removes nucleotides and oligonucleotides less than 40 bp long), and a PCR clean-up kit (which removes oligonucleotides less than 100 bp long). Initially the nucleotide removal kit was used as Qiagen recommended this kit. However, there still appeared to be the 'halo-effect'. Secondly, the PCR clean-up kit was used. The CGH images were better, with no

evidence of the 'halo-effect'. However, the intensity of the images was too low for analysis by the computer.

The amount of Ulysis labelled DNA (which had been 'cleaned' using the PCR clean-up kit) was increased in each hybridisation, proving to be extremely successful. CGH was repeated using DNA extracted from normal paraffin-embedded colonic tissue and labelled with the Ulysis system and cleaned using the PCR clean-up kit. This also proved to be successful and was used as a control in subsequent CGH hybridisations to set the image intensity level for correct analysis. MPE600 fragmented with DNAse was also labelled with the Ulysis system and used as a positive control in subsequent CGH hybridisations. Results confirmed that the method was suitable for detecting the known aberrations of the MPE600 DNA. However, due to the limited resources, Ulysis labelled MPE600 was not available for the final set of CGH experiments.

Consultation with Kreatech confirmed that similar problems had also been experienced by other researchers. They confirmed that the 'halo-effect' was as a result of very small, labelled fragments of DNA hybridising non-specifically to any RNA or cytoplasmic debris on the slides and suggested that the DNA be cleaned twice using the Qiagen columns and titrating the amount of Ulysis labelled DNA used in CGH hybridisations.

CGH was also performed using extracted DNA labelled with a combination of nick translation and Ulysis labelling. However, there appeared to be a lot of background signals as a result of non-specific hybridisations between the very small, labelled fragments of DNA and residual cytoplasm/debris on the slides. It was felt that this avenue of labelling would not be cost effective in either time or reagents. Therefore, it was decided that the Ulysis labelling system offered the best possibility of a successful hybridisation for analysis.

A further setback was encountered, with extensive background fluorescence as a result of non-specific hybridisation to cytoplasmic debris on the normal metaphase target slides, which had been purchased from Vysis. This became a consistent problem with variability in the quality of slides not only existing between differing batches but also within individual batches. This problem had also been encountered by other researchers, both within the department and other institutes. Therefore, in an attempt to reduce the background debris (which was most likely to be cytoplasmic remnants), Vysis slides were pre-treated with pepsin digestion using the same protocol as for FISH

(see Methods and Materials section 2.2.4 Fluorescent *In Situ* Hybridisation - pepsin digestion steps 1-3, with the exclusion of the final PBS-$MgCl_2$ wash). The slides were then dehydrated through an ethanol series and allowed to air-dry at room temperature. Pepsin digestion was performed for 5, 10 and 15 minutes, with CGH being performed as per protocol thereafter, together with a control slide, which had not been pre-treated. Nick translation labelled 46XX and Ulysis labelled colorectal cancer were used for all slides. The results of pre-treatment are shown in table 4.3.

Table 4.3 The results of pepsin digestion pre-treatment for various durations.

	Duration of Pepsin Digestion Pre-treatment			
	Slide 1 0 mins (control)	**Slide 2 5 mins**	**Slide 3 10 mins**	**Slide 4 15 mins**
Level of Background fluorescence	++++	+++	+++	+
Appearance of target metaphases	Normal appearance with good DAPI staining	Metaphases over-digested appearing 'moth eaten'	Increasing over digestion	Very pale metaphases. Unable to distinguish chromosomes
Intensity and quality of hybridisations	Good coverage but unable to analyse because of high background fluorescence.	Poor coverage for both red and green fluorochromes. No analysis possible.	Poor coverage for both fluorochromes. Computer able to analyse hybridisations, but reliability of results questionable.	Extremely poor coverage for both fluorochromes. No analysis possible.

As each batch of Vysis slides would be produced slightly differently as a result of variances in humidity and temperature during the manufacturing process, pre-treatment with pepsin digestion would have to be standardised for each batch, thus resulting in the expenditure of more time, reagents and tumour material.

As a final attempt to obtain usable target metaphase slides, target slides were manufactured in-house using PHA stimulated lymphocytes from healthy male volunteers. Lymphocytes were cultured and harvested by the Department of Genetics, Children's Hospital, Sheffield. The target metaphase slides were prepared as outlined in Materials and Methods section 2.2.1.5 Preparation of Metaphase Chromosome Spreads - steps 12 and 13. The slides underwent pre-treatment with pepsin digestion and fixation as outlined in Materials and Methods section 2.2.4 Fluorescent *In Situ* Hybridisation. The resulting slides were then used to perform CGH using formalin-fixed paraffin-embedded paired tumour samples pCRC18/pLM18 and pCRC8/pLM8 and also fresh-frozen paired tumour samples fCRC1 and fLM1 (see chapter 6.3). CGH was performed in all cases with nick translation labelled 46XX controls. CGH was finally partially successful for these paired tumour samples, although further experiments to refine the technique could not be performed due to time restriction.

4.2.3 Results of CGH Analysis of Tumour Samples pCRC18, pLM18, pCRC8 and pLM8

No chromosomal abnormality was found for the primary colorectal cancer pCRC18, and was thus deemed not to have been successful. Whilst for its paired liver metastasis (pLM18), the abnormalities detected included gains of chromosomes 2 and 3 and chromosome arms 8q and 13q and loss of chromosome arms 8p and 9q.

For tumour sample pCRC8 again no significant chromosomal abnormality was detected, whilst for pLM8, analysis of the hybridisation was not possible due to high background fluorescence.

Figure 4.9 shows a typical two-colour image of CGH for pLM18 with figure 4.10 showing the DAPI stained image of the same metaphase spread. ULS red was used to label the test tumour DNA and Spectrum Green the normal 46XX reference DNA. The ratio of ULS red and spectrum green fluorescence was plotted along each chromosome ideogram, where chromosomal imbalances were detected on the basis of deviation of the

ratio profile from the balance value (ULS red:Spectrum Green = 1:1). Values of 1.25 and 0.75 were used as respective diagnostic cut-off levels to represent amplification and deletion respectively. Figure 4.11 shows the average ratio profile for pLM18. Figure 4.12 shows the average profile from the analysis of several chromosome spreads, where vertical lines to the left of the chromosome ideogram indicates deletion whilst to the right indicates amplification.

Figure 4.9 A typical two-colour image of pLM18 (tumour DNA labelled Ulysis rhodamine and normal reference DNA labelled Spectrum Green).

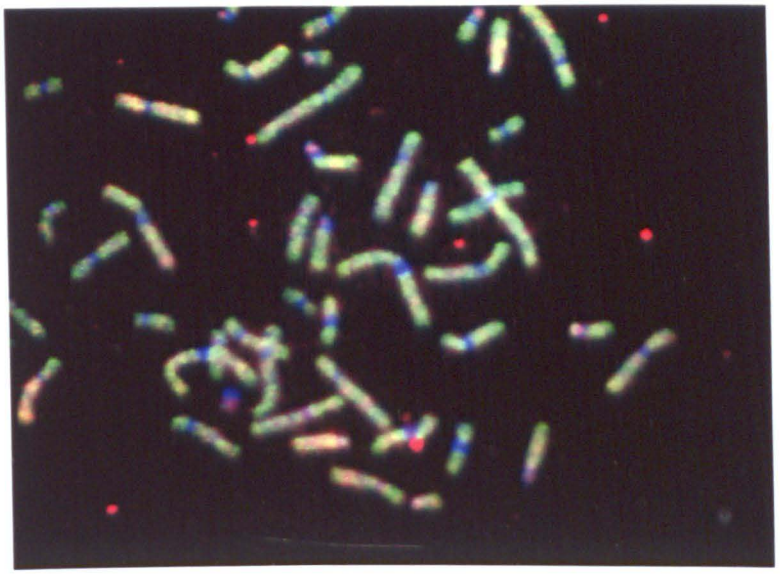


Figure 4.10 The DAPI stained image of the target metaphase spread as seen in figure 4.9, for pLM18.

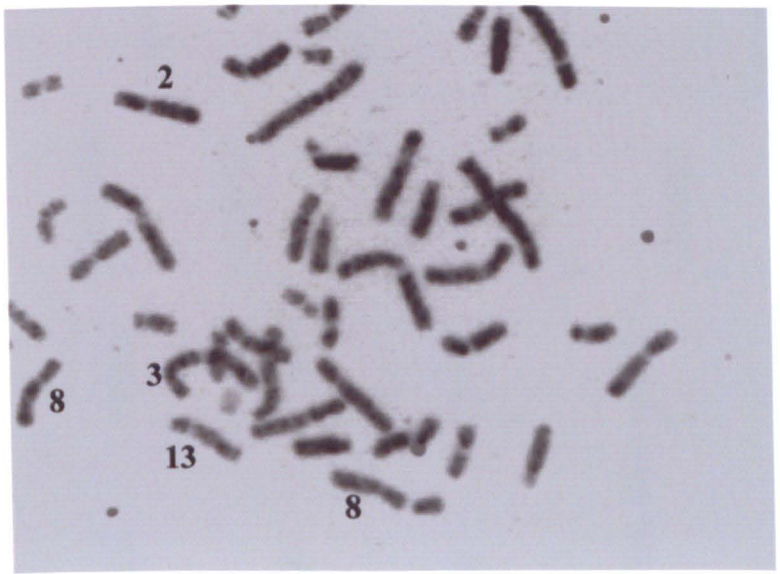


Figure 4.11 The average ratio profile of several metaphase spreads for pLM18.

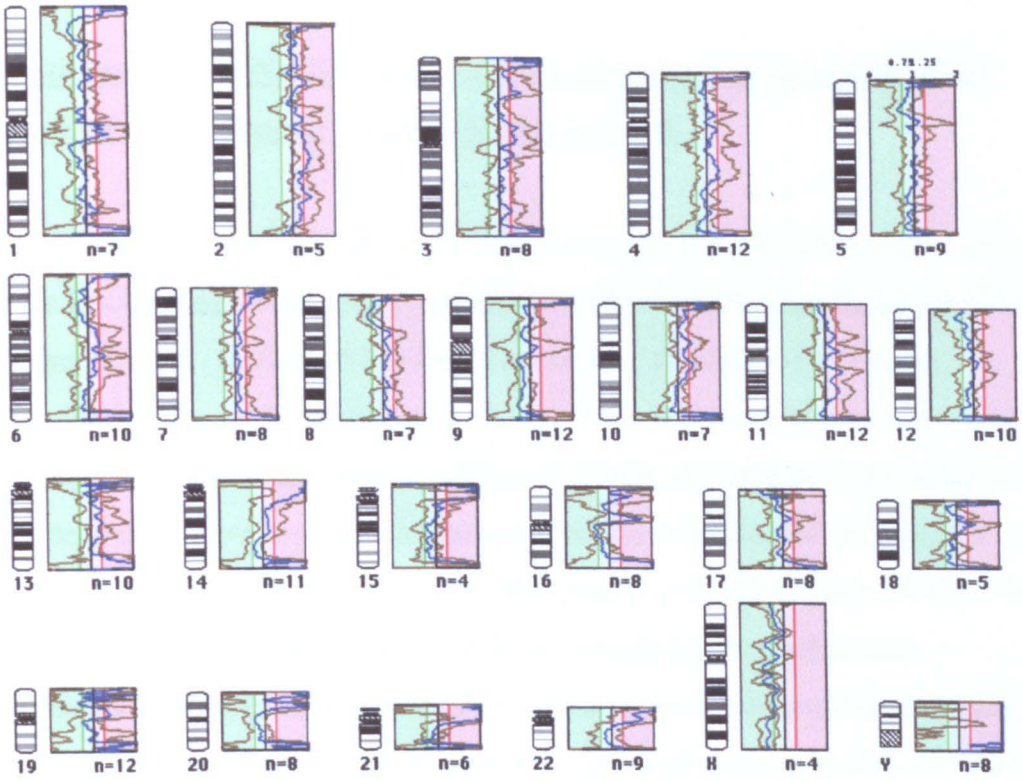
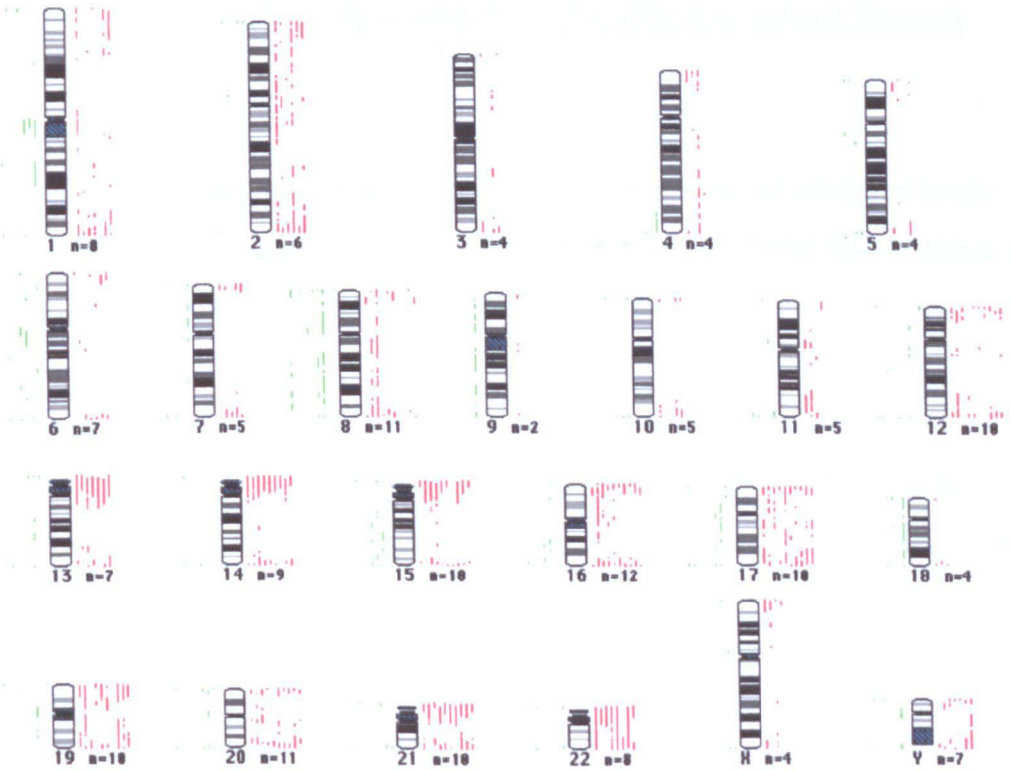


Figure 4.12 The average ratio profile ideogram for pLM18, where vertical lines to the left of the chromosome ideogram indicate deletion whilst to the right indicate amplification.



4.3 DISCUSSION

4.3.1 Optimisation of DNA Extraction and Purification from Formalin-Fixed Paraffin-Embedded Tumour Samples for use in CGH

The foremost problem with extracting DNA from paraffin-embedded tissue material, is that the DNA is rarely well preserved and is usually degraded, resulting in the loss of long fragment DNA (1000-2000 bp) which is essential for CGH, if a uniform hybridisation is to be achieved (Ghazvini et al., 1996). In this study several protocols were utilised with modifications in an attempt to obtain high quality DNA suitable for CGH. Attempts to improve extraction and purification were greeted with limited success, with the optimal protocol utilising the Qiagen QIAamp DNA Mini Kit, which proved to be the most efficient method of DNA extraction and purification. Improvements to the original standard DNA extraction protocol could have included the use of microwave irradiation in order to de-wax the paraffin-embedded tissue material, thus obviating the need for organic solvents and potentially reducing the loss of DNA, although protein contamination would still have been a problem (Banerjee et al., 1995; Diaz-Cano and Brady, 1997; Sato et al., 2001).

4.3.2 Optimisation of CGH on Formalin-Fixed Paraffin-Embedded Tumour Samples

There are several reasons for why more gains or losses were not observed in the tumour specimens, when compare to other studies (Korn et al., 1999; Al-Mullah et al., 1999; Aragane et al., 2001; Nakao et al., 2001). These include:

1. Operator error.
2. Poor image analysis as a result of computer hardware or software deficiencies.
3. Excessive contamination of the extracted tumour DNA samples by normal tissue DNA.
4. Specific gains or losses were so small that the resolution of CGH was unable to visualise such differences.

5. Extremely heterogeneous tumour samples which resulted in any large gains or losses cancelling out.
6. Prolonged tissue fixation resulting in excessively degraded tumour DNA.

Operator error could be discounted because normal 46XX control samples hybridised well and showed normal profiles when analysed. Although, a positive control such as MPE600 was not available for the latter experiments, in the earlier experiments the expected imbalances were observed. With the latter experiments the intensity of the Ulysis rhodamine labelled tumour DNA was not as bright as that of the Spectrum Green labelled normal reference DNA and so initially the captured images were un-analysable by the computer. In an attempt to rectify this, the duration of exposure using each filter during the image capturing process was determined empirically, until an image was obtained which the computer could analyse. This could mean that hybridisation intensity levels along the chromosomes could have been artificially compromised, thus masking regions of gains or losses, the result being an apparently normal tumour DNA analysis. The incorrect pairing of chromosomes due to inadequate DAPI staining could have accounted for the low observance of imbalances, as any significant differences may have been negated when several chromosome ideograms are combined to produce an average profile. Although this was not thought to be a major problem as chromosome pairing was verified by an experienced cytogeneticist (Dr K. Sisley).

Excessive contamination by normal tissue DNA could largely be excluded as tumour tissue was microdissected from slides which were confirmed to contain viable tumour tissue as oppose to necrotic tissue by two independent histopathologists (Dr M. Muzaffer and Dr S. Cross).

An extremely heterogeneous tumour sample could have resulted in non-significant net gain or loss of chromosome material, although this is unlikely as other studies all showed extensive gains and losses of chromosome material (Korn et al., 1999; Al-Mullah et al., 1999; Aragane et al., 2001; Nakao et al., 2001). Conversely, the microdissected tumour sample may have contained material from a specific subclone of cells which only showed minor abnormalities, again this is thought to be unlikely as tumour material was microdissected from several slides from a single paraffin-embedded tissue block. Another possibility, is that the structural changes were so small (<10mb) that the resolution of CGH was unable to visualise such differences.

A final possibility, is that prolonged tissue fixation in an unknown fixative lead to excessive degradation during tumour DNA extraction, resulting in a propensity of short length fragments of DNA (~300-600 bp) which hybridised non-specifically to the target metaphase spread in preference to the longer fragments of DNA (~1000-2000 bp), which in turn would have prevented any significant changes from being visualised (Ghazvini et al., 1996). Long length DNA fragments were confirmed to have been produced as all extracted tumour DNA samples showed a 'smear' when electrophoresed on ethidium bromide stained agarose gels and the use of the Ulysis labelling technique would have prevented DNA fragmentation which is a consequence of the nick translation labelling technique. The use of fresh tumour material would have hopefully eliminated many of these problems.

Thus, a variety of problems could have been responsible for the low numbers of chromosomal aberrations detected, not least the problem with the quality of target metaphase slides, which was in part rectified by the manufacture of in-house target metaphase slides. With additional study successful analysis could have been performed, should time have permitted.

4.3.3 CGH Analysis of Paired Primary and Liver Metastatic Colorectal Cancer

The progression from colorectal adenoma to cancer has been associated with specific chromosomal changes with gains in chromosome arms 13q, 8q and 20q and losses in chromosome arm 18q occurring more frequently in cancers than in adenomas (Meijer et al., 1998). With nearly 90% of all colorectal cancers showing chromosomal abnormalities only a minority exhibit a normal karyotype (Muleris et al., 1990; Bardi et al., 1995). The most common numerical abnormalities are loss of chromosome 18 (26%) and gain of chromosome 7 (25%). Other common abnormalities include losses of chromosomes Y (15%), 17 (13%), 14 (12%) and 22 (12%), and common gains include those of chromosomes 20 (14%) and 13 (12%). Less frequent findings include loss of 1p and gain of 1q, loss of 8p and gain of 8q, loss of 13p and gain of 13q and loss of 17p and gain of 17q (Bardi et al., 1997). More recently, allelic imbalance with loss of chromosome arms 8p (Halling et al., 1999) and 18q (Martínez-López et al., 1998) have been reported to be significantly associated with reduced survival in patients with Dukes B and C staged cancers.

Due to the problems encountered using the CGH technique, significant results were only obtained for pLM18, showing gain of chromosomes 2 and 3, and chromosome arms 8q and 13q, and loss of chromosome arms 8p and 9q. These findings are similar to the results obtained by Korn et al. (1999), who found significant gains of chromosome arms 8q and 13q and loss of 8p amongst others, on examining 26 paired tumour samples using CGH. Al-Mullah et al. (1999), on examining 7 paired samples with CGH, also found gains of chromosome arms 8q and 13q with loss of chromosome 8p. Similarly, Aragane et al. (2001) also found a gain of 8q, in particular 8q23-24, and also associated gain of 8q with a significantly reduced disease-free survival period. Nakao et al., (2001) examined 35 primary tumours, 16 of which had liver metastases and assessed whether there were specific genetic changes associated with either the presence or absence of liver metastases. Although they did not specifically examine the liver metastases for chromosomal abnormalities, they found significant gains in chromosome arms 8q and 13q in primary tumours with liver metastases, whereas they found no changes in 6q or 7q in those primary tumours without evidence of liver metastases. Although only one tumour sample appeared to have successfully hybridised in our study, the results did concur with these other studies.

Of the other abnormalities found by Korn et al. (1999), gain of chromosome arm 20q and loss of chromosome 18 and chromosome 1p were also significant; suggesting that chromosome arm 20q may harbour a candidate oncogene responsible for metastasis. Al-Mullah et al. (1999) also came to a similar conclusion, associating gains of 20q with advanced colorectal cancers and liver metastasis. They also found a significant association between loss of 17p and gains of 6p and 17q with liver metastasis, and postulated that these regions may also harbour genes involved in liver metastasis. Aragane et al. (2001) also found frequent gains of 7p, 7q13-36, 15q21-26, 19p and 20q and loss of 18q1-23 and 5q21. As well as gains of 8q, they associated gains of 20q and loss of 18q with a significantly reduced disease free period. Nakao et al. (2001) found a significant difference in the frequency of chromosomal abnormalities in 6q, 7q, 8q, 13q and 20q, when comparing primary colorectal cancers with and without liver metastases, stating that loss of 17p and 18q and gain of 7q, 8q, 13q and in particular 20q, may influence the occurrence of liver metastases.

Of the studies already mentioned investigating chromosomal abnormalities in primary and liver metastatic colorectal cancer (Korn et al., 1999; Al-Mullah et al., 1999;

Aragane et al., 2001), only Al-Mullah et al. (1999), used formalin-fixed paraffin-embedded tissue, analysing seven paired samples, whereas the other studies were able to analyse fresh-frozen tumour specimens. Al-Mullah et al. (1999) used a phenol-chloroform DNA extraction technique and a random-primed labelling technique with indirect detection when performing CGH. This would have enabled repeat detection washes to enhance the intensity of any hybridisation signals. Although, CGH has been successfully performed on a variety of formalin-fixed paraffin-embedded tumour tissues such as breast (Nishizaki et al., 1997), oesophageal cancer (Van Dekken et al., 1999), uveal melanoma (Aalto et al., 2001) and malignant gliomas (Paunu et al., 2000) to name a few, using a variety of DNA labelling techniques; nick translation (Nishizaki et al., 1997; Aalto et al., 2001; Paunu et al., 2000), and ULS (Alers et al., 1999; Van Dekken et al., 1999; Naus et al., 2001), CGH is still dependant on good quality extracted DNA and target metaphase slides. A technique which may have improved analysis of paraffin-embedded material is DOP-PCR (**d**egenerated **o**ligonucleotide **p**rimer –PCR), which universally amplifies extracted DNA. The amplified DNA can then subsequently be labelled using either nick translation or ULS methods (Zitselsberger et al., 2001). However, meticulous laboratory technique and negative controls are essential to ensure that DNA contamination has not taken place (Lichter et al., 1995), and the fluorescent ratios along the chromosomes can be more variable (Speicher et al., 1993). Time and resources permitting, the DOP-PCR (universal DNA amplification) method would have been the next avenue to be explored in amplifying the extracted DNA from the paired tumour specimens followed by labelling by either technique. Again however, CGH would only be successful if good quality target metaphase slides were available.

4.3.4 Summary

The technique of CGH was developed to analyse the genomic changes in primary colorectal cancers and their liver metastases. Several hurdles were overcome in the development of this technique, including the poor quality of DNA obtained from the formalin-fixed paraffin-embedded tissue samples and the highly variable quality of the purchased target metaphase slides. Time permitting a more extensive analysis of the paired samples would have been undertaken. Though, from the limited data obtained,

which was comparable with other reports, gain of chromosome arms 8q and 13q may be involved in the development of liver metastases.

CHAPTER 5 – MICROSATELLITE ANALYSIS OF PRIMARY AND LIVER METASTATIC COLORECTAL CANCER

CONTENTS

CHAPTER 5

5.1 INTRODUCTION

As mentioned previously, there appears to be two major mechanisms involved in the tumourigenesis of colorectal cancer, chromosomal instability and microsatellite instability (Vogelstein et al., 1988; Fearon and Vogelstein, 1990; Aaltonen et al., 1993; Peltomäki et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993). Although microsatellite instability was initially described in association with hereditary non-polyposis colorectal cancer, up to 20% of sporadic colorectal cancers are also thought to exhibit MSI (Thibodeau et al., 1993; Aaltonen et al., 1993; Kim et al., 1994; Rüschoff et al., 1995). Colorectal cancers with MSI express a distinct phenotype characterised by mucinous growth, location to the right side of the colon, poor histological differentiation and paradoxically a more favourable prognosis (Kim et al., 1994). The presence of MSI has been further categorised to MSI-H (MSI at >30% of microsatellites examined) and MSI-L (<30% of microsatellites examined) (Boland et al., 1998). Whereas MSI-H tumours tend to be associated with inactivation of DNA mismatch repair genes, MSI-L tumours appear to behave similarly to tumours with CIN, with some reports suggesting that most colorectal cancers will exhibit some degree of MSI depending on the selection and number of loci examined (Halford et al., 2002; Laiho et al., 2002). There is also continuing debate as to whether MSI-L tumours really represent a distinct clinicopathological entity (Gonzalez-Garcia et al., 2000; Tomlinson et al., 2002).

Several studies have associated the presence of MSI colorectal tumours in patients with a more favourable prognosis compared to patients with microsatellite stable (MSS) tumours (Thibodeau et al., 1993; Lothe et al., 1993; Gafa et al., 2000; Gryfe et al., 2000; Hemminki et al., 2000). While other studies have stated that any survival advantage is not independent from the stage of the disease i.e. the TNM classification remained the best indicator for prognosis (Messerini et al., 1999; Salahshor et al., 1999; Johannsdottir et al., 1999; Feeley et al., 1999; Curran et al., 2000; Gervaz et al., 2002). Recently, a number of studies have also implicated that individuals with MSI-H tumours are at greater risk of developing metachronous tumours (Sengupta et al., 1997; Masubuchi et al., 1999; Shitoh et al., 2002). Moreover, some studies only selected certain categories of patients (<50 years of age), and few studies assessed the prognostic significance of

MSI in the presence of LOH of 17p and 18q, which are thought to adversely affect prognosis (Jen et al., 1994; Choi et al., 2002). The incidence of MSI in sporadic colorectal cancer has also been found to be related to disease staging, with the incidence increasing proportionately with stage, the incidence having been reported to be 12% for Dukes A cancers and between 20-34% for Dukes B and C cancers (Aaltonen et al., 1994; Bubb et al., 1996; Chen et al., 1997).

The relevance of staging classifications is not limited to prognosis, such systems also determine the administration of any adjuvant therapy. Currently, adjuvant chemotherapy with 5-fluorouracil and levimasole has been limited to patients with Dukes C (TNM stage III) cancers as no survival benefit has been proven for patients with Dukes B (TNM stage II) cancers (Moertel et al., 1990; NIH Consensus Conference, 1990). However, Dukes B staged cancers represent approximately 50% of all colorectal cancers and have a 5-year survival rate of 60-75% with surgery alone, which would suggest that 15-20% of all patients with colorectal cancer, will eventually die of metastatic disease without even being considered for adjuvant therapy (Moertensen et al., 1992). However, identifying this subgroup of patients with genetic techniques has proven to be extremely difficult (Chung, 1998; Rosty et al., 2001).

Liver metastases when present at the time of diagnosis are associated with significant morbidity and mortality, with the median survival time being about 10-15 months (Ballantyne and Quin, 1993). Although surgical resection of the liver metastases improves overall survival, surgery is only indicated in localised metastases which account for only 20-25% of all cases, thus chemotherapy plays an important role in these patients (Geoghegan and Scheele, 1999). Previous studies have indicated that *p53* mutations alter the response to chemotherapeutic agents, conferring resistance to certain agents (Weller, 1998). The presence of MSI has also been implicated in resistance to chemotherapeutic agents (Kinzler and Vogelstein, 1996; Wright et al., 2000).

5.1.1 Aim of this Study

The aim of the study was to assess the presence or absence of MSI in primary colorectal cancer and it's paired liver metastasis (whether present synchronously or metachronously) and compare it to overall survival, specifically to assess whether the

presence of MSI in either the primary or metastatic tumour is associated with a better prognosis.

5.2 RESULTS

A series of 31 paired tumour samples (of both the primary colorectal cancer and its liver metastasis) from 31 patients undergoing liver resection for liver metastases, between January 1997 and December 1999 were analysed for MSI using the PCR technique. Of the 31 patients, 18 were male and 13 were female, the median age was 63 years with an age range 0f 41-82 years. Of the primary tumours analysed, 29 showed moderate histological differentiation, the other two being well differentiated. Five primary tumours were situated in the right colon, 3 in the left colon, 12 in the sigmoid colon and 11 in the rectum. Eighteen had synchronous liver metastases and were thus staged Dukes D (TNM stage IV). Of those which had metachronous liver metastases, the median time between diagnosis of the primary tumour and diagnosis of the liver metastasis was 16.9 months (range 6.3-102.9 months), all being diagnosed by routine ultrasound surveillance. Of the primary tumours with metachronous metastases, 7 were initially staged as Dukes B (TNM stage II) and 7 as Dukes C1 (TNM stage III). Ten patients with tumours staged as either Dukes C or Dukes D received adjuvant chemotherapy and /or radiotherapy, one patient with a Dukes B staged tumour received adjuvant chemotherapy. None of the patients had received neo-adjuvant chemo/radiotherapy. There were no mortalities within 30 days of the liver resection surgery. The median duration of follow-up after resection of the primary tumour was 92 months (range 13-186 months), with 17 patients having died by the end of the study as a result of recurrent disease and one from a myocardial infarction (table 5.1).

The presence of a family history of colorectal cancer could not be assessed as this data was incomplete.

A reference panel of microsatellite primers recommended for the detection of MSI in colorectal cancer were used (Dietmaier et al., 1997; Boland et al., 1998). These included three mononucleotide repeats (BAT25, BAT26 and BAT40) and five dinucleotide repeats (D2S123, APC (D5S346), Mfd15 (D17S250), D10S197, D18S58 and D18S69) and one tetranucleotide repeat (MYCL1) located to loci on chromosome 2p, 4q12, 1p13.1, 2p16, 5q21/22, 17q11.2-q12, 10qter, 18q22.3, 18q21 and 1p32 respectively. (Unfortunately, primers BAT26, D2S123 and MYCL1 failed to amplify, this will be discussed in section 5.3.1 Optimising Conditions for PCR for use on Formalin-Fixed Paraffin-Embedded Tumour Specimens).

MSI-H was defined as instability at 2 or more loci.

Statistical Analysis

Independent samples t-test, Fisher's exact test and the Wilcoxon Mann Whitney U test were performed to determine any significant differences in clinicopathological variables between tumours (primary and/or metastatic) with MSI-H and those without MSI (microsatellite instability stable - MSS). The log-rank test was performed to compare survival, which was represented by Kaplan-Meier survival curves. Logistic regression analysis was performed to predict whether MSI-H tumours (either primary or metastases) were significantly associated with specific clinicopathological variables (age, sex, primary tumour location, stage of the primary tumour, the presence of solitary or multiple metastases, the presence of synchronous or metachronous metastases, the administration of adjuvant chemotherapy or radiotherapy and pre-operative (liver resection) CEA levels). Statistical analysis was performed using SPSS statistical software package on a PC computer, with a p value for significance set at <0.05, but for logistic regression analysis a p value for significance was set at <0.008 using the Bonferroni correction, due to the small sample size.

5.2.1 Optimising Conditions for PCR for use on Formalin-Fixed Paraffin-Embedded Tumour Specimens

Initially 1% agarose gels were used in order to detect any microsatellite instability, however, the resolution obtained was extremely poor (figure 5.1). In order to improve the resolution and to produce tightly defined bands, a 2% agarose gel was used, however even this failed to improve the resolution. As a shift of 1 base pair was needed to be detected in order to define microsatellite instability, it was felt that agarose gels would not provide the necessary resolution. It was therefore decided to run the PCR products on polyacrylamide gels and then to use silver staining to detect the products. These gels did provide a vast improvement on agarose gels, however even using a variety of gels at various concentrations (6%, 8% and 10%) failed to provide the resolution of 1 base pair required (figure 5.2).

Figure 5.1 PCR products electrophoresis on a 1% agarose gel, using primers D18S58, BAT40, APC, BAT25 and D2S123 on samples from case pCRC14. T=primary tumour; N= normal colonic tissue; M= liver metastasis. A 1kb marker has been run in the far left hand lane.

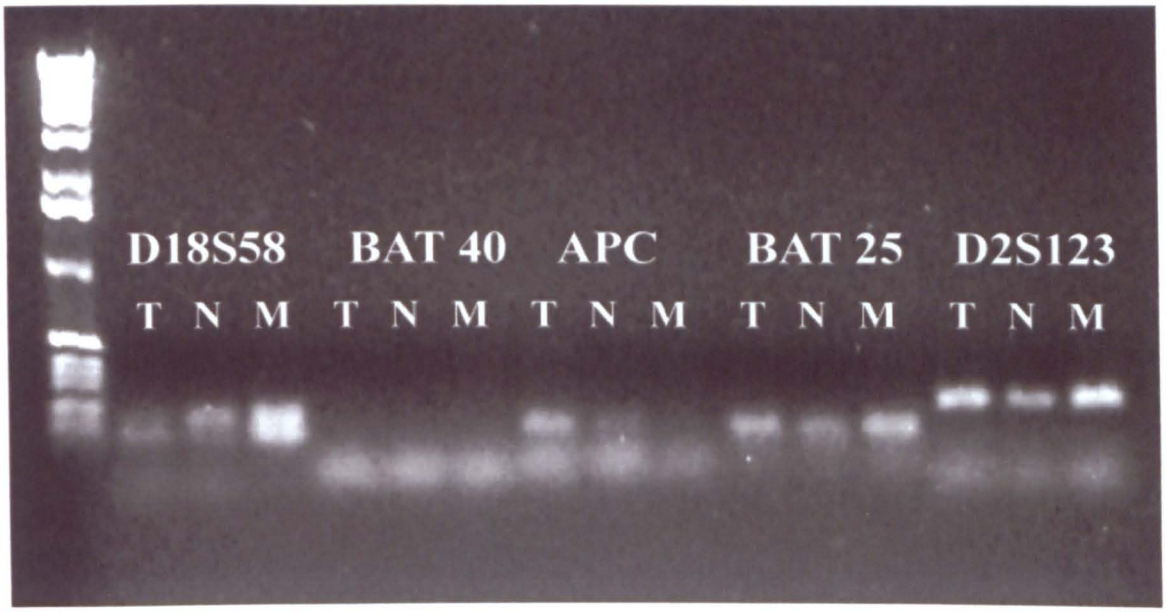
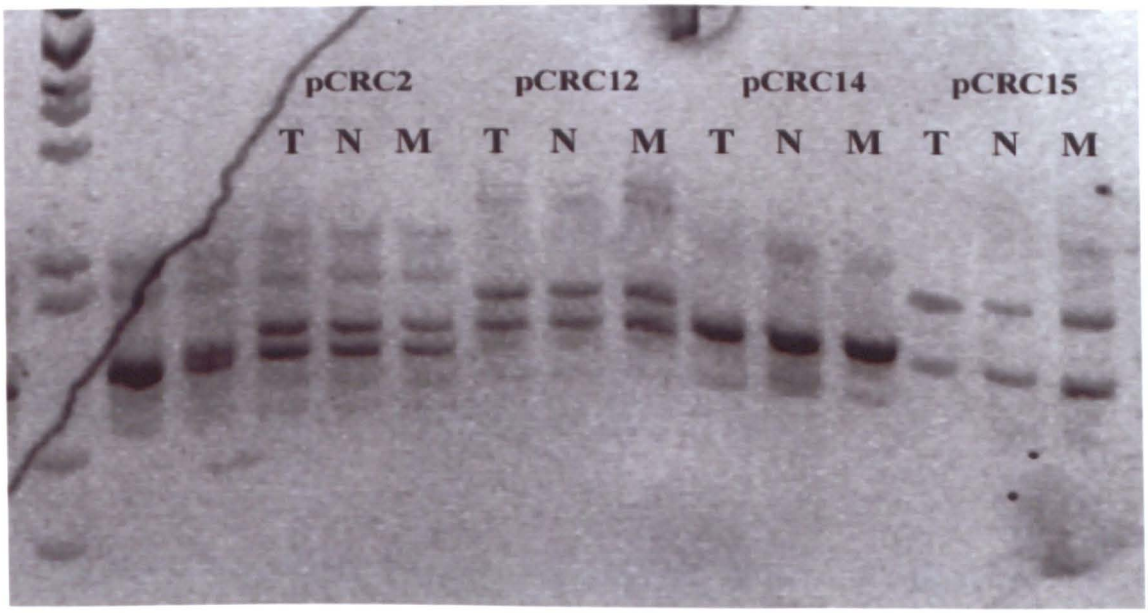
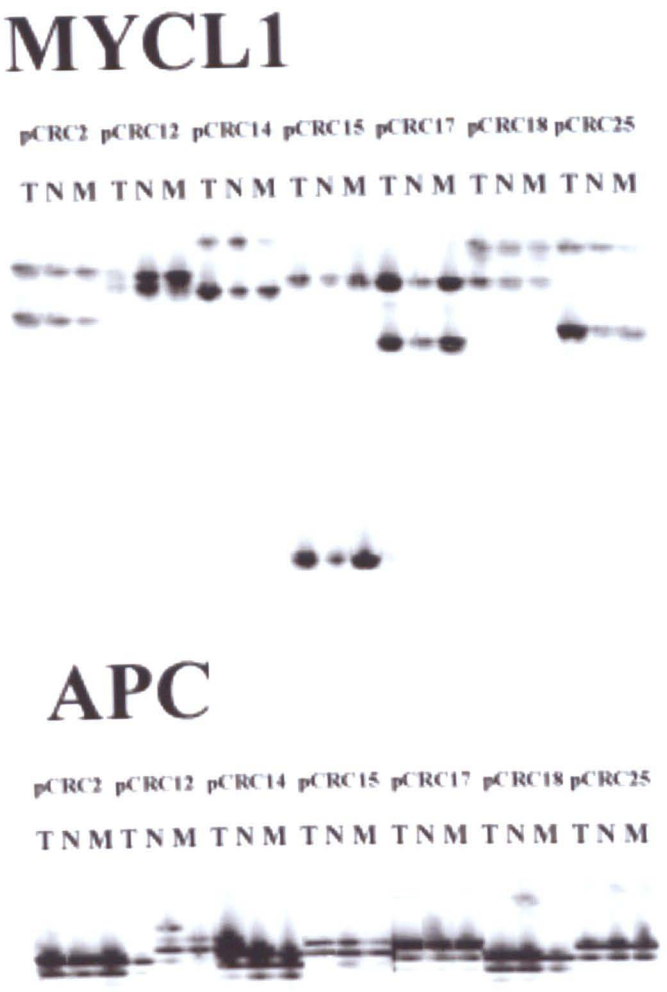


Figure 5.2 PCR products electrophoresis on 8% polyacrylamide gel using the MYCL1 primer on samples from cases pCRC2, pCRC12, pCRC14 and pCRC15. T=primary tumour; N= normal colonic tissue; M= liver metastasis. A 1kb marker has been run in the far left hand lane.



At this point the department purchased a LiCor 4200 LongReadIR automated sequencer which used primers labelled with either IRD 700 or IRD 800 with electrophoresis performed on 25cm LongRanger gels. The resolution produced was to a single base, sufficient to detect any microsatellite instability. Seven paired samples, consisting of the primary colorectal cancer and its paired liver metastases and a normal tissue control microdissected from either normal colonic mucosa or liver parenchyma, were used as test samples (figure 5.3).

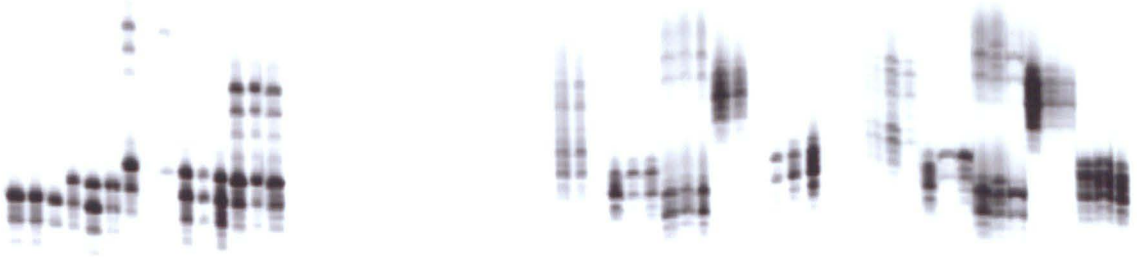
Figure 5.3 PCR products electrophoresis on a 25cm LongRanger gel using primers MYCL1 and APC with samples pCRC2, pCRC12, pCRC14, pCRC15, pCRC17, pCRC18 and pCRC25. T=primary tumour; N= normal colonic tissue; M= liver metastasis.



Once the PCR reactions had been optimised for use on the LiCor automated sequencer, the rest of the tissue samples were microdissected and digested according to

the protocol. However, these samples failed to amplify even after increasing the number of cycles to 35. Subsequently, the volume of DNA added to the PCR reaction was increased from 1µl to 2µl, this produced a very slight improvement with the number of cycles set at 30. So the volume of DNA was increased further to 5µl, however this had the opposite effect and no amplified products were visualised at all. As a final measure, the ph of the original and the recently digested tumour samples were assessed using ph strips. The original samples had a ph of 8.5 whereas the recently digested samples had a ph of 11, which was confirmed using a new ph meter. The ph of the digested samples were corrected, and the PCR reactions repeated. These samples were divided into three groups, one without correcting the ph, a second group using 1µl of the corrected ph and a third group of samples using 2µl of the corrected ph. These were then assessed against the original test samples acting as positive controls. All samples amplified except for the fist group of samples, which had not been ph corrected, thus confirming that the high ph was the reason for the PCR reactions failing to amplify. Both the 1µl and 2µl DNA volumes produced amplified products however, the use of the larger volume of DNA produced an increase in non-specific products, thus 1µl was sufficient for most reactions (figure 5.4).

Figure 5.4 Results of the pH correction experiment performed on the second batch of microdissected formalin-fixed paraffin-embedded tumour samples. PCR reaction performed using the APC primer with electrophoresis on 25cm LongRanger gels, with the original samples as controls in the far left hand lanes.



Original samples – 1µl of DNA used for each reaction

Second batch samples without pH correction

Second batch samples + 22µl 3M Tris HCl – 1µl of DNA used for each reaction

Second batch samples + 22µl 3M Tris HCl – 2µl of DNA used for each reaction

Having successfully produced amplified products using the IRD labelled primers with LongRanger gel electrophoresis, three of the primers subsequently failed to amplify these were BAT26, D2S123 and MYCL1. The other primers continued to amplify suggesting that the problem possibly existed with primers. Since they had previously amplified a possible explanation was that continual freeze-thawing of the primers (during retrieval from storage at -20°C), had somehow cleaved the fluorescent IRD label off the primers, another explanation being gradual degradation of the tumour DNA samples, as they had been stored at 4°C. A further batch of labelled BAT26 was purchased as some studies have suggested that BAT26 is the perhaps the most sensitive marker for MSI assessment in colorectal cancers (Dietmaier et al., 1997; Cravo et al., 1999), however, even this new batch failed to amplify with our DNA, suggesting indeed that the DNA had started to degrade. Due to limited resources and time, further tumour samples could not be microdissected or labelled primers be purchased. Thus, only seven microsatellite markers were successfully analysed (BAT25, BAT40, APC (D5S346), Mfd15 (D17S250), D10S197, D18S58 and D18S69).

5.2.2 Results of Microsatellite Analysis of Primary Colorectal Cancers and their Liver Metastases

Of the 31 primary tumours analysed 3 tumours (9.7%) exhibited microsatellite instability at two or more loci, thus were considered as MSI-H, six tumours (19.4%) exhibited MSI at one locus and were considered to be MSI-L (table 5.2). When considering MSI-H and MSS primary colorectal cancers, there was no significant statistical difference in age ($p=0.721$), sex ($p=>0.95$), pre-operative CEA levels ($p=0.749$), Dukes stage ($p=0.349$), TNM stage ($p=0.598$), histological differentiation ($p=0.875$), the site of the primary tumour ($p=0.122$), the presence of either synchronous ($p=>0.95$) or metachronous liver metastases ($p=>0.95$), presence or absence of recurrence ($p=0.101$) or in survival ($p=0.3702$). However, there was a significant difference in the presence of either solitary or multiple liver metastases and MSI-H tumours, where the presence of MSI-H in primary tumours was significantly associated with solitary liver metastasis ($p=0.049$), (table 5.3).

Of the 31 liver metastases analysed 4 tumours (12.9%) were considered as MSI-H, whereas 11 tumours (35.5%) were considered as MSI-L (table 5.2). When considering

MSI-H and MSS colorectal cancer liver metastases, there was no significant difference in age (p=0.523), sex (p=>0.95), pre-operative CEA levels (p=0.635), the presence or absence of recurrence (p=0.333) or in survival (p=0.3972). However, there was also a significant difference in the presence of either solitary or multiple liver metastases and MSI-H tumours, where again the presence of MSI-H in liver metastases was significantly associated with solitary liver metastasis (p=0.016), (table 5.3).

Three primary tumours exhibited MSI-H (CRC17, CRC89 and CRC94) of these the paired liver metastases of CRC17 and CRC89 also exhibited MSI-H with MSI present at the same loci in both the primary and metastasis, whilst the paired liver metastasis of CRC94 exhibited MSI-L with MSI present at one locus similar to that of the primary tumour (figure 5.5). CRC17 was a Dukes D staged tumour located in the left colon with a solitary liver metastasis. The patient did not receive any adjuvant therapy and survived for 96 months after the original colonic surgery before succumbing to recurrent liver disease. CRC89 was a Dukes C1 staged caecal tumour with a solitary metachronous liver metastasis diagnosed 41.2 months after the primary tumour had been resected. The patient did receive adjuvant chemotherapy after the original surgery and is currently alive and well. While CRC94 was staged as a Dukes D sigmoid tumour with a solitary liver metastasis, the patient did not receive any adjuvant therapy and is currently alive and well. The liver metastases from CRC15 and CRC34 also exhibited MSI-H, where the primary tumours were staged as Dukes C1 and Dukes B, and situated in the left colon and rectum respectively. Primary tumour CRC15 exhibited MSI-L, whereas CRC34 was MSS. Both liver metastases were solitary, diagnosed 2.5 months and 22.9 months after the original colorectal surgery, with patient CRC34 receiving adjuvant chemotherapy after the original rectal surgery and is currently alive and well. However, patient CRC15 died 67 months after the original colonic surgery, succumbing to peritoneal recurrence.

There appeared to be a trend for improved survival in patients with primary tumours exhibiting MSI-H compared to those without MSI (MSS), although this was not statistically significant (p=0.3702), (figure 5.6), nor was there any significant difference in survival between metastatic tumours exhibiting MSI-H and those without MSI (MSS), (p=0.3972), (figure 5.7). When considering the initial stage of the disease for all the primary colorectal cancers, there also appeared to be a trend for improved survival in patients with Dukes B staged primary tumours, although as expected, since all patients

at some point developed metastases, this was not statistically significant (p=0.3454), (figure 5.8).

Logistic regression analysis of the relationship between the presence of MSI-H in either the primary or metastatic tumour and possible risk factors such as age, sex, primary tumour location, stage of the primary tumour, the presence of solitary or multiple metastases, the presence of synchronous or metachronous metastases, the administration of adjuvant chemotherapy or radiotherapy and pre-operative CEA levels showed that MSI-H tumours were significantly associated with the presence of solitary metastases (p=0.007), (table 5.4).

Table 5.1 Results of MSI status and clinicopathological characteristics.

Case (pCRC)	Sex	Age	Primary site	Dukes stage	TNM stage	Pre-op CEA levels (mg/ml)	Differentia -tion	Adjuvant chemo-therapy	Adjuvant radio-Therapy	Synchronous/ metachronous liver metastases	Time to metachronous metastases (months)	Number of metastases: solitary/ multiple (number of tumours)	Recurrence - site	Status (Dead/ Alive)	Duration of survival (months)	Primary (MSS/ MSI-L/ MSI-H)	Metastasis (MSS/ MSI-L/ MSI-H)
2	F	53	Sigmoid	D	T3N0M1	0.9	Moderate	No	No	Synchronous	N/A	Multiple (2)	Yes - lung	Dead	119	**MSS**	**MSS**
9	F	62	Rectum	B	T3N0M0	25.0	Moderate	No	No	Metachronous	16.9	Solitary	Yes - liver	Dead	170	**MSS**	**MSS**
11	M	74	Rectum	C	T4N2M0	2350.0	Moderate	Yes	Yes	Metachronous	15.1	Multiple (3)	Yes - liver	Dead	66	**MSS**	**MSS**
12	M	70	Sigmoid	D	T3N1M1	7.7	Well	Yes	Yes	Synchronous	N/A	Multiple (4)	Yes - abdo	Dead	49	**MSS**	**MSS**
14	M	54	Rectum	D	T3N2M1	500.0	Moderate	Yes	No	Synchronous	N/A	Multiple (5)	Yes - pelvis	Dead	44	**MSS**	**MSS**
15	F	79	Left	C	T4N1M0	17.4	Moderate	No	No	Metachronous	2.5	Solitary	Yes - abdo	Dead	67	**MSI-L**	**MSI-H**
17	F	74	Left	D	T3N1M0	742.0	Moderate	No	No	Synchronous	N/A	Solitary	No	Dead	96	**MSI-H**	**MSI-H**
18	M	57	Rectum	D	T3N1M1	31.0	Moderate	No	Yes	Synchronous	N/A	Solitary	No	Alive	140	**MSS**	**MSS**
19	M	82	Rectum	D	T3N0M1	385.0	Moderate	No	No	Synchronous	N/A	Multiple (3)	Yes - abdo	Dead	46	**MSS**	**MSS**
25	M	66	Rectum	B	T3N0M0	6140.0	Moderate	No	No	Metachronous	15.7	Multiple (2)	Yes - lung	Dead	150	**MSS**	**MSI-L**
27	F	69	Sigmoid	C	T4N1M0	6.0	Moderate	Yes	No	Metachronous	5.3	Multiple (3)	No	Dead	36	**MSS**	**MSS**
30	F	61	Sigmoid	D	T3N2M1	20.0	Moderate	No	No	Synchronous	N/A	Multiple (2)	Yes - liver	Dead	52	**MSS**	**MSS**
32	M	67	Sigmoid	D	T3N1M1	6.0	Moderate	Yes	No	Synchronous	N/A	Multiple (2)	Yes - liver	Dead	88	**MSS**	**MSS**
34	M	65	Rectum	B	T2N0M0	4.0	Well	Yes	No	Metachronous	22.9	Solitary	No	Alive	181	**MSS**	**MSI-H**
35	M	49	Rectum	D	T2N1M1	47.8	Moderate	Yes	No	Synchronous	N/A	Solitary	No	Alive	170	**MSS**	**MSS**
38	F	68	Sigmoid	D	T3N0M1	6.9	Moderate	No	No	Synchronous	N/A	Multiple (3)	No	Dead	13	**MSS**	**MSI-L**
43	M	55	Rectum	D	T4N2M1	60.2	Moderate	No	No	Synchronous	N/A	Multiple (5)	Yes - lung	Dead	71	**MSI-L**	**MSI-L**
46	F	76	Right	D	T3N0M1	2.9	Moderate	No	No	Synchronous	N/A	Solitary	No	Alive	129	**MSS**	**MSI-L**
47	F	47	Rectum	C	T4N1M0	2842.0	Moderate	No	Yes	Metachronous	3.7	Multiple (3)	Yes - liver	Dead	66	**MSI-L**	**MSI-L**
48	F	51	Rectum	B	T2N0M0	7.6	Moderate	No	Yes	Metachronous	21.5	Multiple (2)	No	Alive	158	**MSS**	**MSI-L**
49	M	72	Right	D	T3N1M1	87.9	Moderate	No	No	Synchronous	N/A	Multiple (3)	Yes - liver	Dead	57	**MSS**	**MSI-L**
58	F	47	Sigmoid	D	T3N1M1	3.2	Moderate	No	No	Synchronous	N/A	Multiple (2)	Yes - liver	Dead	38	**MSS**	**MSS**
59	M	52	Right	D	T3N0M1	54.0	Moderate	No	No	Synchronous	N/A	Solitary	Yes - liver	Alive	89	**MSS**	**MSS**
62	F	63	Sigmoid	B	T3N0M0	16.1	Moderate	No	No	Metachronous	14.4	Multiple (2)	Yes - lung	Dead	115	**MSS**	**MSS**
70	M	70	Sigmoid	C	T3N1M0	82.3	Moderate	Yes	No	Metachronous	18.7	Multiple (2)	No	Alive	103	**MSS**	**MSS**
74	M	64	Sigmoid	D	T2N0M1	1.0	Moderate	No	No	Synchronous	N/A	Multiple (2)	No	Alive	97	**MSS**	**MSS**
77	M	45	Sigmoid	D	T3N0M1	1.9	Moderate	No	No	Synchronous	N/A	Multiple (2)	No	Alive	92	**MSI-L**	**MSI-L**
89	M	41	Right	C	T2N1M0	0.8	Moderate	Yes	No	Metachronous	41.2	Solitary	No	Alive	186	**MSI-H**	**MSI-H**
94	M	63	Sigmoid	D	T3N1M1	5.6	Moderate	No	No	Synchronous	N/A	Solitary	No	Alive	80	**MSI-H**	**MSI-L**
95	M	58	Left	C	T3N1M0	276.0	Moderate	Yes	Yes	Metachronous	35.4	Solitary	No	Alive	171	**MSI-L**	**MSI-L**
96	F	52	Right	B	T3N0M0	929.1	Moderate	No	No	Metachronous	25.0	Solitary	No	Alive	142	**MSI-L**	**MSI-L**

Table 5.2 Results of the microsatellite marker analysis showing the number and loci affected for each case (+ = MSI; - = MSS).

Case (pCRC)	Primary BAT25	Metastasis BAT 25	Primary BAT40	Metastasis BAT 40	Primary APC	Metastasis APC	Primary Mfd15	Metastasis Mfd15	Primary D10S197	Metastasis D10S197	Primary D18S58	Metastasis D18S58	Primary D18S69	Metastasis D18S69	Primary MSI-L (no. loci)	Primary MSI-H (no. loci)	Metastasis MSI-L (no. loci)	Metastasis MSI-H (no. loci)
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	+	-	-	+	+	-	-	+(1/7)	-	-	+(2/7)
17	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	+(2/7)	-	+(2/7)
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+(1/7)	-
27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+(2/7)
35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+(1/7)	-
43	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+(1/7)	-	+(1/7)	-
46	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+(1/7)	-
47	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+(1/7)	-	+(1/7)	-
48	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+(1/7)	-
49	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-
58	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
59	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
62	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
74	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
77	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+(1/7)	-	+(1/7)	-
89	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	+(4/7)	-	+(2/7)
94	-	-	-	-	+	-	-	-	+	+	-	-	-	-	-	+(4/7)	+(1/7)	-
95	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+(1/7)	-	+(1/7)	-
96	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+(1/7)	-	+(1/7)	-

Figure 5.5 PCR products electrophoresis for cases pCRC15, pCRC17, pCRC34, pCRC89 and pCRC94 on a 25cm LongRanger gel. Shifts in bands suggesting MSI are exhibited in the metastasis at locus Mfd15 and in both the primary and metastasis at locus D18S58 in case pCRC15, whilst case pCRC17 exhibits MSI in both the primary and metastasis. Case pCRC34 exhibits MSI in the metastasis only at loci BAT25 and BAT40, whilst pCRC89 exhibits MSI in both the primary and metastasis at loci BAT25, BAT40, APC and D18S58. Case pCRC94 exhibits MSI in the primary only at locus APC but in both the primary and metastasis at locus D10S197. T=primary tumour; N= normal colonic tissue; M= liver metastasis.

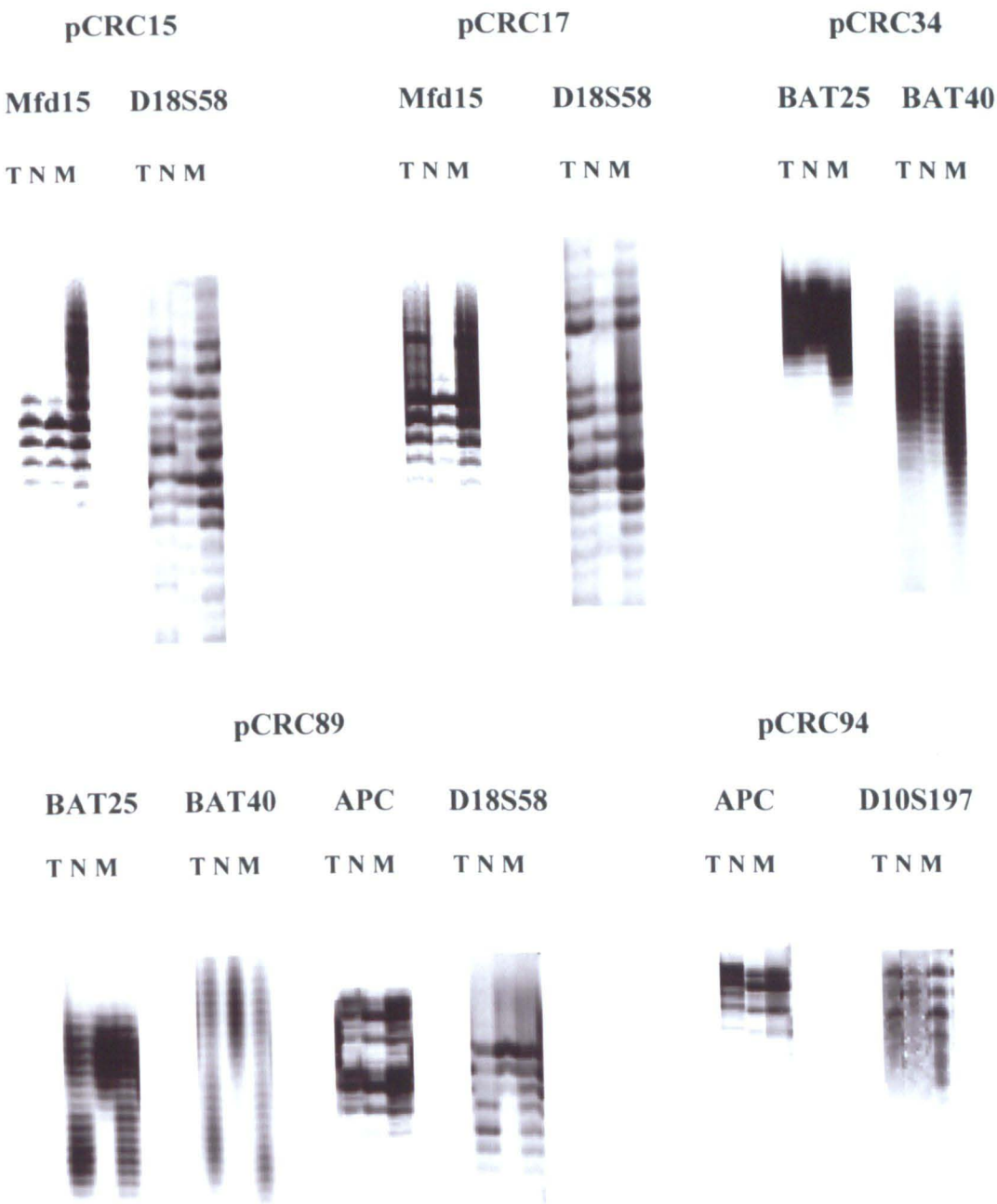


Table 5.3 Results of univariate statistical analysis.

	All cases	Primary tumour				Liver metastasis			
		MSS	MSI-L	MSI-H	p value for significance	MSS	MSI-L	MSI-H	p value for significance
Total No.	31	22 (71%)	6 (19%)	3 (10%)		16 (52%)	11 (35%)	4 (13%)	
Mean age (SD)	61.5 (10.7)	63.3 (9.4)	56 (12.3)	59.3 (16.8)	**0.721**	62.1 (9.7)	59.4 (10.4)	64.8 (16.9)	**0.523**
Gender									
Male	18	13	3	2	**>0.95**	10	6	2	**>0.95**
Female	13	9	3	1		6	5	2	
Primary site									
Right	5	3	1	1	**0.122**	N/A	N/A	N/A	**N/A**
Left	3	0	2	1					
Sigmoid	12	10	1	1					
Rectum	11	9	2	0					
Proximal/Distal[1]	5/26	3/19	1/5	1/2	**0.422**				
Dukes Stage									
B	6	5	1	0	**0.349**	N/A	N/A	N/A	**N/A**
C	7	3	3	1					
D	18	14	2	2					
Mean Pre-op CEA levels (mg/ml), (SD)	472.9 (1242)	444.8 (1367)	687.7 (1112)	249.5 (426.6)	**0.749**	221.0 (586.2)	941.8 (1922)	191.1 (367.4)	**0.635**
Differentiation									
Well	2	2	0	0	**0.875**	N/A	N/A	N/A	**N/A**
Moderate	29	20	6	3					
Synchronous metastases	18	14	2	2	**>0.95**	N/A	N/A	N/A	**N/A**
Metachronous Metastases	13	8	4	1					
Solitary metastases	12	6	3	3	**0.049**	4	4	4	**0.016**
Multiple metastases	19	16	3	0		12	7	0	
Recurrence									
Absent	15	9	3	3	**0.101**	13	8	4	**0.333**
Present	16	13	3	0		3	3	0	
Status									
Alive	13	8	3	2	**0.558**	5	6	2	**>0.95**
Dead	18	14	3	1		11	5	2	

[1]Proximal colon is defined as sites proximal to the splenic flexure.

Table 5.4. Results of logistic regression analysis of the clinicopathological variables.

	MSI-H Primary tumour		MSI-H Liver metastasis	
Variable	**Roa's score statistic**	**p value for significance**	**Roa's score statistic**	**p value for significance**
Age	0.138	**0.710**	0.442	**0.506**
Sex	0.101	**0.751**	0.123	**0.726**
Primary site (right sided tumours)	0.727	**0.394**	0.267	**0.605**
Dukes' Stage	0.839	**0.360**	N/A	**N/A**
TNM Stage	0.416	**0.519**	N/A	**N/A**
Pre-operative CEA levels	0.111	**0.739**	0.244	**0.621**
Histological differentiation	0.229	**0.632**	N/A	**N/A**
Synchronous/Metachronous metastases	0.101	**0.751**	2.062	**0.151**
Solitary/Multiple metastases	5.259	**0.022**	7.272	**0.007**

Figure 5.6 Kaplan-Meier survival curves for all deaths in patients with primary colorectal cancers exhibiting MSI-H and primary colorectal cancers classified as MSS or MSI-L.

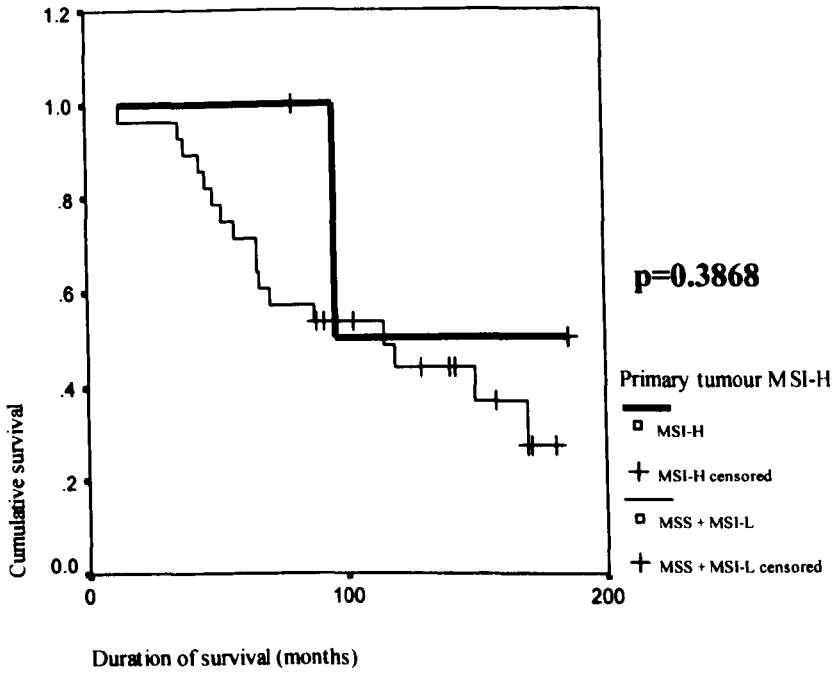


Figure 5.7 Kaplan-Meier survival curves for all deaths in patients with primary colorectal cancers exhibiting MSI-H and primary colorectal cancers classified as MSS or MSI-L.

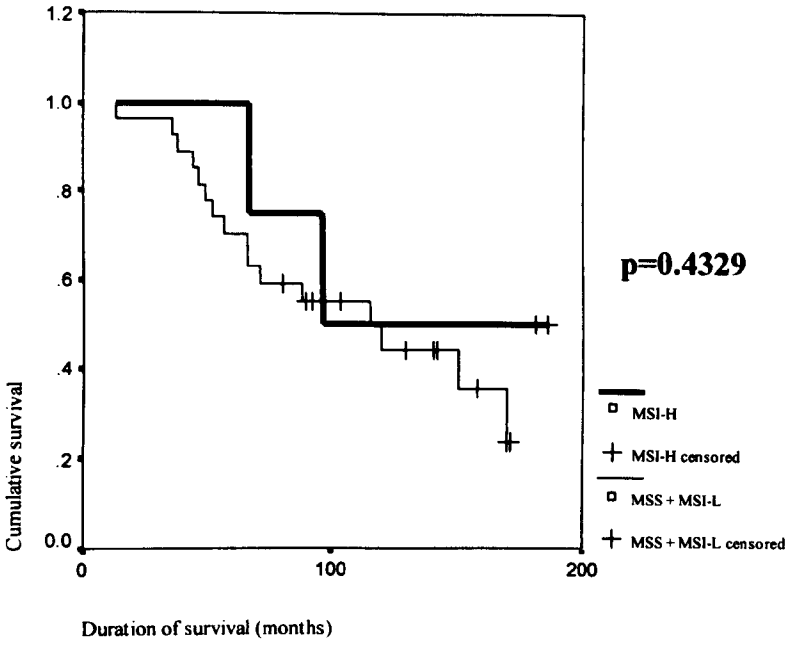
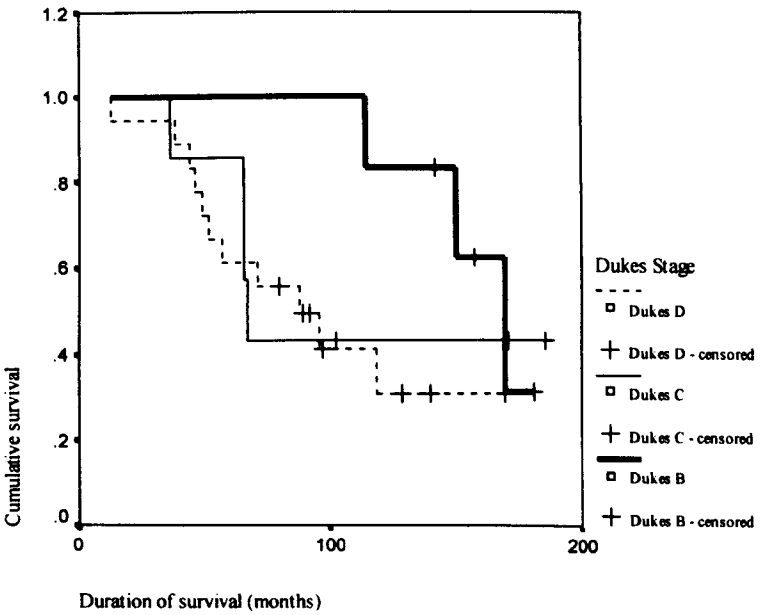


Figure 5.8 Kaplan-Meier survival curves for all deaths in patients with originally staged Dukes B, C and D primary colorectal cancers.



5.3 DISCUSSION

The incidence of MSI-H in this study was 9.7% (3/31) for primary tumours and 12.9% (4/31) for liver metastases. The only significant association in this study being the presence of MSI-H in the primary tumour and the development of solitary liver metastasis; however, this did not translate into an improved prognosis.

Several studies have already been undertaken exploring the presence of microsatellite instability in primary and metastatic colorectal cancer (Ishimaru et al., 1995; Chen et al., 1997; Kochhar et al., 1997; Schneider et al., 2000).

Ishimaru et al. (1995), examined 80 primary fresh-frozen primary tumours and 36 fresh-frozen liver metastases, in 14 cases the primary tumour and liver metastases were obtained from the same patient. The incidence of MSI was examined using 8 radiolabelled microsatellite markers; one mononucleotide repeat (RB intron 21), five dinucleotide repeats (D3S1284, D14S65, D14S78, LPL and D8S167), one tetranucleotide repeat (RB intron 20) and one pentanucleotide repeat (*p53* intron 1). MSI was detected in 20.1% (17/80) primary tumours including five which showed MSI at two or more loci, while the incidence in liver metastases was 22.2% (8/36), however, there was only one case where MSI was present at more than one locus in the liver metastases group. Of the 14 paired primary and metastatic tumours, three pairs showed evidence of MSI in both the primary and metastatic tumour, but only at one locus, where MSI was present at the same locus in both the primary and metastasis. There were no cases where MSI was present in the metastasis but not in the primary tumour. The authors concluded that MSI occurred early in colorectal cancer tumourigenesis and that clones of cells with instability at different loci metastasized and grew preferentially at different sites, whether this is in the colon or liver. They found no significant relationship between the presence or absence of MSI and clinicopathological variables. Although when comparing the MSI-L (12 primary and 1 metastasis) and MSI-H (5 primary and 1 metastasis) groups, significant associations were found between MSI-H and right sided tumour location and allelic retention (i.e. no LOH) on chromosomes 17p and 18q. However, they concede that the number of tumours in these subgroups were small.

Chen et al. (1997), examined 30 patients with Dukes D staged primary tumours and their paired liver metastases, extracting DNA from paraffin-embedded tissue and examining MSI at 4 loci; Mfd-27, Mfd-26, TP53 and D2S123 representing sites on the *APC*, *DCC* and *p53* genes and on chromosome 2 respectively. They detected MSI in 30% (9/30) primary tumours and in 43.3% (13/30) of the liver metastases. Although there appeared to be a trend for better survival in Dukes D staged patients with MSI compared to those without MSI, this was not statistically significant. In 8 patients, MSI was found at the same loci in both the primary and metastatic tumour, but more interestingly they also found MSI present only in the liver metastasis in four cases. They noted that MSI was detected at the TP53 and D2S123 loci in these four cases, which tend to be involved in the later stages of colorectal cancer tumour progression. At first glance the results of Chen et al. (1997) are contradictory to those of Ishimaru et al. (1995), where Chen et al. (1997) found a higher incidence of MSI in the metastatic tumours (a similar finding in our study), but this may partly be attributed to sample size or the number and selection of microsatellite loci. Chen et al. (1997) concluded that MSI was associated with progression in disease staging and in the metastatic process in sporadic colorectal cancer as a consequence of either increased genetic instability with tumour progression, or clonal selection by the metastatic process, which has been demonstrated in gastric cancer (Chong et al., 1994) and small cell lung cancer (Merlo et al., 1994).

The incidence of MSI in liver metastases was reported to be 2.5% in a study by Kochhar et al. (1997), after examining 141 liver metastases and examining MSI with markers located to chromosomes 5q, 8p, 10q, 15q, 17p, 18p and 18q. Concluding that this extremely low frequency may have been as a result of selection of patients with Dukes D primary tumours who had potentially curative resection for metastatic colorectal cancer and who represent a small fraction (20-25%) of all patients with metastatic disease. However, one would assume that in the studies by Ishimaru et al. (1995) and Chen et al. (1997) those patients had also undergone potentially curative liver resection. They also reported that allelic imbalance (LOH) was present with at least one marker in 87% of all tumours analysed and that allelic imbalance on 17p was significantly associated with extra-hepatic disease an extra-hepatic lymph node involvement, thus resulting in reduced survival. Allelic imbalance in primary colorectal cancer and liver metastases has also been investigated by Thorstensen et al. (1996), who

examined the presence of allelic imbalance in 12 primary tumours, 15 local recurrences and 22 distant metastases (20 liver metastases and 2 lung metastases), assessing allelotypes at 43 microsatellite loci throughout the genome. Two of the twelve primary tumours (16.7%) showed evidence of allelic imbalance, whereas more than 20% of the distant metastases exhibited allelic imbalance, with the greatest incidence of allelic imbalance in these metastases being on chromosome arms 14q, 17p, 18p and 18q. However, the authors stated that the frequency of allelic imbalance was influenced by the percentage of normal cells present in the biopsy from which the DNA had been extracted, where the proportions of normal cells was indirectly related to the frequency of allelic imbalance seen, which may have accounted for the low frequency seen in primary tumours, where the percentage of tumour cells was less than fifty percent. Allelic imbalance could not be reliably assessed in our study as the concentration of DNA in each sample of primary tumour, normal colonic and metastatic tumour tissue was variable.

A more recent study by Schneider et al. (2000), found the incidence of MSI in primary tumours to be 15% (6/39). However, no MSI was detected in 29 liver metastases examined, when using a panel of 25 microsatellite markers. However, the authors stated that a major shortcoming of the study was that primary and metastatic tumours were not from the same patient and tumours had been resected in different centres in different countries.

It is not immediately apparent why patients with tumours exhibiting microsatellite instability should develop fewer metastases, as compared to patients with MSS tumours, a possible reason is statistical error due to the small number of tumours analysed. Interestingly, Bocker et al. (1996), reported a significantly lower proliferative capacity in microsatellite unstable sporadic colorectal cancers as oppose to MSS tumours, speculating that the genetic instability initiated by defective DNA mismatch repair, leads not only to tumour formation but also affects genes involved in tumour progression and possibly leads to early cell death as a result of lethal mutations. Defective mismatch repair could result in the production of dysfunctional cell-cycle proteins or novel cell-associated neoantigens, thus activating an immune response (Nicholl and Dunlop, 1999). Concordant with this suggestion is the marked lymphocytic infiltration seen in colorectal cancers exhibiting MSI, which has been indicated as a positive prognostic indicator, similarly gastric cancers with MSI are also associated with lymphocytic

infiltration and also appear to be associated with an improved prognosis (Seruca et al., 1995). Thus, a possible explanation for the significant association between the presence of MSI and solitary liver metastases is that MSI not only acts to contribute to tumour initiation it also paradoxically restraints tumour progression and if it is assumed that liver metastases can themselves metastasize (Fidler, 1990), the presence of MSI in tumours could possibly diminish the occurrence of metastases from the initial liver metastasis, thus accounting for solitary metastatic tumours.

5.3.1 Summary

The incidence of MSI-H in primary tumours (9.7%) and liver metastases (12.9%) in this study were slightly less than those reported in other studies (Ishimaru et al., 1995; Chen et al., 1997), however, this may have been reflected in the number and selection of markers used. The finding that the incidence of MSI in metastases being higher than in primary tumours is in agreement with Chen et al. (1997). This study also found a significant association between MSI-H status and the likelihood of solitary metastases, although this did not translate into a statistically significant survival advantage. Of the five patients with MSI-H tumours (one primary tumour MSI-H, three primary and liver metastases MSI-H and one liver metastasis MSI-H) two patients had died, both from recurrent disease. Shortcomings of this study include the small sample size, which may have concealed a clinically important difference, and possibly the small number of microsatellite markers used. Ideally, the amplification of all ten primers, especially BAT26, would have assured that no tumour had been classified as MSS when instability was in fact present (Cravo et al., 1999). The administration of adjuvant chemotherapy to patients CRC34 and CRC89 may have selected for clones with MSI-H, thus tending to corroborate the conclusions drawn by Fink et al. (1996) and Carethers et al. (1999), both observing that the presence of defective DNA mismatch repair in both *in vitro* and animal studies was associated with 5-fluorouracil and platinum chemotherapeutic drug resistance. However, both these patients continue to be alive and well and so it is unclear whether this possibility of drug resistance, really does have any clinically detrimental affect. Whether, MSI-H liver metastases behave differently to MSS tumours and lead to differing prognoses, will only be borne out by larger prospective studies.

CHAPTER 6 - FISH, CGH AND MICROSATELLITE ANALYSIS OF A SINGLE FRESH-FROZEN PRIMARY COLORECTAL CANCER AND ITS LIVER METASTASIS

CONTENTS

CHAPTER 6

During the course of the study ethical approval was sought and obtained to commence the prospective collection, storage and analysis of fresh tumour samples of primary colorectal cancers with synchronous liver metastases. But as a consequence of a subsequent change in policy within the North Trent region, surgeons were advised not to sample liver metastases as the possibility for curative resection of liver metastases could be compromised. Thus, only one fresh-frozen sample of a primary colorectal cancer with its liver metastasis was available for analysis. Nevertheless, analysis of this one paired sample allowed us to assess the full potential of the various techniques already discussed for any future prospective studies. The following is a discussion of the results obtained for fresh-frozen tumour sample fCRC1 and fLM1.

The tumour specimens were obtained intra-operatively and stored at -80°C.

Clinicopathological data

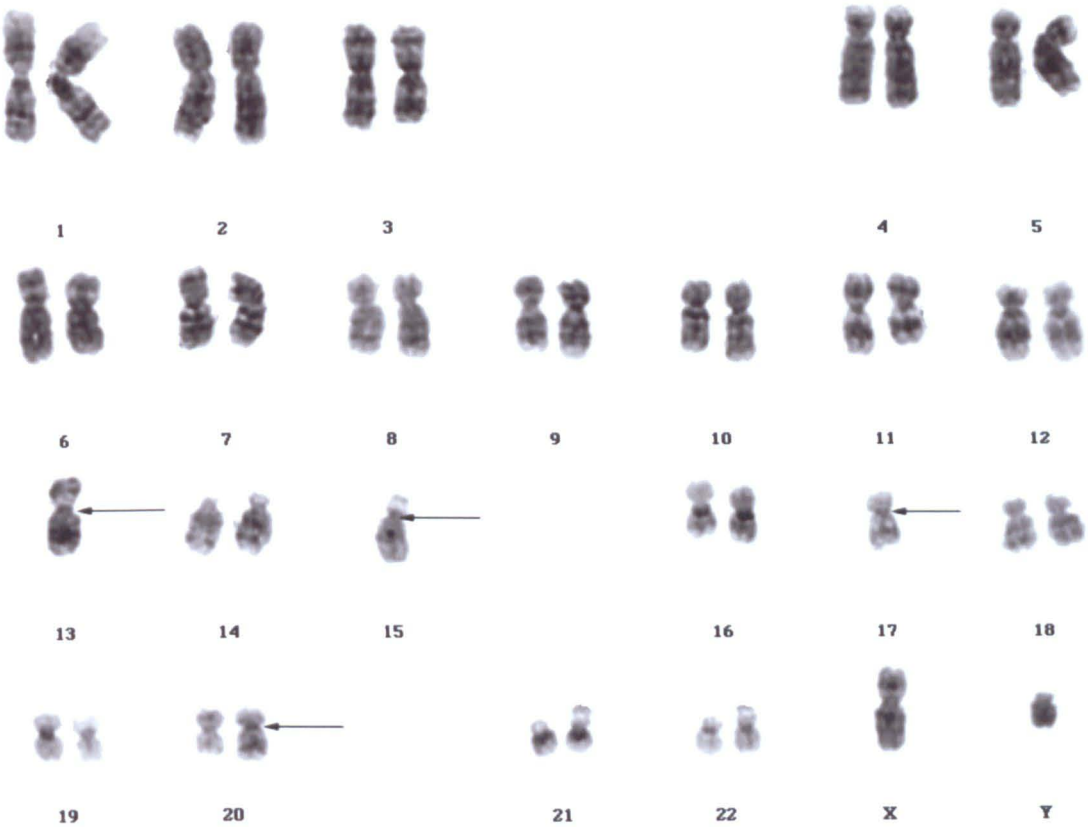
The patient was a male aged 51 years old with a rectal carcinoma associated with synchronous multiple (>10) liver metastases localised to the right lobe of the liver. The patient underwent a palliative anterior resection, the primary tumour being staged as T4, N2, M1 (Dukes D) with moderate histological differentiation. The patient had not received any neo-adjuvant therapy but did receive adjuvant chemotherapy (5-FU/folinic acid). The patient survived 8 months before succumbing to metastatic disease.

6.1 CELL CULTURE AND KARYOTYPIC ANALYSIS OF fCRC1 AND fLM1

Both the primary colorectal cancer and its synchronous liver metastasis were amenable to cell culture as outlined in Materials and Methods section 2.2.1.1-2.2.1.6. However, obtaining analysable metaphase spreads from short-term cultures proved to be a more difficult problem to solve. Initially, using a protocol which had been previously established for uveal melanomas within the Department of Ophthalmology and Orthoptics, University of Sheffield, very few metaphase spreads were obtained, and if any spreads were visualised, the chromosomes were extremely condensed, precluding any practical analysis. A number of changes were made to the protocol including

changes to the concentration of colcemid and the time of harvesting. The most efficacious solution was to add 10μg/ml colcemid to a T25 cell culture flask and leave for 24hr before harvesting. Even with these changes only fCRC1 produced metaphase spreads which were amenable to analysis. The result of one typical analysis is shown in figure 6.1, namely loss at chromosome 13, a 13p translocation t(13;17)(p11;q11) and a loss of chromosome 15 and a derivative chromosome 20 add(20q). These findings will be discussed later in this chapter.

Figure 6.1 A typical karyotype for fCRC1, showing a loss at chromosome 13q, a 15p:17p translocation and a gain at chromosome 20q (←=abnormality).



6.2 FLUORESCENT *IN SITU* HYBRIDISATION OF fCRC1 AND fLM1

Using the protocol standardised for the FISH analysis of fresh-frozen uveal melanoma samples, FISH analysis of fCRC1 and fLM1 proceeded without

complications. In order to assess the effect of cell culture on tumour samples, FISH analysis was performed on both fresh-frozen and cell cultured samples at passage seven.

For each sample, 300 cells were counted. Cells were excluded if they were clumped together or if they appeared to have been cut. The results of the FISH analysis are shown in table 6.1.

Table 6.1 Results of the FISH analysis.

	fCRC1		fCRC1w1p7		fLM1		fLM1w1p7	
Results	2:2=175	58.3%	2:2=239	79.6%	2:2=162	54%	2:2=263	87.7%
(Chromosome 3	1:3=40	13.3%	1:2=19	6.3%	2:3=46	15.3%	1:2=10	3.3%
hybridisation	1:2=38	12.6%	4:4=13	4.3%	1:3=44	14.6%	2:4=10	3.3%
signals:	2:3=22	7.3%	2:3=8	2.6%	1:2=24	8%	2:1=4	1.3%
Chromosome 8	3:3=12	4%	3:3=6	2%	2:4=10	3.3%	2:3=4	1.3%
hybridisation	2:4=6	2%	1:3=5	1.6%	3:3=9	3%	4:4=4	1.3%
signals)	3:2=4	1.3%	2:1=5	1.6%	2:1=2	0.6%		
	1:4=3	1%	3:2=1	0.3%	3:2=2	0.6%		
			4:3=1	0.3%	1:4=1	0.3%		

Analysing the FISH results of fCRC1 and fLM1, the majority of cells show normal constitutional target hybridisation signals for both chromosomes 3 and 8 (58.3% and 54% respectively) and at first glance there appears to be no similarity to uveal melanomas (where gross abnormalities of chromosomes 3 and 8 are associated with metastasis to the liver). However, both fCRC1 and fLM1 show a variety of other abnormalities in the number of hybridisation signals for chromosomes 3 and 8, where gains of chromosome 8 appear to be the second commonest finding when all the abnormalities are grouped together. Monosomy 3 is also a frequent finding within both fCRC1 and fLM1. This would all tend to support the original hypothesis that an abnormality of chromosomes 3 and 8 are related to liver metastasis.

Comparing the FISH analysis results between the fresh-frozen tumour samples (fCRC1 and fLM1) to the cell cultured sample fCRC1w1p7 and fLM1w1p7 (which

represented a single wash of the original tumour sample at passage 7), there appears to be a larger proportion of cells with normal constitutional number of hybridisation signals in the cell cultured sample as compared to the fresh-frozen sample. This is not entirely surprising as cell culture characteristically leads to the selection of clones suited to an *in vitro* environment and thus not necessarily representing the clones present *in vivo*, where a number of clones will co-exist together. This clearly demonstrates one of the limitations of genetic studies using cultured cells.

6.3 COMPARATIVE GENOMIC HYBRIDISATION OF fCRC1 AND fLM1

DNA was extracted from each tumour sample using the Qiagen QiaAmp protocol for fresh-frozen tissue as outlined in Materials and Methods section 2.2.5.3. The DNA was labelled using the nick translation technique as outlined in Materials and Methods section 2.2.6.1. Tumour DNA was labelled red and was co-hybridised with Spectrum Green labelled reference 46XX DNA onto metaphase target slides which had either been purchased from Vysis or produced in-house.

No results were obtained for either tumour sample when using the Vysis slides, whereas using slides made in-house, CGH was partially successful with results being obtained for fCRC1 (figures 6.2 and 6.3). The hybridisation for fLM1 could not be analysed as a result of high background fluorescence, most likely due to non-specific hybridisation to cytoplasmic remnants on the slide. However, the analysis of fCRC1 showed gains of chromosome arms 20q with loss of chromosome arms 13q (figure 6.4). Although not present in all the metaphases analysed there were also potential gains of chromosome arms 4p and 17p and loss of chromosome arms 4q and 2q. The results using CGH were comparable to the karyotype analysis, where there was evidence of loss of chromosome arm 13q and gains at 20q. Interestingly, changes in chromosome arms 4p and 4q were not evident using conventional cytogenetic analysis, but not entirely surprising as CGH analysis not only allows a greater degree of resolution but also amalgamates variations from a population of tumour cells. However, a shortfall of CGH is that translocations cannot be observed, as is evident by the 13p:17q translocation seen with karyotypic analysis but missed by CGH. This translocation could possibly have resulted in either the inactivation of a tumour suppressor gene or the activation of an oncogene (Sheer, 1997).

Figure 6.2 A typical two-colour image of fCRC1 (tumour DNA labelled Ulysis rhodamine and normal reference DNA labelled Spectrum Green).

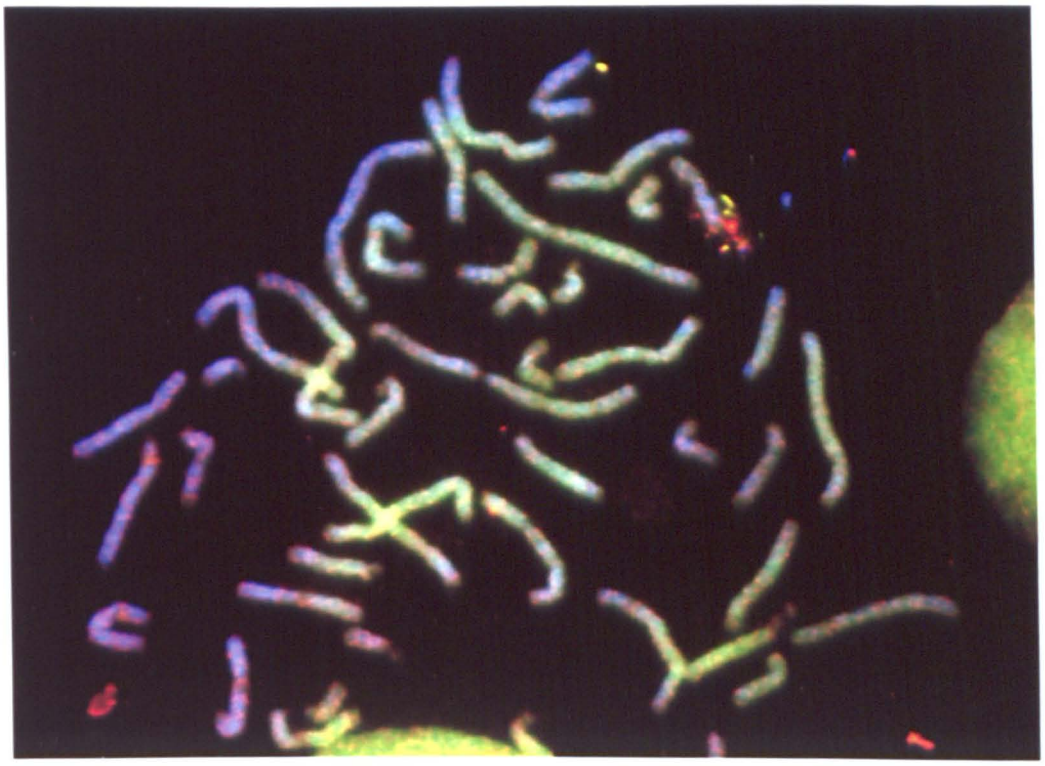


Figure 6.3 A DAPI stained image of the target metaphase spread as seen in figure 2, for fCRC1.

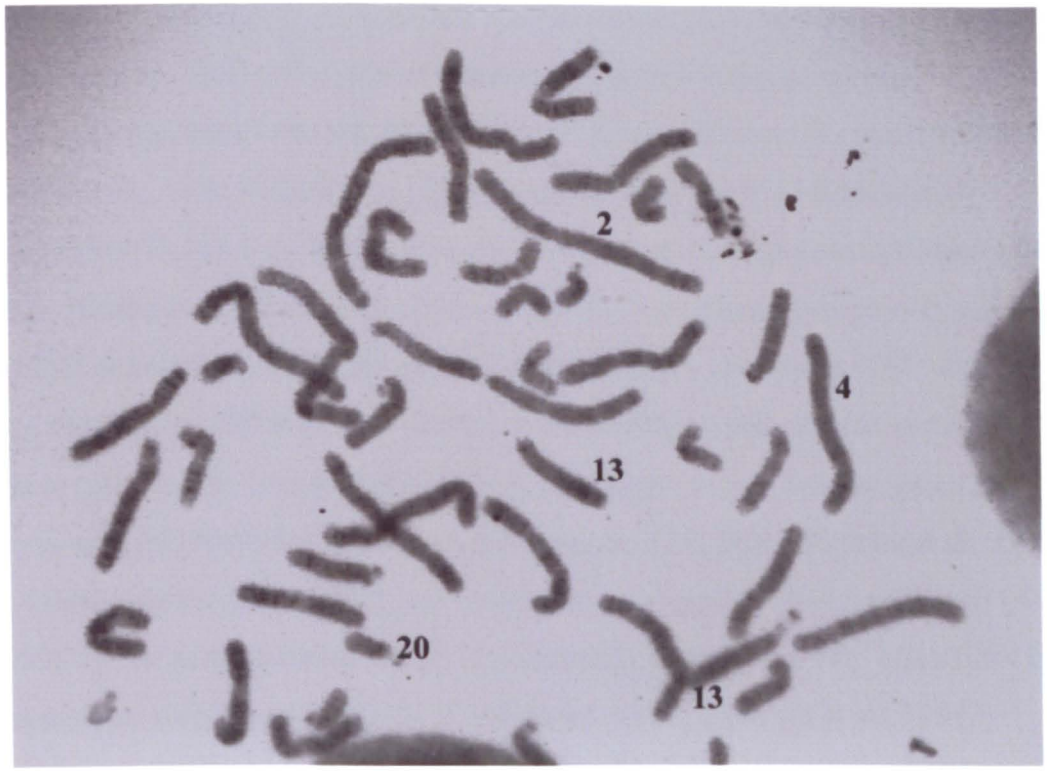
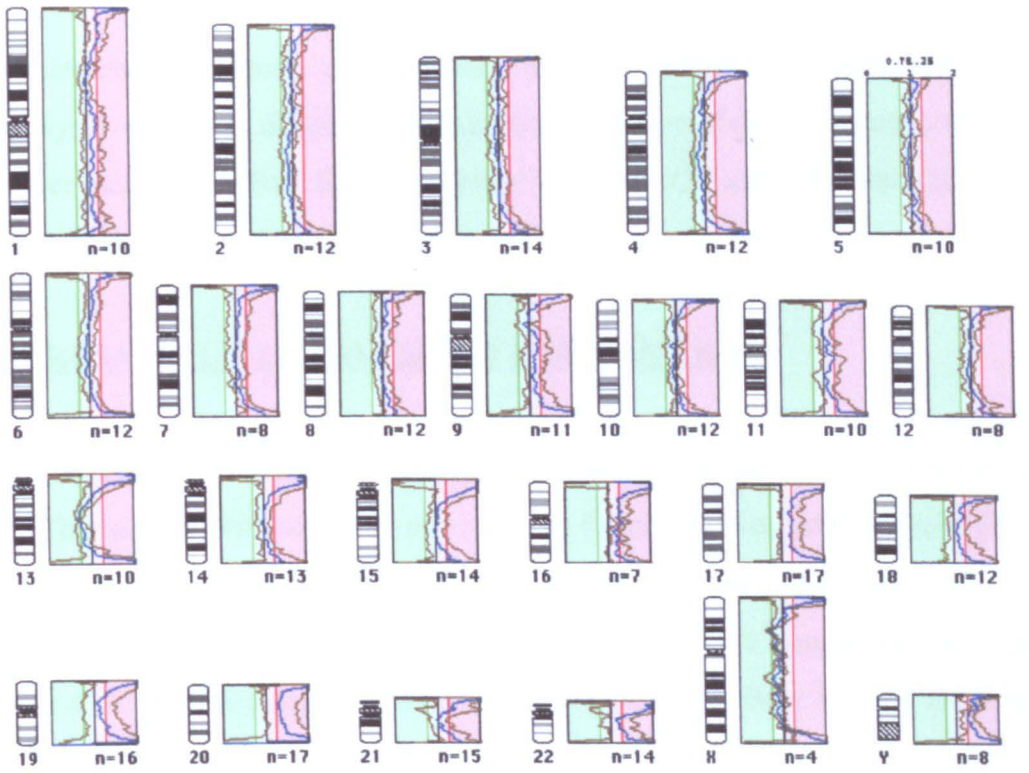


Figure 6.4 An average ratio profile of several metaphase spreads for fCRC1.



Several studies have implicated the possibility of a tumour suppressor gene on chromosome 4q, although as yet none has been identified (Niketeghad et al., 2001; Zitselsberger et al., 2001). The gain of chromosome arm 20q has previously been reported to be associated with the development of liver metastases (Korn et al., 1999; Al-Mullah et al., 1999; Hidaka et al., 2000; Aragane et al., 2001), with a region localised to 20q13.2 as a possible site of an oncogene involved in metastasis (Korn et al., 1999; Hidaka et al., 2000). Nanashima et al (1997) performed cytogenetic analyses using FISH on primary colorectal cancers and their hepatic metastases. The study found gains of chromosome 20 as a frequent aberration in both the primary and metastatic regions in patients with liver metastases from colorectal cancers. Several genes are known to exist on chromosome 20 and which include *E2F*, *BCLX* (Stokke et al., 1995), *SRC* (Asimakopoulos et al., 1994), and proliferating cell nuclear antigen (*PCNA*) (Ku et al., 1989). Of the genes mentioned only an increased expression of *SRC* and *PCNA* has been associated with liver metastases of colorectal cancer (Teixeira et al., 1994, Nakamura et al., 1996; Irby et al., 1999). Analysis of breast cancers using CGH,

suggests that chromosome 20q13 was the locus for another as yet unknown gene associated with metastases in these cancers (Tanner et al., 1995).

It is reassuring that there is a concordance of results between karyotypic analysis and CGH analysis of fCRC1, although unfortunately results could not be obtained for the paired liver metastasis fLM1. The comparison between CGH and karyotypic analysis demonstrates the advantages and shortfalls of CGH.

6.4 MICROSATELLITE ANALYSIS OF fCRC1 AND fLM1

Using the protocol described in Materials and Methods section 2.2.8.2 PCR using the Abgene Thermo-Fast 96 low profile plate and the Primus 96 Plus thermo cycler for use on LongRanger sequencing gels microsatellite analysis of fCRC1 and fLM1 was performed using IRD labelled primers with electrophoresis on a LongRanger gel, using the LiCor 4200 DNA sequencer with analysis using the LiCor Base ImagIR software.

All PCR reactions were performed with positive and negative controls and were successful. All ten primers were used; BAT26, BAT40, Mfd15, D2S123, APC, Bat25, D10S197, D18S58, D18S69 and MYCL1. No microsatellite instability was detected with any of the primers for either fCRC1 or fLM1, using DNA extracted from normal colon mucosa as the normal control. This is not entirely surprising as the incidence of MSI in sporadic colorectal cancers is up to 20% (Thibodeau et al., 1993; Aaltonen et al., 1993) and in our study (Chapter 5) the incidence of MSI-H was 9.7% in primary colorectal cancers and 12.9% in the liver metastases. The primary colorectal cancer in this study was a rectal cancer, and as stated in other studies MSI tends to be associated with right sided colonic tumours (Thibodeau et al., 1993; Lothe et al., 1993; Kim et al., 1994).

6.5 SUMMARY

All the fore mentioned techniques were successfully applied to the analysis of a single paired sample of fresh-frozen primary colorectal cancer and its liver metastasis, albeit partial success using CGH analysis.

CHAPTER 7 – GENERAL DISCUSSION AND FUTURE RESEARCH

CONTENTS

CHAPTER 7

7.1 GNERAL DISCUSSION

The majority of patients with cancer die as a consequence of metastatic disease rather than the effect of the primary tumour, more so in the cases of colorectal cancer and uveal melanoma. Thus, metastases pose a significant cause of morbidity and mortality in these patients (Ballantyne and Quin, 1993).

A number of genetic changes will take place in the development and metastasis of the primary tumour to a distant site (Schirmacher, 1985; Klein and Klein, 1985), with only those cells with the necessary genetic or phenotypic adaptations being able to survive and progress at this distant site (Fidler and Kripke, 1977; Fidler, 1990; Gregoire et al., 1993; Radinsky, 1995; Singh et al., 1997; Nicolson and Moustafa, 1998).

The aim of the research was to elucidate novel genetic abnormalities, which would be predictive for the development of liver metastases in patients with colorectal cancer. The information could then be used to institute adjuvant therapies or, enhance the surveillance for liver metastases, with the view to offer potentially curative liver resection.

Patients with uveal melanoma are also known to develop a high frequency of liver metastases and thus the study was designed to compare the two malignancies, and to establish whether colorectal cancers capable of metastasising to the liver shared common genetic abnormalities with posterior uveal melanoma. It has already been established by cytogenetic analysis that monosomy 3 and gain of chromosome 8q has been particularly associated with reduced survival and the development of liver metastases in patients with uveal melanoma (Prescher et al., 1996; Sisley et al., 1997; White et al., 1998). Thus, the analysis of uveal melanoma tumour specimens provided a starting point from which to conduct this study. The prospective collection of uveal melanoma tumours had already been established in the Department of Ophthalmology and Orthoptics, University of Sheffield, while no protocol existed for the collection, storage and analysis of colorectal cancers and their liver metastases and so initial investigations were dependant on the analysis of formalin-fixed paraffin-embedded tumour specimens, while ethical approval was sought.

The initial part of this investigation involved the fluorescent *in situ* hybridisation examination of uveal melanoma specimens using alpha centromeric probes for chromosomes 3 and 8, where long term follow-up data was available. FISH examination using the same probes were also used to investigate paired samples of colorectal cancers and their liver metastases, but due to the variability in length of formalin-fixation of both the primary and secondary colorectal cancer specimens, FISH analysis of the archival paraffin-embedded tissue samples was unsuccessful. In contrast, FISH examination of the fresh-frozen uveal melanoma tissue samples proved more successful with results showing a statistically significant association between the presence of abnormalities in copy numbers of chromosomes 3 and 8, with reduced survival and the development of liver metastases. The results concurred with previous studies, suggesting the possibility of a putative oncogene on chromosome 8 and a tumour suppressor gene on chromosome 3 (Prescher et al., 1996; Sisley et al., 1997; White et al., 1998). The unavailability of liver metastatic tissue from patients with uveal melanoma meant that FISH analysis for genetic imbalance could not be assessed.

FISH analysis for chromosomes 3 and 8, proved to be a reliable and efficient technique in the analysis of fresh-frozen tumour specimens and valuable in the prediction of prognosis in patients with uveal melanoma. Further large scale prospective studies will be required to define the sensitivity and specificity of this technique in clinical practice.

In an attempt to examine the archival primary colorectal and liver metastatic tissue specimens, comparative genomic hybridisation was utilised, to allow a genome-wide search for regions of amplification and deletion. The technique was optimised for the analysis of formalin-fixed paraffin-embedded tissue samples, by improving DNA extraction methodology as well as labelling techniques. Problems in the variable quality of target metaphase slides were overcome by the production of target metaphase slides in-house. Unfortunately, only partial results were obtained from the analysis of a single liver metastatic tissue sample, showing gain of chromosomes 2, 3, 7 and of chromosome arms 8q and 13q, with loss of chromosome arm 8p. These findings concurred with other studies Korn et al., 1999; Al-Mullah et al., 1999; Aragane et al., 2001; Nakao et al., 2001). Further refinements and development of the technique could have allowed the

complete analysis of all the samples, but limitations in time prevented this and until more extensive studies have been undertaken, only limited conclusions can be drawn.

Microsatellite instability in primary colorectal cancers has been linked to improved patient survival (Thibodeau et al., 1993; Lothe et al., 1993; Gafa et al., 2000; Gryfe et al., 2000; Hemminki et al., 2000), although not all studies have confirmed this (Messerini et al., 1999; Salahshor et al., 1999; Johannsdottir et al., 1999; Feeley et al., 1999; Curran et al., 2000; Gervaz et al., 2002). The presence of microsatellite instability in the paired samples of primary and liver metastatic colorectal cancer tissue was assessed using polymerase chain reactions, using a reference panel of microsatellite markers. The analysis of 31 paired samples showed an incidence of high level MSI (MSI-H), in 9.7% of primary tumours and 12.9% of liver metastases, which is in accordance with other reports (Ishimaru et al., 1995; Chen et al., 1997). A significant association was found with the presence of MSI in either primary or secondary tumours with the likelihood of solitary liver metastases, although this did not translate into a significantly improved prognosis.

In an analogous study by a colleague (Mr Neil Cross), MSI was essentially found to be absent in the primary uveal melanomas studied (unpublished data), and as no liver metastases from uveal melanoma patients was available to study, it was not possible to assess whether MSI existed in the liver metastases, as was found in colorectal cancer liver metastases. Certainly, the primary uveal melanomas examined did not exhibit MSI and in this respect appear to differ from colorectal cancers.

In the final part of the study a fresh paired sample of a primary colorectal cancer and its synchronous liver metastasis was obtained. FISH analysis was particularly interesting, where both the primary and liver metastasis showed evidence of genetic imbalance for chromosomes 3 and 8, in a manner consistent with the findings for uveal melanomas. Although, more variability was seen compared with most uveal melanomas, abnormalities of chromosomes 3 and 8 were present, in particular gain of chromosome 8 and monosomy 3 being a frequent finding. If confirmed, this finding would suggest that colorectal cancers metastasising to the liver do share some common genetic changes with uveal melanomas, possible implying the involvement of similar genes, however, it is too early to draw such conclusions on the basis of one paired sample. CGH analysis of

the primary colorectal cancer corroborated karyotype findings with evidence of gain of 20q and loss of 13q, which were in agreement with other studies (Nanashima et al., 1997; Korn et al., 1999; Al-Mullah et al., 1999; Hidaka et al., 2000; Aragane et al., 2001). Further paired samples were not available for study, due to a change in policy in the Trent Region, where sampling liver metastases was discouraged, as future liver resection surgery could be compromised. Nonetheless, the analysis of this single paired sample demonstrated that the techniques could be applied successfully in the genetic analysis of cancer specimens.

7.2 FUTURE RESEARCH

Recent developments in the fields of genomics and molecular biology have unleashed an array of new techniques to explore the development and treatment of cancer. The human genome will shortly be sequenced, with the promise of increasing the understanding of how genes and the environment interact to give rise to the cellular mechanisms that underlie the biological process and the genetic variations between individuals, providing scope for new therapeutic developments such as pharmacogenomics and gene therapy, with the potential to eventually provide opportunities for disease prevention.

Genetic screening for familial diseases, may allow for the identification of susceptible individuals, thus allowing diagnostic interventions or removal of the target organ to be offered. Stratification with reference to pharmacogenetic consideration will allow more appropriate, effective and safer drug treatments to be offered (Sadée, 1999). Choi et al. (2002) and Rosty et al. (2001) have already tried to stratify patients into groups according to LOH and MSI status and relate this to response to chemotherapeutic agents. The development of cDNA microarray technology allows for the assessment of numerous polymorphisms on a single glass plate or 'gene chip'. Thus, genome-wide screening for polymorphisms could identify important polymorphisms associated with a particular clinical response or adverse reaction, thus enabling chemotherapy to be either directed to a specific group of patients, who would positively respond or be avoided in those in whom severe toxicity could result. Microarray analysis could also be used to predict survival in those patients with particular polymorphisms, in response to chemotherapy (Wei et al., 2002). Further large scale studies will expand on these

preliminary results and incorporate novel colorectal cancer pathway molecules. However, within the genome little is known of the non-coding DNA sequences, of the mechanisms responsible for replication, RNA splicing, the control of cell cycle and gene expression or of the roles of centromeres and telomeres. The complexity of genetic analysis is compounded further because of ethnic and racial variations. Moreover, variable expressivity and incomplete penetrance are already seen in relatively simple monogenic disorders such as retinoblastoma and furthermore, the expression of similar phenotypes can arise from genetic heterogeneity, whether in the form of allelic heterogeneity (different mutations at the same allele) or locus heterogeneity (different mutations at the same locus). Similarly, different mutations in the same gene can result in differing clinical manifestations, such as in the *RET* oncogene, where activating mutations can result in multiple endocrine neoplasia type II (identified by familial phaeochromocytomas, parathyroid and medullary thyroid cancers), whilst inactivating mutations are associated with Hirschsprung's disease (Mulligan et al., 1993; Romeo et al., 1994; Edery et al., 1994). Thus, the identification of mutations is not necessarily the be all and end all.

The initial speculation that gene therapy would revolutionise medicine has been over-optimistic. A number of problems still need to be overcome before gene therapy can be widely used in routine clinical practise. Despite these difficulties a number of trials are already underway assessing the use of gene therapy in treating cancer (Harris and Sikora, 1996). Another expanding field has been in stem cell technology. Haematopoietic stem cells have already been used in the treatment of various haematological conditions. The difficulty with using solid tissue stem cells has been in reproducing the three-dimensional arrangements and cell-cell and cell-extracellular matrix that exists in solid tissues. The existence of hepatic stem cells could revolutionise liver resection surgery for the treatment of both primary liver and metastatic cancers, allowing more extensive resections to be performed followed by tissue regeneration (Allain et al., 2002; Danet et al., 2002).

7.3 CONCLUSION

The research undertaken in this study has utilised a number of techniques in the investigation of the genetic changes involved in liver metastasis. Fluorescent *in situ* hybridisation allowed for the targeting of specific DNA sequences, while CGH allowed for a genome-wide search of regions of chromosomal amplification or loss. Possible chromosomal rearrangements were visualised with karyotypic analysis, whereas, PCR allowed for the analysis of microsatellite instability. It has been shown that all the techniques used have had their advantages and disadvantages, but by using several techniques complementing each other genetic analysis could be successfully performed. The prospective collection of fresh-frozen tissue will allow for genetic analysis to be correlated to clinical outcome and other clinicopathological variables. The short-term culture of primary colorectal cancers and liver metastases will allow for the correlation of genetic analysis to phenotypic expression, from assessing the production and function of proteins to cell-cell or cell-extracellular matrix interactions. The development of new techniques such as the DNA microarray technology could be applied to assess the relevance of genetic polymorphisms and the effects of various chemotherapeutic agents to cultured cells, with the aim of providing each patient with a tailor-made therapeutic regimen, thus maximising efficacy and minimising side-effects. The development of gene-therapy to either halt the progression of cancer cells or augment their response to therapeutic agents is also an area that could be developed from the genotype-phenotype analysis.

This study has shown the ways in which emerging technologies can be applied to the investigation of both uveal melanomas and colorectal cancers. Continued investigations may subsequently lead to the identification of specific genetic changes related to the development of liver metastases, the aim of which would be to allow clinicians to determine prognosis for their patients, augment current surveillance protocols for recurrence or metastasis, instigate adjuvant treatment, or to specifically target these genetic abnormalities with novel therapies.

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Appendix I: Summary of treatment options for colorectal cancer.

Stage	Treatment Options
0	1. Local excision of polyps or colon resection for larger tumours
I (Dukes A)	1. Wide surgical resection and anastomosis
II (Dukes B)	1. Wide surgical resection and anastomosis 2. ?Adjuvant chemotherapy
III (Dukes C)	1. Wide surgical resection and anastomosis with adjuvant chemotherapy of either 5-FU/levamisole for 12 months or 5-FU/leucovorin for 6 months 2. ?Adjuvant radiotherapy
IV (Dukes D)	1. Surgical resection and anastomosis or by-pass surgery for selected patients with obstructing cancers 2. Surgical resection of isolated metastases (liver/lung) 3. Chemotherapy 4. Radiotherapy 5. Clinical trials evaluating new drugs and biologic therapy

Appendix II: The various staging classifications used for colorectal cancer.

Dukes Staging

- **A -** Tumour confined to the bowel wall
- **B -** Tumour grows beyond the bowel wall
- **C -** Lymph node metastases present
- **(D -** Distant metastases present - added by Turnbull)

Aster-Coller

- **A -** Tumour limited to mucosa - carcinoma in situ
- **B1 -** Tumour extends through the muscularis mucosae but not beyond the muscularis propria
- **B2 -** Tumour extends beyond muscularis propria
- **C1 -** B1 with regional lymph node metastases
- **C2 -** B2 with regional lymph node metastases
- **D -** Distant metastases

TNM Classification

T assesses the tumour stage

- **TX -** Primary tumour cannot be assessed
- **T0 -** No evidence of primary tumour
- **Tis -** Carcinoma in situ - limited to mucosa
- **T1 -** Tumour invades submucosa but does not extend beyond muscularis propria
- **T2 -** Tumour invades muscularis propria but not beyond
- **T3 -** Tumour extends beyond muscularis propria but not T4
- **T4 -** Tumour perforates visceral peritoneum or invades adjacent organs or structures (including loops of bowel)

N assesses regional lymph node stage

- **N0 -** No regional lymph node metastases
- **N1 -** 1-3 regional lymph node metastases
- **N2 -** 4 or more regional lymph node metastases
- **N3 -** Regional lymph node metastases along named vascular trunks

M assesses distant metastases

- **MX -** Distant metastases cannot be assessed
- **M0 -** No distant metastases
- **M1 -** Distant metastases present

Appendix III: Primer sequences, annealing temperatures and chromosomal location.

DNA Primer	Primer Sequence 5'--3'	Repeat Motif	Annealing Temp.	Size (bp)	Chromosomal location
BAT-25	TCG CCT CCA AGA ATG TAA GT TCT GCA TTT TAA CTA TGG CTC	mononucleotide $TTTT.TTTT.(T)_7.A(T)_{25}$	58°C	~90	4q12
BAT-26	TGA CTA CTT TTG ACT TCA GCC AAC CAT TCA ACA TTT TTA ACC C	mononucleotide $(T)_5.....(A)_{26}$	58°C	~80-100	2p
BAT-40	ATT AAC TTC CTA CAC CAC AAC GTA GAG CAA GAC CAC CTT G	mononucleotide $TTTT.TT..(T)_7.....TTTT.(T)_{40}$	58°C	~80-100	1p13.1
APC	ACT CAC TCT AGT GAT AAA TCG AGC AGA TAA GAC AGT ATT ACT AGT T	CA dinucleotide $(CA)_{26}$	55°C	96-122	5q21/22
Mfd15CA	GGA AGA ATC AAA TAG ACA AT GCT GGC CAT ATA TAT ATT TAA ACC	CA dinucleotide $(TA)_7.....(CA)_{24}$	52°C	~150	17q11.2-12
D10S197	ACC ACT GCA CTT CAG GTG AC GTG ATA CTG TCC TCA GGT CTC C	CA dinucleotide $CACCAGA(CA)7.A.A(CA)_{12}(AGAAA)_2$	65°C	161-173	10qter
D18S69	CTC TTT CTC TGA CTC TGA CC GAC TTT CTA AGT TCT TGC CAG	CA dinucleotide $(CA)_4AA(CA)_{14}(T)_6$	60°C	~110	18q21
D2S123	AAA CAG GAT GCC TGC CTT TA GGA CTT TCC ACC TAT GGG AC	CA dinucleotide $(CA)_{13}TA(CA)_{15}(T/G\ A)_7$	60°C	197-227	2p16
D18S58	GCT CCC GGC TGG TTT T GCA GGA AAT CGC AGG AAC TT	CA dinucleotide $(GC)_5GA(CA)_{17}$	53°C	144-160	18q22.3
MYCL1	TGG CGA GAC TCC ATC AAA G CTT TTT AAG CTG CAA CAA	tetranucleotide $GAAAA(GAAAA)_2TAAAA(A/G)_{10}GAAAGA(GAAA)_{14}$	53°C	140-209	1p32

Appendix IV: Publications and Abstracts.

Publications:

Prediction of prognosis in patients with uveal melanoma using fluorescence in situ hybridisation.

Patel KA, Edmondson ND, Talbot F, Parsons MA, Rennie IG, Sisley K.

British Journal of Ophthalmology. 85: 1440-1444, 2001.

Abstracts:

Prediction of liver metastases in patients with uveal melanoma using FISH.

Patel KA, Edmondson N, Talbot F, Sisley K, Parsons A, Rennie IG.

Clinical and Experimental Metastasis. 17(9): 753, 2000.