



MOLECULAR DETECTION AND SIGNIFICANCE OF CIRCULATING COLORECTAL CANCER CELLS

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ABSTRACT

At present, up to 30% of patients who present with early stage colorectal cancer (CRC) die of disease within 5 years of 'curative' resection. This suggests that an occult metastatic disease process was already under way, or that tumour cells with metastatic potential were released at the time of resection. These circulating cells may be present in very low numbers and would not be detected by conventional means. Detection of micro-metastatic disease at presentation may have prognostic implications independent of established staging factors and would result in the identification of 'high risk' patients who might benefit from adjuvant therapy.

This thesis describes the development of a sensitive technique, combining immuno-magnetic bead isolation of epithelial cells with polymerase chain reaction (PCR)-based detection of tissue-specific markers. A pilot study of 27 colorectal cancer patients, using a *K-ras* mutation identified in the tumour as a marker, showed that detection of tumour cells in blood at diagnosis was significantly associated with shortened disease-free survival ($p=0.0001$). This marker was only applicable however in a proportion of CRC patients whose tumour carried a *K-ras* mutation. To overcome this problem, epithelial-specific expression of cytokeratin 19 and 20 and mucin genes, detected by reverse transcription-PCR, were used as markers for epithelial-derived cells in blood. Kaplan-Meier survival analysis showed that the group in whom marker-positive cells were detected had a significantly poorer prognosis than marker-negative patients ($p=0.001$). The analysis for post-operative blood showed no difference in survival suggesting that in some patients normal colon cells were also released as a direct result of colorectal resection. Interestingly, normal colon cells were also detected pre-operatively in 3 patients with adenoma and one with diverticulitis. In order to

differentiate between colon cancer cells and normal colon cells in the circulation post-resection, metastasis-associated markers were sought. Preliminary results showed that of 8 patients who were positive for epithelial markers in blood, 5 were also positive for metastasis-associated markers. Although follow-up of these patients is less than 12 months, one stage B patient has died of metastatic disease 2 months post-resection. Future research will be directed at finding the most appropriate marker(s) for the specific detection of metastatic cancer cells.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Signed:

Date: 21st December, 1998

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LIST OF ABBREVIATIONS

BM	-	bone marrow
bp	-	base pair(s)
cDNA	-	complementary DNA
CK	-	cytokeratin
CRC	-	colorectal cancer
DNA	-	deoxyribonucleic acid
dNTP	-	deoxynucleoside triphosphate
ECM	-	extracellular matrix
EMA	-	epithelial membrane antigen
GAPDH	-	glyceraldehyde phosphate dehydrogenase (house-keeping gene)
LAM γ 2	-	laminin γ 2
MAT	-	matrilysin
MNC	-	mononuclear cells
mRNA	-	messenger RNA
MUC	-	mucin
NS	-	not significant
PB	-	peripheral blood
PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
RNA	-	ribonucleic acid
RT-PCR	-	reverse transcription-polymerase chain reaction
SSCP	-	single strand conformation polymorphism

PUBLICATIONS

Refereed publications arising from this thesis

Hardingham JE, Kotasek D, Farmer B, Butler RN, Mi J-X, Sage RE, Dobrovic A. Immunobead-PCR: A technique for the detection of circulating tumor cells using immunomagnetic beads and the polymerase chain reaction. *Cancer Research* 53:3455-58, 1993

Hardingham JE, Kotasek D, Sage RE, Eaton MC, Pascoe VH, Dobrovic A. Detection of circulating tumour cells in colorectal cancer by immunobead-PCR is a sensitive prognostic marker for relapse of disease. *Molecular Medicine* 1: 789-94, 1995.

Eaton MC, Hardingham JE, Kotasek D, Sage RE, Dobrovic A. Immunobead RT-PCR. A sensitive method for detection of circulating tumour cells. *Biotechniques* 22: 100-105, 1997.

Hardingham JE, WJ Butler, D Roder, A Dobrovic, RE Sage, IC Roberts-Thomson. Somatic mutations, acetylase status and prognosis in colorectal cancer. *Gut* 42:669-72, 1998.

Other publications arising from this thesis

Hardingham JE. Detection of circulating tumour cells using immunobead-PCR, *in* *Clinical Applications of PCR*, ed. Dennis Lo, Humana Press, 1998.

Eaton MC, Hardingham JE, Kotasek D, Sage RE, Dobrovic A. Immunobead RT-PCR. A sensitive method for detection of circulating tumour cells, *in* *The PCR Technique: RT-PCR* ed. Paul Siebert, Biotechniques Books, Natick, 1998.

Submitted for publication

Hardingham JE, Hewett PJ, Sage RE, Kotasek D, Dobrovic A. Prognostic significance of circulating colon cancer cells detected by immunobead isolation and RT-PCR.

Walsh D, Hardingham JE, Sage RE, Kotasek D, Norman JE, Maddern G, Dobrovic A. The prognostic significance of breast cancer cells in peripheral blood stem cell collections detected by immunobead RT-PCR.

CHAPTER 1

INTRODUCTION

AND

LITERATURE

REVIEW



1.1 COLORECTAL CANCER - THE PROBLEM OF EARLY RELAPSE

At present, a significant proportion of patients diagnosed with early stage colorectal cancer (CRC), without evidence of tumour dissemination, relapse and die of metastatic disease often 1-2 years after 'curative' surgical resection. Currently there are no procedures in use for the staging of colorectal cancer that will detect micro-metastatic disease, thus the main objective of this thesis was to establish a sensitive and specific assay to detect circulating colorectal cancer cells in patients' blood samples and to establish whether this was useful as a prognostic marker. I planned to firstly isolate epithelial cells by using magnetic beads coated with an anti-epithelial antibody, and secondly to use molecular techniques to identify the isolated cells on the basis of a tumour-specific mutation. Since only a proportion of tumours carry a suitable tumour-specific mutation as a marker, I subsequently developed reverse-transcription polymerase chain reaction (RT-PCR) assays for epithelial-specific and metastasis-associated gene expression as markers of colon cancer cells so that all the patients could be included in a longitudinal survival analysis.

1.1.1 Epidemiology

Colorectal cancer is the second most common internal cancer in Australia with about 8,000 new cases reported annually. On the basis of current incidence rates, one in 20 Australians can expect to develop colorectal cancer during their lifetime, with similar rates being found in other Western countries (Giles 1989, Bonett et al., 1990). The incidence is increasing slowly world-wide, probably as a result of changing dietary patterns in the developing countries and the effects of environmental toxins (Hill, 1989).

Despite effective surgical resection of tumours, relapses will occur in about 50% of patients with colorectal carcinoma within 5 years following initial diagnosis, including patients diagnosed with early stage disease. This could be due to the following reasons:

(1) Late presentation: Currently there are no reliable and cost-effective means of screening to ensure early diagnosis of colorectal cancer. Community screening programs using faecal occult blood testing and colonoscopies are expensive and the current recommendation for screening is confined to high-risk groups only, such as a family history, previous polyps, or inflammatory bowel disease (Ransohoff et al., 1991; Winawer et al., 1991). Many patients therefore present with advanced disease with metastatic (or micrometastatic) disease already present.

(2) Release of tumour cells into the circulation: It is thought that tumour cells escape into the circulation and invade other tissues once the tumour has reached a certain size (>2mm) and angiogenesis (new blood vessel formation) has occurred (reviewed in Folkman, 1990). Another equally important consideration is the possibility that tumour cells with metastatic potential are released into the blood (and lymph) circulation at the time of surgery for colorectal malignancy. Irrespective of the source of circulating tumour cells, some at least are likely to be responsible for recurrence at the primary site and development of metastases. The major site of metastases is the liver which drains venous blood from the gastro-intestinal tract via the portal system. Detection of tumour cells circulating in the blood may be of great value in predicting later relapses and development of metastases.

The expected survival of colorectal cancer patients according to different pathological stages is summarised in Table 1.1. The staging system in use by the South Australian Cancer Registry is the Australian Clinical Pathological Staging system (ACPS) and is based on the extent of invasion of the tumour and whether or not lymph

nodes contain metastatic deposits, as depicted in Figure 1.1. One of the major problems for clinicians treating this disease is to determine which patients are at greatest risk of cancer recurrence. As can be seen from Table 1.1, a marked reduction in survival rates is seen in the more advanced stages. However, despite undergoing a 'curative' resection, up to 10% of patients diagnosed with stage A tumours and 30% of patients with stage B relapse and die of their disease within 5 years (South Australian Cancer Registry, 1997). This suggests that a micro-metastatic disease process was already under way, or that tumour cells with metastatic potential were released into the circulation at the time of resection. These cells may be present in very low number and would not be detected by conventional means, thus a more sensitive detection system is needed.

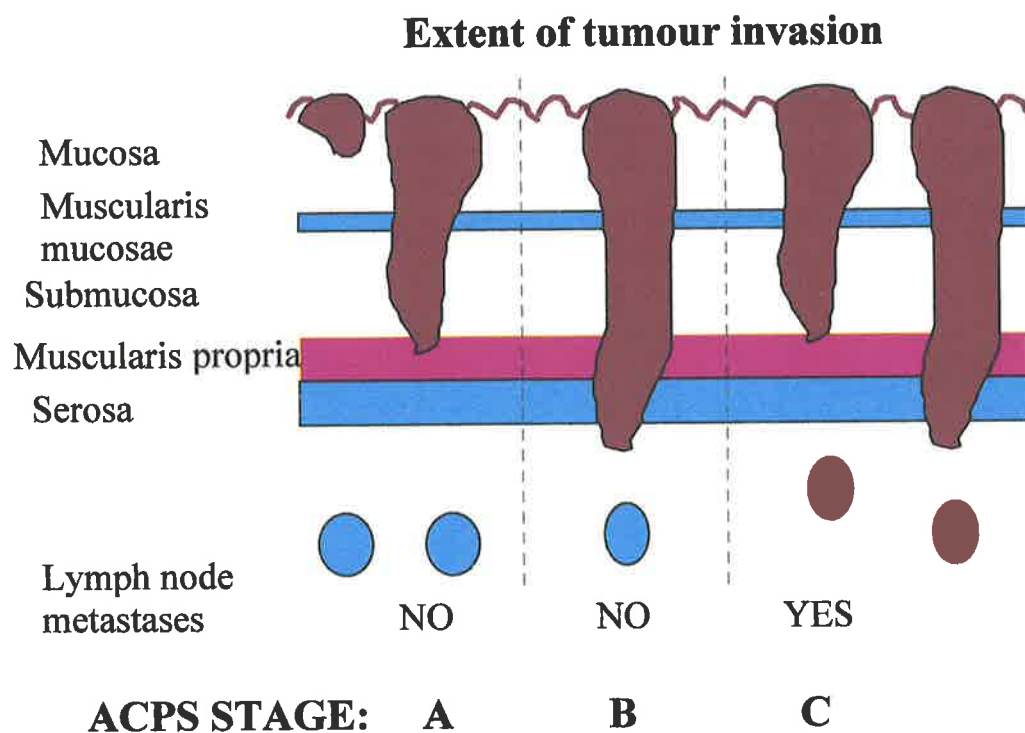
Detection of micro-metastatic disease at presentation may have prognostic implications independent of established staging factors and would result in the earlier identification of 'high risk' patients who might benefit from adjuvant therapy. Adjuvant chemotherapy, currently recommended for Dukes' stage C patients, has resulted in a significant reduction in both cancer recurrence and mortality in this group (Moertel et al., 1990, Moertel, 1994). A trial using systemic treatment with the monoclonal antibody 17-1A in Dukes' C patients has also been effective in reducing the overall death rate and recurrence rate (Riethmuller et al., 1994, 1998). In rectal carcinoma, local recurrences occur with an even greater incidence than for colon carcinoma, and distant metastases (mainly to the liver) are also common. Adjuvant local radiation therapy together with chemotherapy has reduced both local and distant recurrences in patients with high-risk rectal cancer (Krook et al., 1991). Detection of circulating tumour cells may therefore allow an earlier and more appropriate selection of patients for adjuvant therapy.

Table 1.1 Colorectal tumour stage and 5 year survival rates

ACPS	5 year survival (%)
A (not beyond muscularis propria)	90
B (invasion beyond muscularis propria)	70
C (metastases in 1 or more lymph nodes)	45
D (metastases to distant organs)	8

Adapted from data from the South Australian Cancer Registry, 1997.

Figure 1.1 The ACPS classification system for the staging of colorectal carcinoma



Adapted from the book Gastroenterology p236 (Heuman DM, Mills AS, McGuire HH, 1997). ACPS stage D has distant metastases.

1.2 CARCINOMA CELLS IN BLOOD

The possibility that cancer cells could be detected in blood was first raised in 1869 by Ashworth who described cells in the blood of a cancer patient at post mortem similar in appearance to those of the primary tumour. In the early 1930s Pool and Dunlop (1934), using cytological techniques, found "atypical" cells in the peripheral blood in 17/40 patients with cancer but were uncertain as to whether these cells were in fact cancer cells.

Later, Fisher and Turnbull (1955) performed a cytological analysis of blood samples from patients undergoing surgery for CRC. Blood was collected from the efferent vessels draining the tumour to determine the presence of tumour cells. They found tumour cells in 8/25 (32%) cases of CRC including 4 cases in which histopathologic evidence of venous extension was not observed. Further, 1/8 cases was staged as a Dukes' A tumour, and 6/8 as Dukes' B. Before resecting the colon to remove the tumour they had taken the precaution of ligating the arteries and veins in the most proximal region of the mesentery and also the collateral veins to prevent the escape of most of the venous blood from the tumour area. Following mobilisation of the tumour-bearing colon segment, a second ligature was applied to the mesenteric veins closer to the tumour so that a column of blood was trapped between the two. This blood was subsequently flushed out into a tube and the cells stained for cytological assessment. This study was the basis from which they developed their "no-touch isolation" technique for colorectal resection, where preliminary venous ligation is used to avoid the possibility of venous dissemination of tumour cells. A subsequent review of 5 year survival data of a large controlled prospective trial using this technique showed a tendency for reduction in the number of liver metastases occurring in the "no-touch" group (Wiggers et al., 1988). Fisher and Turnbull's original study soon lead to many similar studies such that by 1965,

40 different investigators were testing cancer patients for tumour cells in blood. However, many of these initial studies reported a high positivity rate that was later shown to be due to the low specificity of the cytological methods employed (reviewed in Christopherson, 1965).

More recently Graves et al. (1988) used an immunofluorescence method, employing the anti-cytokeratin antibody AE3, to detect carcinoma cells in blood from the renal vein in 8/10 patients undergoing nephrectomy for renal carcinoma. Three of the 8 patients had detectable metastases prior to the operation, while 5/8 remained disease-free 35 months post-surgery. Moss and Sanders (1990) used immunohistochemistry (IHC) to detect neuroblastoma cells in the mononuclear cell (MNC) fraction of blood samples drawn from a central venous catheter. They found that the presence of neuroblastoma cells in samples from 6/13 patients, taken during a course of therapy, correlated with subsequent relapse. Their reported sensitivity was 2 tumour cells per 100,000 MNCs.

Leather et al. (1993) were the first to report the detection of colorectal cancer cells in blood using cytology and IHC. They found circulating tumour cells in 4/42 patients, including 2/4 with early stage (Dukes' B) disease. Their low positivity rate was attributed to the fact that strict morphological criteria were applied in the determination of malignant cells.

At the time of commencing my project there had only been two reports in the literature using molecular techniques to detect carcinoma cells in the peripheral circulation. Smith et al. (1991) used reverse-transcription-PCR (RT-PCR) to detect melanoma cells in peripheral blood by virtue of their tyrosinase expression, a gene only actively transcribed in melanocytes. Blood samples from 4/7 patients with melanoma gave positive results, whereas all 8 control samples were negative. In the second study, Mattano et al. (1992) used RT-PCR to detect neuroblastoma cells in peripheral blood of

patients with neuroblastoma. Their detection system used expression of a neuroendocrine-specific gene PGP 9.5 in RT-PCR assays, with a sensitivity of 1 neuroblastoma cell per 10^7 MNCs. Eight of 18 patients were positive by RT-PCR, 4 of which were also positive by immunohistochemistry. They concluded that the RT-PCR assay was much more sensitive than immunohistochemistry. Since this time there have been many reports of the detection of carcinoma cells in blood using molecular markers which will be reviewed in more detail later.

Thus, there had been only been a single attempt to identify colorectal cancer cells in blood (Leather et al., 1993) using the technique of IHC which has been shown to be less sensitive than molecular assays (2 tumour cells per 100,000 MNCs compared to 1 tumour cell per 10^7 MNCs) (Mattano et al., 1992). Our approach focused on the use of molecular techniques for the detection of colon cancer cells in blood.

1.3 MOLECULAR MARKERS OF CANCER CELLS

1.3.1 Cancer - a multistep process

Cancer is considered to be the result of an accumulation of genetic alterations within a cell, affecting the expression or function of genes controlling cell growth and differentiation. Clonal expansion occurs from a single cell, allowing further genetic events to occur to provide a growth and survival advantage, and finally to facilitate metastasis (reviewd in Nowell, 1976).

The proliferation and differentiation of normal cells is thought to be regulated by the balanced effects of growth promoting proto-oncogene products and growth inhibiting tumour suppressor gene products (reviewed in Bishop 1987 and 1991, Sager 1989,

Weinberg 1989). Activating mutations in proto-oncogenes result in oncogene products that up-regulate the growth of cells. Inactivation of tumour suppressor genes, either by loss of the chromosome in part or whole encompassing both alleles of the tumour suppressor gene, or by a mutation or transcriptional silencing in one allele and loss of the other allele, liberates the cell from growth restraints and results in uncontrolled proliferation (reviewed in Weinberg, 1991; Siegfried and Cedar, 1997).

1.3.2 Genetic alterations as tumour markers

1.3.2.1 Mutations in oncogenes and tumour suppressor genes

The genetic alterations causing cancer are of two sorts: dominant mutations, affecting proto-oncogenes, and recessive mutations, affecting tumour suppressor genes. Dominant mutations in proto-oncogenes, resulting in activated oncogenes, cause changes in either normal gene expression, or in the gene product itself, and result in up-regulation of growth. Loss of tumour suppressor gene function has been described as a recessive change since both copies of the gene need to be inactivated for the neoplastic phenotype to be uncovered. Both types of genetic damage have frequently been found in a variety of human malignancies (reviewed in Bishop, 1991).

1.3.2.2 Oncogene activation

Proto-oncogenes are normal cellular genes that encode proteins involved in the cascade of signal transduction by which growth factors stimulate normal cell division. Oncogenes were first described as retroviral genes that transformed cells in culture and produced tumours in laboratory animals. These genes were subsequently shown to be the dominant mutated forms of host genes (proto-oncogenes) that had been incorporated or

"transduced" into the viral genome (reviewed in Bishop, 1987; Varmus, 1990). Proto-oncogenes have been conserved throughout evolution suggesting they play a fundamental role in cellular function (reviewed in Cantley et al., 1991 and Hunter, 1991). An activating mutation in one such gene, *K-ras*, has been implicated in the earliest stages of colon cancer progression (Bos et al., 1987).

1.3.2.3 Tumour suppressor gene inactivation

Loss of part or all of one allele, coupled with mutational inactivation of the remaining allele, has become the hallmark of the tumour suppressor gene (reviewed in Weinberg, 1991). The classic example of a tumour suppressor gene is the retinoblastoma (RB) gene. Individuals developing RB inherit a mutant "loss of function" allele from one parent and then a somatic event inactivates the other normal allele. In contrast, in sporadic cases, both events are somatic and the second event must occur in a cell that is clonally derived from the first. These properties led Knudson to postulate his now classical "two hit" theory of tumorigenesis in which he proposed that all types of retinoblastoma involve two separate mutations (Knudson, 1971). It was shown that there was a constitutional deletion in all somatic cells at band 13q14, and this deletion was also seen in sporadic cases (Cavenee et al., 1983). Loss of heterozygosity has now been found in many human tumours, including colorectal, lung and breast cancer.

1.3.3 Colorectal tumour progression - activation of oncogenes and inactivation of tumour suppressor genes

Colorectal tumours progress through easily recognisable clinical stages that has allowed study of tumour progression at both the histological and molecular genetic level. Tumorigenesis is preceded by cellular hyper-proliferation of the colonic epithelium, from

which foci of benign adenomas arise. The adenomas may then progress in size and dysplasia, often changing in morphology from tubular to villous. If an adenoma acquires sufficient genetic changes it will progress to become a carcinoma. Further progression involves the capacity to metastasise to distant sites (Sugarbaker et al., 1985). This progression may take decades. Extensive studies by Vogelstein's group (Fearon et al., 1987; Vogelstein et al., 1988, 1989) have resulted in the formulation of a genetic model for colorectal tumour progression (Figure 1.2). However, it is thought that the accumulation of genetic changes rather than the precise order in which they occur is responsible for tumour progression; fewer changes being required for benign tumorigenesis (Fearon and Vogelstein, 1990).

1.3.4 A genetic model for colorectal tumorigenesis

Colorectal tumours develop as a result of the mutational activation of oncogenes coupled with the mutational inactivation or loss of tumour suppressor genes (Vogelstein et al., 1988). Fearon and colleagues (1987) have demonstrated that all tumours examined were monoclonal in composition suggesting that they arose from a single cell that underwent clonal expansion.

One of the earliest changes leading to tumour progression may be epigenetic i.e. changes in DNA methylation. In mammals about 70% of cytosine residues at dinucleotide GC sites are methylated in normal, differentiated cells. Goelz et al. (1985) compared methylation in DNA from benign polyps and colon carcinomas to DNA from adjacent normal tissue and found that the polyps and carcinomas were hypomethylated to a similar degree in 23/23 patients suggesting that this change precedes malignancy. More recently, Sharrard and colleagues (1992) showed that hypomethylation of a CCGG site of

the third exon of c-myc occurred in the earliest stages of colon polyp formation and that the extent of hypomethylation increased with tumour progression. Makos and colleagues (1992) have shown localised increased methylation in the region of loss on chromosome 17p, and that this precedes the allelic loss on 17p (involving the p53 gene) that is characteristic of colorectal tumour progression. *De novo* methylation of CpG islands in the promoter region of tumour suppressor genes reduces or prevents (silences) transcriptional activity and thus may be another mechanism for inactivating tumour suppressor genes (reviewed in Jones and Gonzalgo, 1997).

Loss of genetic information from chromosome 5q21, associated with inheritance of familial adenomatous polyposis (FAP), has also been found in over 40% of sporadic cases (Solomon et al., 1987; Ashton-Rickardt et al., 1989, 1991) suggesting the presence of one or more tumour suppressor genes at this locus. The genes APC (adenomatous polyposis coli) (Grodin et al., 1991) and MCC (mutated in colon cancer) have been localised to 5q21 (Kinzler et al., 1991a) and have been found to be tightly linked to the FAP syndrome (Kinzler et al., 1991b).

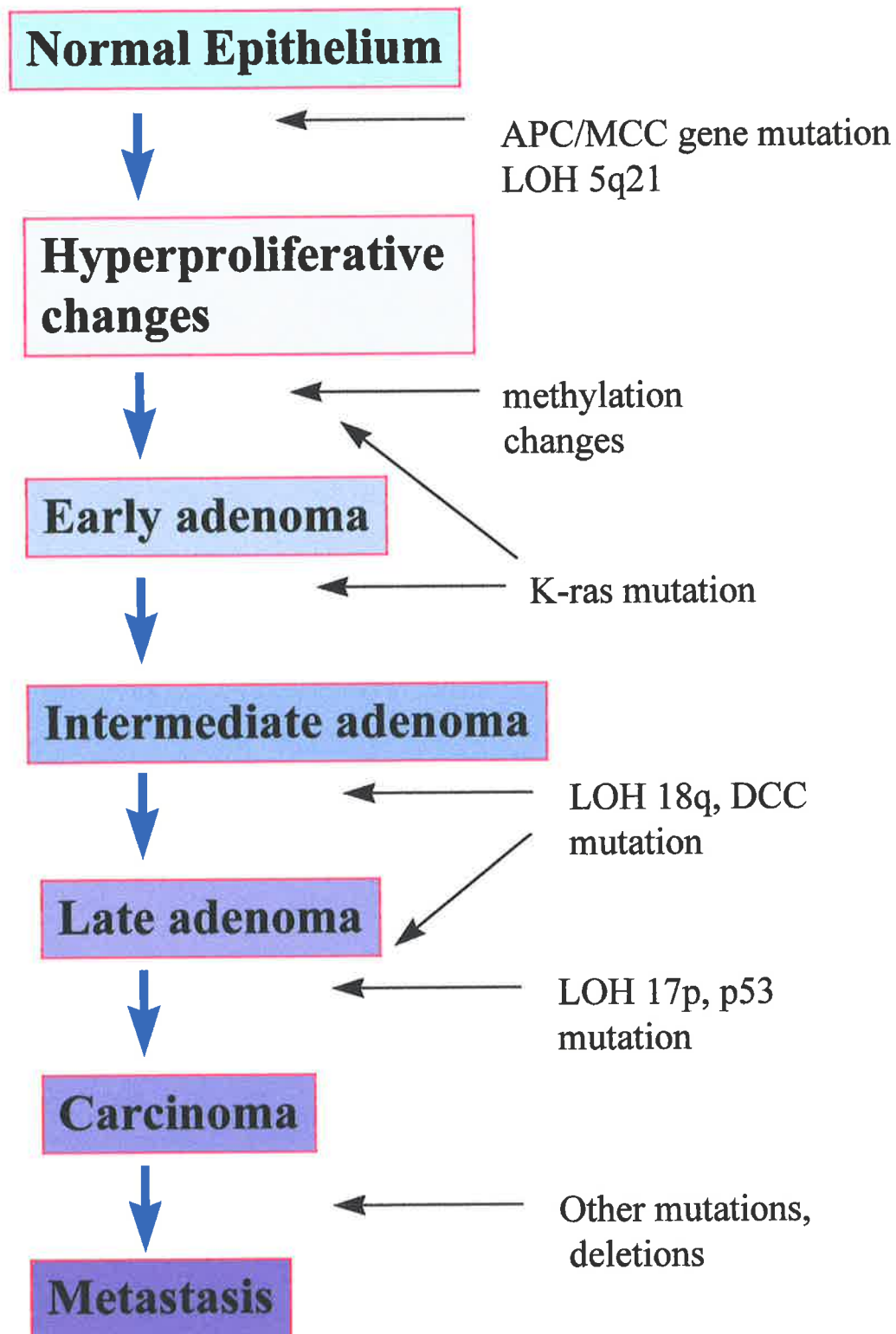
Two chromosomal regions 17p and 18q are lost in over 75% of colorectal carcinomas (Vogelstein et al., 1988; Delattre et al., 1989) and at least 6 additional loci are each lost in over a quarter of carcinomas (Vogelstein et al., 1989). The tumour suppressor genes on 17p and 18q have been identified as p53 and DCC (deleted in colorectal carcinoma) respectively (Baker et al., 1989; Fearon et al., 1990).

A small proportion of colorectal cancers (5%) arise as a result of an inherited mutation to any one of four mismatch repair genes. This syndrome is referred to as hereditary nonpolyposis colorectal cancer (HNPCC) or Lynch syndrome and affected individuals develop CRC at a relatively early age (before 45 years of age) and may also have a predisposition to develop carcinoma of the endometrium, ovary, breast, stomach

or pancreas. Inactivating mutations of both alleles of any one of the mismatch repair genes means the cell cannot repair replication errors, thus resulting in an accumulation of mutations in the daughter cells. (reviewed in Heuman et al., 1997).

Allelic deletions of the NM23-H1 locus may be associated with development of distant metastases. Cohn et al. (1991) studied 21 patients prospectively and found that metastases had developed in 8/11 patients with NM23-H1 deletions but in only 2/10 without deletions, after a median follow-up of 25 months. They suggest that NM23-H1 may be a late acting tumour suppressor gene in colorectal carcinoma. However, more recently, allelic imbalance at the NM23-H1 (also known as NME1) locus in colorectal cancers was found *not* to be linked to nodal or liver metastases (Lamb et al., 1996).

Figure 1.2. Progression of Colorectal Cancer
(adapted from Fearon & Vogelstein, 1990.)



1.4 MUTATIONS AS TUMOUR-SPECIFIC MARKERS

The model of colorectal cancer progression (Figure 1.2) indicates a role for both oncogene activation and inactivation of several tumour suppressor genes. The role of two of these mutations is described in more detail below.

1.4.1 *Ras oncogene mutations*

The name *H-ras* and *K-ras* derived from the sarcoma viruses from which they were first identified i.e. *v-H-ras* is the transforming gene of Harvey, BALB and Rasheed sarcoma viruses, while *v-K-ras* is the transforming gene of Kirsten sarcoma virus (Barbacid, 1987). Mutated *ras* oncogenes were first identified by their ability to transform immortalised NIH3T3 fibroblasts in DNA transfection experiments (reviewed in Weinberg, 1984).

Ras is the only oncogene known to be involved in a relatively large percentage of colorectal tumours. In particular, codon 12 of the *K-ras* gene is a frequent target of point mutations, and such mutations are found in about 40% of intermediate and late stage adenomas and carcinomas of the colon (Bos et al., 1987; Forrester et al., 1987; Vogelstein et al., 1988). No difference in frequency of *K ras* mutations was observed between proximal and distal tumours (Delattre et al., 1989). More recently, gene "knockout" technology has been used to disrupt the mutant *K-ras* allele in colon cancer cell lines which results in slowing of the growth rate and reversal of the cell lines' ability to form tumours in nude mice (Shirasawa et al., 1993). Other evidence supporting the role of *K-ras* in development of malignancy has come from work with non-small cell (NSC) lung cancer. Slebos et al. (1990) showed that in NSC lung cancer patients the presence of *K-ras* mutations is a negative prognostic indicator for both disease-free and overall survival.

Mukhopadhyay et al. (1991) showed that in NSC lung cancer cell lines, antisense inhibition of mutant *K-ras* expression reduced both the growth rate and tumorigenicity.

The *ras* gene family, *H-ras*, *K-ras* and *N-ras*, encode highly similar 21 kilodalton proteins (p21) that are involved in signal transduction pathways critical for normal proliferation and differentiation. These proteins belong to the GTPase superfamily and show significant homology to G proteins suggesting that they too are involved in signal transduction across the cell membrane to the nucleus (the term G proteins is reserved for the classical heterotrimeric signal transducing proteins). *Ras* genes are highly conserved throughout evolution, suggesting they play a fundamental role in cell biochemistry (reviewed in Barbacid, 1987).

Normal *ras* p21 acts as a molecular switch whose on/off states are triggered by binding and subsequent hydrolysis of GTP. Ras is active in the GTP bound form and is inactivated as GTP is hydrolysed to GDP by its intrinsic GTPase activity (Bourne et al., 1990). The active and inactive conformational states of Ras are regulated by two kinds of proteins: guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs increase the dissociation rate of GDP, allowing GTP to bind Ras. GAPs inactivate the Ras-GTP complex by accelerating the slow intrinsic rate of GTP hydrolysis thus switching off the ras signal (Scheffzek et al., 1997).

Ras proteins are located at the inner surface of the cell membrane and are activated in response to receptor tyrosine kinases or other growth factor dependent stimuli, generating a signal for cell proliferation i.e. they transmit signals from upstream tyrosine kinases to downstream serine/threonine kinases ultimately leading to changes in gene expression (reviewed in Waldmann and Rabes, 1996).

It has been shown that a functional *ras* protein is essential for cells to leave the G0 stage of the cell cycle and pass through the G1/S transition and that this is mediated by the

retinoblastoma (Rb) tumour suppressor gene protein (Peeper et al., 1997). Recently, Ras and Rho (another member of the Ras superfamily of small GTP-binding proteins) have been reported to be the proteins involved in signalling pathways driving expression of molecules that regulate apoptosis (reviewed in Gomez et al., 1998).

Point mutations in codons 12, 13 and 61 of *ras* genes convert normal *ras* p21 to the oncogenic form (reviewed in Barbacid, 1987) e.g. the presence of a glycine residue at position 12 appears to be necessary for the normal α -helical structure (and hence normal GTP binding site) of the protein and substitution by any other amino acid (except proline) results in oncogenic activation (Seeburg et al., 1984). Oncogenic mutations, present in some 25-30% of human tumours, reduce the intrinsic GTPase activity of the *ras* protein and render it insensitive to GAPs (McCormick, 1989). Mutant *ras* p21 remains in the "switched on" GTP bound state for a much longer period and inappropriately transduces signals to downstream effector molecules (reviewed in Waldmann and Rabes, 1996).

Ras mutations could occur as a result of tumour progression: the "mutator phenotype" hypothesis of Loeb (1991) suggests that tumour cells encode altered enzymes of DNA metabolism that make for an increased mutation rate. Others suggest that acquisition of a *ras* mutation may precede development of malignancy (Kumar et al., 1990). In colorectal carcinoma, mutations in codon 12 of K-*ras* were present in both aneuploid and diploid tumour subpopulations (sorted by flow cytometry) suggesting that these mutations precede ploidy alterations in the progression of these tumours (Burner et al., 1989). Others have also demonstrated mutations of the K-*ras* oncogene in adenomatous polyps suggesting that this mutation occurs early in progression (Bos et al., 1987, Forrester et al., 1987, Burner and Loeb 1989). It is thought that *ras* may be a target of bowel carcinogens that cause point mutations through adduct formation (Barbacid,

1987). An example is the formation of mutagenic products in the bowel from heterocyclic amines derived from cooked meat and fish (Nagao and Sugimura, 1993).

1.4.2 Mutational inactivation of the p53 gene

The p53 protein was discovered through its association with the large T antigen of SV40 (Lane and Crawford, 1979; Linzer and Levine, 1979). The p53 gene was originally thought to be a dominant acting oncogene, able to transform primary rat cells in culture (Eliyahu et al., 1994; Parada et al., 1984). However, this interpretation was found to be incorrect, since the cloned p53 gene used in these experiments contained a point mutation; normal p53 cDNA clones were unable to cooperate with *ras* in transformation (reviewed in Stanbridge, 1990). Others have since shown that the wild-type gene serves to suppress the growth (i.e. acts as a tumour suppressor gene) of transformed murine cells (Eliyahu et al., 1989; Finlay et al., 1989) and human cells (Baker et al., 1990; Casey et al., 1991).

The importance of p53 as a tumour suppressor gene has been well illustrated in the Li-Fraumeni syndrome, a rare familial autosomal dominant syndrome characterised by the occurrence of a diversity of childhood and adult tumours in affected individuals. In one of the earliest studies, germ line p53 mutations were detected in all 5 Li-Fraumeni families (Malkin et al., 1990). Another family study identified the same point mutation in codon 245 of the p53 gene in 4 family members (Srivastava et al., 1990). These findings suggested that the inherited p53 mutation may predispose affected family members to develop cancer.

The p53 gene encodes a 393 amino acid, 53 kilodalton nuclear phosphoprotein. The gene is comprised of 11 exons within a span of 20 kilobases on the short arm of chromosome 17. A model for the normal function of p53 suggests that p53 acts as a

negative regulator of cell division (Lane 1992). More recent studies have shown that wild-type p53 functions to regulate the transition from G1-S phase of the cell cycle as a checkpoint control. In the presence of DNA damage, p53 protein accumulates and promotes expression of downstream genes. One of these genes is WAF1/Cip1, the product of which, p21, binds to cyclin dependent kinases and inhibits their activity thereby preventing phosphorylation of the retinoblastoma protein required for cell cycling.

Another gene target is GADD45 (growth arrest DNA damage), the protein product of which binds to PCNA (proliferating cell nuclear antigen). In this way DNA replication is prevented and the cell cycle is arrested. This allows DNA repair to take place before the synthesis of new strands of DNA which would have incorporated a mutation (reviewed in Chang et al., 1995). Alternatively, if the damage cannot be successfully repaired, wild-type p53 induces programmed cell death (apoptosis) (Lane, 1993). It is thought that this regulating function requires sequence specific binding of the protein to DNA. Evidence exists that wild-type p53 binds to DNA and activates expression of genes adjacent to the p53 DNA binding site, whereas mutated forms inhibit this activity (Raycroft et al., 1990; Kern et al., 1992). All the mutated p53 proteins analysed to date have lost this sequence specific binding; furthermore many of these mutant proteins form oligomeric complexes with the wild-type proteins to abolish this function (Nigro et al., 1989; Lane, 1992). The function of the wild-type protein can also be abrogated by other oncogene products that bind to it and inhibit p53-mediated transactivation e.g. E6 of the human papilloma virus, EA1 of adenovirus, and the mdm-2 cellular oncogene product (reviewed in Momand et al., 1992).

Loss of heterozygosity (LOH) of chromosome 17p has been documented in 75% (45/60) of colorectal carcinoma specimens, but this finding was infrequent in adenomas.

Vogelstein's group suggest that 17p losses are associated with progression from adenoma to carcinoma (Vogelstein et al., 1988; Fearon et al., 1987). In several of these carcinomas, the remaining allele contained a point mutation in the p53 gene (Baker et al., 1989; Nigro et al., 1989). The data are consistent with the hypothesis that the wild-type p53 gene inhibits colorectal tumour growth, and the removal of the wild-type gene is the selective pressure underlying 17p allelic losses in these tumours. Loss of the wild-type allele uncovers the effect of an existing mutation in the remaining p53 locus, which allows further tumour progression (Fearon and Vogelstein, 1990). LOH involving the short arm of chromosome 17 occurs with high frequency in astrocytomas, breast, lung and colorectal carcinomas (Nigro et al., 1989). Baker et al. (1989) have defined the region that was commonly lost in colon cancers as 17p12 to 17p13.3, which encompasses the p53 gene. Experiments with p53 "knockout" mice (p53-/p53-) showed development of multiple tumours after a few months of age, underscoring the role of p53 as a tumour suppressor gene (Donehower et al., 1992).

Inactivation of p53 by mutation (or loss) or by its interactions with oncogene products of tumour viruses results in loss of growth regulating properties (Levine et al., 1991; Hollstein et al., 1991). A point mutation within the coding sequence of the p53 gene appears to be the most common change in p53 in human cancers, giving rise to a protein with a changed conformation, longer half-life and altered function (Hollstein et al., 1991; Levine et al., 1991; Chang et al., 1993). The majority of point mutations (which are mostly missense mutations) are clustered in 4 conserved domains of the p53 gene, in exons 5 to 8 (Nigro et al., 1989). At least four mutation "hot spots" have been localised at amino acid residues 175, 248, 273, and 282 in several human cancers (Harris, 1991). P53 gene mutations seem to be the most common genetic changes in human cancers (Vogelstein, 1990) and are found in about one half of adult cancers of the colon, stomach,

lung, oesophagus, breast, liver, brain, reticuloendothelial, and haematopoietic tissues (reviewed in Chang et al., 1995). To date some 570 different mutations in p53 have been identified in human cancers (Hainaut et al., 1998).

1.5 EPITHELIAL-SPECIFIC EXPRESSION AS A MARKER OF CARCINOMA CELLS

Although markers such as *K-ras* or p53 mutations are tumour-specific, they are only found in a proportion of tumours, thus many patients did not have a suitable marker with which to detect tumour cells in blood. To overcome this problem, I used expression of epithelial-specific markers to detect epithelial cells in blood, using the premise that colonic epithelial cells do not have access to the circulation under normal circumstances. Thus detection of epithelial cells in blood prior to surgical resection for colorectal malignancy implies the presence of a metastatic process.

1.5.1 Expression of cytokeratins as markers of epithelial cells

The intestinal epithelium is an example of a rapidly self-renewing simple epithelium. In the adult intestinal mucosa, a pool of relatively un-differentiated epithelial cells in the crypts steadily proliferates and gives rise to sub-populations which undergo terminal differentiation to either enterocytes, goblet cells, neuroendocrine cells, or Paneth cells. The process of differentiation takes place in a timed and orderly migration from the crypt up to the villus tip where the cells eventually undergo apoptosis (programmed cell death) and are shed into the bowel lumen. An essential part of the differentiation program is the assembly of specific structures of the cell, notably the complex stable filamentous

cytoskeletal structure, comprised of intermediate-sized (8-14 nm) filaments of the cytokeratin family (reviewed in Moll et al., 1993).

Cytokeratins (CKs) represent a complex multigene family of proteins comprising 20 distinct CK polypeptides identified from various human epithelial cell types (Moll et al., 1992). The authors had previously recognised that intestinal cells expressed CKs 8, 18 and 19 (Moll et al., 1982). Subsequently they reported another CK, which had been present in 2 dimensional gels as a prominent protein band at 46 kilodalton MW, in cytoskeletal preparations from human intestinal mucosa but not from other tissues; they named this new protein CK 20 (Moll et al., 1990).

The cytokeratins were originally defined according to their molecular weight and isoelectric pH so that CK1-19 were numbered according to decreasing MW and iso pH values (Moll et al., 1982). Cytokeratin 20, identified much more recently, has a remarkably large sequence divergence from all known CKs, with only 58% amino acid homology in the conserved α -helical rod domain between CK 20 and the closest other member, CK14 (Moll et al., 1990).

1.5.1.1 Distribution of CK 20

Expression of CK20 is almost entirely confined to the gastric and intestinal epithelium, urothelium and Merkel cells (Moll et al., 1992). Using immunohistochemistry with CK 20 specific antibodies, Moll et al. (1993) showed that CK 20 synthesis first appears at embryonic week 8 in individual developing simple epithelial cells of the intestinal mucosa. In later foetal stages, CK 20 synthesis was detected in most goblet cells and a variable number of villus enterocytes, compared to adult tissue in which all goblet cells and enterocytes as well as certain low differentiated columnar cells contained CK 20. Neuroendocrine and Paneth cells were negative. The authors also used immunohisto-

chemical staining in 52 cases of adenocarcinoma of the colon and rectum to look for the presence of CKs 20, 7, 17, 18, 19, 4, and 13. The sections were almost all negative for CKs 4, 7, and 13, variably positive for 14 and 17, 100% uniformly positive for 18 and 19, while for CK 20 50/52 were positive. Of these cases 20 (38%) exhibited a nearly uniform staining with all cells being positive, however the remaining CK 20 positive tumours showed heterogeneous but usually extensive staining, with more than 50% of the tumour positive. Reduction or loss of morphological features did not result in decreased CK 20 immunostaining hence poorly differentiated tumours expressed CK 20 at significant levels. The 2 negative cases were graded as grade 4 (undifferentiated).

1.5.1.2 Distribution of CK 19

CK 19 has been reported to be present in a broad range of epithelial tissues as a major component of the cytoskeleton in simple epithelia and as a minor component in stratified epithelia (Moll et al., 1982). In that study, CK19 was not detected in hepatocytes nor epidermis or related tissues. Further, normal lymph node tissue did not contain any of the cytokeratins, the sole type of intermediate filament being vimentin, whereas adenocarcinomas of the gastrointestinal tract and breast were found to strongly express CK19.

1.5.2 Expression of mucins as markers of epithelial cells

Expression of mucin (MUC) genes, detected by RT-PCR, may also provide likely candidate markers for carcinoma cells in blood. The mucins are high MW glycoproteins with O-glycosidic-linked carbohydrate side chains. They are synthesised by glandular epithelial tissue as membrane-bound or secreted forms. The human MUC1 gene codes for a mucin glycoprotein found to be over expressed in breast carcinoma tissue (Gendler et al., 1990; Noguchi et al., 1994; McGuckin et al., 1995). The MUC2 gene codes for a

high MW glycoprotein that is polymerised end to end to form a large polymeric molecule (Allen et al., 1998). Both MUC 1 and MUC 2 have been found to be over-expressed by many human colon carcinoma cell lines (Devine et al., 1992) and primary mucinous or "colloid" colon tumours but not in well differentiated tumours (Ho et al., 1993). Excessive and abnormal mucin synthesis has been observed in cancers of the breast and colorectum. In cancer, changes to the carbohydrate side chains and excessive sialylation result in altered cell adhesion, immunological profile so as to escape immune destruction, and increased invasiveness (reviewed in Verma, 1994). Bresalier et al., (1996a) showed that colon cancer metastases expressed a decrease in mucin core structures and an increase in sialyl T, Tn and Le(x) compared to the primary tumours. Sialylated carbohydrate structures on mucin play a role in adhesive interactions involving both basement membrane and endothelial associated ligands.

Additional evidence that mucins may play an important role in metastasis was provided by Schwartz et al. (1992). They showed that cell lines producing high levels of mucin demonstrated increased adherence to basement membrane proteins and invaded a reconstituted basement membrane to a greater extent than their low mucin producing counter parts. Specific inhibition of O-glycosylation significantly ameliorated each of the metastasis related events with the greatest effect being on the high mucin producing cell lines.

1.6 METASTASIS-ASSOCIATED MARKERS

Although epithelial-specific markers proved to be reliable markers for epithelial cells in pre-operative blood, they were not able to distinguish malignant colon cells from normal colon cells that may have been shed into the blood stream during colorectal resection

(Chapter 10). As I had found that normal colon cells are sometimes released into the blood during bowel resections for benign conditions such as diverticulitis, the question of whether tumour cells are shed at the time of resection for malignancy was not able to be adequately addressed using the current markers. Thus I undertook a search for carcinoma or metastasis-associated markers to use in RT-PCR assays to specifically detect malignant colon cells.

Metastatic tumour spread is the culmination of a series of steps whereby tumour cells detach from the primary tumour, transgress the extracellular matrix, invade blood and lymph vessels and finally arrest in distant organs where some are able to grow as secondary or metastatic deposits (Liotta et al., 1991; Nicolson, 1988, 1989, 1991). In order to accomplish this, tumour cells within the primary neoplasm must acquire motile and invasive properties. Cells with these properties represent a subpopulation within the heterogeneous tumour mass (Kerbel, 1990). The first experimental evidence for this was provided by Fidler and Kripke (1977) using the murine B16 melanoma, they showed that different tumour cell clones differed in their ability to form lung tumour nodules in recipient mice. This work was extended by Fidler et al. (1981) who isolated cell lines from a parental neoplasm with differing capacities to metastasise; some being tumorigenic but not metastatic with others being both tumorigenic and highly metastatic. Many other studies have since confirmed the heterogeneity of tumours with respect to metastatic potential (reviewed in Fidler, 1991).

The major steps in the process of metastasis are as follows:

(1) Angiogenesis - the formation of new blood capillary networks around the growing tumour. Small ($2\text{-}3\text{mm}^3$) solid tumours may persist *in situ* for months maintained by simple diffusion of oxygen and nutrients, however growth beyond this size requires a blood supply (Folkman, 1990).

- (2) Detachment of the tumour cell from the primary mass and degradation of the surrounding interstitial stroma.
- (3) Adherence to and degradation of components of the extracellular matrix (ECM) including the basement membrane and intercellular junctions thus permitting intravasation into lymph and blood vessels.
- (4) Tumour cells that survive the circulation must arrest in the capillary beds of a distant organ, extravasate into the organ parenchyma and proliferate to form secondary tumours (reviewed in Fidler, 1991). This process is outlined in Figure 1.1.

These functions are not unique to tumour cell behaviour since detachment and adhesion, proteolysis and migration are steps of trophoblast implantation, mammary gland involution, embryonic morphogenesis and wound repair. The difference between normal and tumour cell invasion is one of regulation, the normal process of invasion being tightly controlled (Aznavorian et al., 1993).

1.6.1 Expression of metastasis-associated molecules as markers of carcinoma cells

The acquisition of the metastatic phenotype must be accompanied by an altered expression of genes whose products are involved in this process. These include adhesion molecules, both ligands and receptors, and proteolytic enzymes such as those of the matrix metalloproteinase (MMP) family and serine proteinases. Each of the steps of metastasis is a necessary component; some of the molecules involved are described in the following sections.

1.6.2 Angiogenic proteins.

The induction of angiogenesis is mediated by angiogenic factors secreted by both host and tumour cells. To date there have been at least 12 angiogenic proteins described

(Blood and Zetter, 1990; Folkman, 1996). The two most abundant found in tumours are basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). Expression of VEGF and its receptor KDR was found to be higher in metastatic than in non-metastatic tumours and directly correlated with the extent of new blood vessel formation and degree of proliferation, whereas expression of bFGF and its receptors did not differ among different tumour types (Takahashi et al., 1995).

1.6.3 Cell adhesion molecules

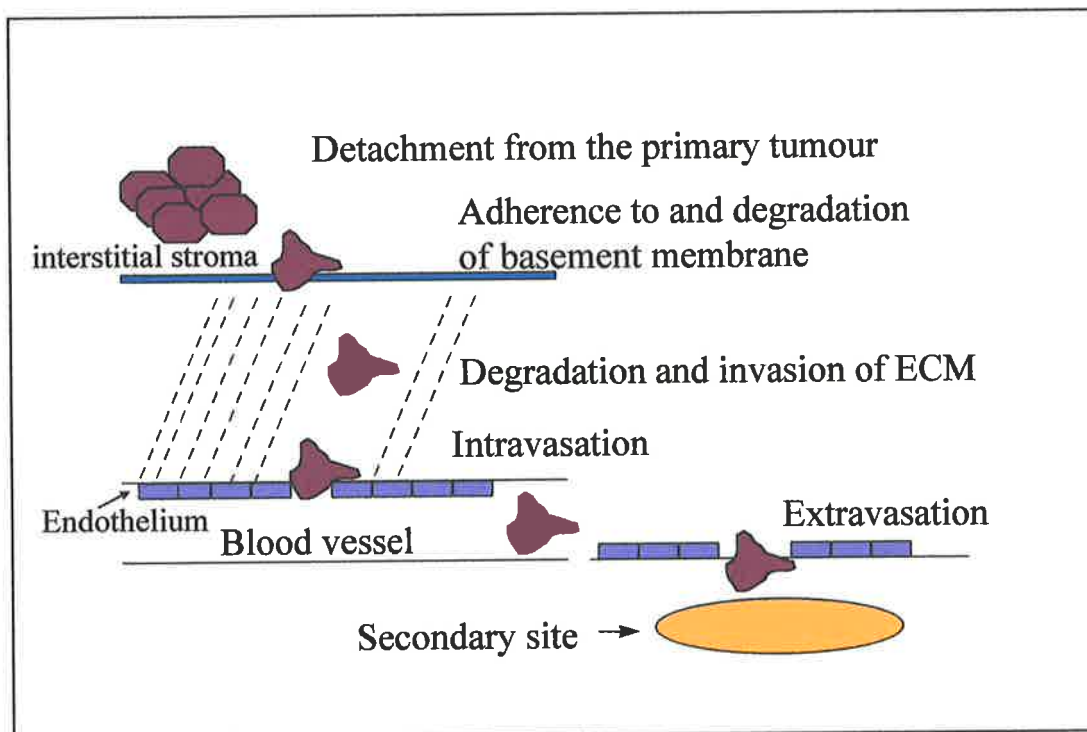
Many steps in the metastatic sequence involve cell/cell and cell/matrix interactions that are mediated by specific cell surface adhesion receptors of the integrin and non-integrin type. Integrins are a large class of $\alpha\beta$ heterodimeric receptors for adhesion molecules with broad specificity. Integrins provide the link between components of the ECM and the cytoskeletal components (talin, vinculin and actin microfilaments) of the cell (reviewed in Aznavoorian et al., 1993).

1.6.3.1 Cadherins

Cadherins are calcium ion dependent cell adhesion molecules that mediate cell-cell binding (reviewed in Takeichi, 1990). A reduction in expression of E (epithelial) cadherin facilitates detachment of tumour cells and is thought to play a causal role in progression from adenoma to carcinoma (Perl et al., 1998). Evidence for involvement of this receptor in invasion has come from analysis of carcinoma cell lines in culture which revealed an association between loss of E-cadherin expression, loss of adherent phenotype, and acquisition of the ability to invade collagen gels (reviewed in Hart and Saini, 1992). Interestingly, the APC gene product normally associates with E-cadherin and β -catenin in stabilising intercellular junctions. Mutations in the APC gene have

been found in the germline of patients with familial adenomatous polyposis (FAP) syndrome and in some 70% of sporadic colon carcinomas. APC mutations, although an early event in colorectal tumour progression, result in abnormal APC protein which may interfere with normal cell-cell adhesion thus contributing to the invasive phenotype (Bresalier, 1996b).

Figure 1.1 The metastatic cascade



1.6.3.2 Laminin-5 γ 2

In 1992, Kallunki et al. described the cloning and chromosome localisation of another adhesion molecule, the laminin γ 2 chain, which is one of three chains comprising the laminin-5 molecule. Laminin-5 (also known as kalinin, epiligrin or nicein), composed of a heterotrimeric molecule of γ 2, β 3 and α 3 chains, is an isoform of the laminin family. Members of this family are large basement membrane glycoproteins which function in cell growth, differentiation, adhesion and locomotion, as well as being true structural components of the basement membrane (Tryggvason, 1993). The 3 subunits, the α 3, β 3 and γ 2 chains have been found to be differentially expressed relative to each other in various human tissues and cell lines by Northern blotting. The co-expression of all three however, was required for the expression of cell-scattering activity (migration) as observed in culture. Most gastric and squamous cell carcinoma cell lines constitutively expressed all three subunits, while other carcinoma cell lines expressed one or two. Interestingly, addition of the tumour promoter TPA (12-O-tetradecanoylphorbol-13-acetate) or epidermal growth factor (EGF) to culture media greatly increased the expression of the 3 subunits and the observed cell scattering activity further suggesting that laminin-5 is associated with cell motility and migration. High levels of EGF have been reported in colon and gastric cancers. (Mizushima et al., 1996).

The gene for the γ 2 chain has been localised to chromosome 1q25-q31 by *in situ* hybridisation and the cDNA has been sequenced (Kallunki et al., 1992). The same authors used Northern blotting to examine expression of laminin chains in 20 different human foetal tissues including colon. Expression of the γ 2 chain was restricted to epithelial tissue and was highest in lung and skin while being undetectable in colon. Expression in adult tissues was not examined in this paper. Evidence for the importance

of this molecule in adhesion of epithelial cells to the basement membrane was demonstrated in a patient with junctional epidermolysis bullosa, a severe skin blistering disease. Mutations in the $\gamma 2$ chain gene were found in skin tissue from this patient (Pulkkinen et al., 1994). Laminin-5 can behave as a motility factor or as an adhesion factor dependent on the presence of tissue plasminogen activator (tPA), which converts plasminogen to plasmin. In cultures of epithelial cells in which plasmin is not present, laminin-5 in the ECM permits cell motility. After treatment of the culture with plasmin, laminin-5 subunits are processed into a form which not only impede motility but are now capable of assembling hemidesmosomes (cell-matrix adhesive complexes) (Goldfinger et al., 1998).

1.6.3.3 Carcino-embryonic antigen

The carcino-embryonic antigen (CEA) family, members of the immunoglobulin superfamily, comprise related glycoproteins encoded by many different CEA-related genes on the long arm of chromosome 19. CEA is a membrane glycoprotein consisting of a single polypeptide chain with multiple carbohydrate side-chains, varying in size and tissue distribution (Thompson et al., 1992). CEA and related glycoproteins function as calcium-independent intercellular adhesion molecules, and altered expression may lead to altered tissue architecture at the primary tumour site (reviewed by Bresalier, 1996b).

1.6.3.4 DCC

The DCC (deleted in colon cancer) gene is a member of the immunoglobulin superfamily and encodes a cellular adhesion molecule (reviewed in Marshall, 1991; Weinberg, 1991). The gene, a candidate tumour suppressor gene, was identified on chromosome 18q due to the fact that this region is the target of LOH in colorectal tumours, being lost in more than 70% of carcinomas and in almost 50% of late stage adenomas (Vogelstein et al., 1988;

Delattre et al., 1989; Fearon et al., 1990). Distant metastases from colorectal tumours have also been associated with high 18q allelic loss and this was an independent predictive factor of poor prognosis in colorectal cancer patients (reviewed in Bresalier, 1996b).

1.6.3.5 Expression of CD 44 variants

CD44 glycoproteins are trans-membrane receptors widely distributed on various cell types including lymphocytes, mesenchymal cells, neuroectoderm cells, and defined epithelia. They function as lymphocyte homing receptors of activated circulating lymphocytes and as adhesion molecules of epithelial cells (reviewed in Herrlich et al., 1993). Isoforms of CD44 arise from alternative splicing of exons 6-15 (v1-v10) into a site between exons 5 and 16 (reviewed in Bolodeoku, 1998), the standard (haemopoietic) form consisting of exons 1-5 and 16 (encoding the extracellular domain), exon 17 (the transmembrane domain) and exons 18-20 (the cytoplasmic domain).

It is thought that both activated "homing" lymphocytes and metastasising cancer cells make use of the same CD44 variant (v6) to bind to specific ligands in lymph nodes and in the endothelium to ensure passage into and through these barriers. The CD44 variant v6 has also been found to confer metastatic potential to non-metastatic rat tumour cells, suggesting that expression of this variant played a role in metastasis (reviewed in Herrlich et al., 1993). More recently, Mulder et al. (1994) showed, using immunohistochemistry, that overexpression of CD44v6 in colorectal tumours was an independent prognostic variable associated with tumour-related death.

A potential problem exists, however, in using CD44 expression as a marker of tumour cells in blood in that activated lymphocytes and monocytes also express CD44v6 (Salles et al., 1993; Mackay et al., 1994). In our laboratory, a project was undertaken using

RT-PCR and primers spanning the variant region, to determine expression of CD44 isoforms in breast cancer cells (Eaton, 1997). It was found that two predominant isoforms of CD44 were detected in breast cancer cell lines; the standard isoform and a higher MW band corresponding to the epithelial isoform and that the relative proportions of each of these was extremely variable between duplicate cell dilutions, or even from a single cancer cell isolated from fresh tumour tissue by micropipetting. The conclusion from these studies was that the epithelial variant was not exclusively expressed by cancer cells. Also, the presence of occasional blood lymphocytes or monocytes isolated from blood concomitantly with tumour cells, which also express variant exons, could not be excluded. This meant that distinguishing cancer cells from other blood cells using CD44 expression would not be possible (Eaton, 1997).

1.6.4 Proteolysis of the extra cellular matrix (ECM) during invasion

The ECM is composed of a variety of molecules including collagen type 1V, fibronectin, laminin, vitronectin and elastin (Jiang et al., 1994). Proteolysis and cell migration are normal physiological processes that occur during wound healing, blastocyte implantation, embryo morphogenesis, and angiogenesis (formation of new blood vessels). Tumour cells are able to recruit the same enzymatic pathways for the inappropriate invasion of tissue compartments. For a tumour cell to invade, it must induce local proteolysis of the ECM either by expressing proteolytic enzymes or by stimulating the tumour stromal cells to express them. Cyclic attachment and release must then occur as the tumour cell 'crawls' through the area of degradation. Proteolysis must therefore be tightly regulated to permit subsequent reattachment to intact ECM – it is only at the point of pseudopodial projection of the tumour cell that proteolysis occurs

(Pyke et al., 1995). Proteolysis is regulated by a balance of activated proteolytic enzymes and their inhibitors (reviewed in Liotta et al., 1991).

The proteolytic enzymes (proteases) include the serine, aspartyl, cysteinyl and metal atom-dependent (metalloproteinases). The latter group are secreted in latent form (zymogens) and are activated once the interaction between the metal atom (zinc) and the sulphhydryl side chain is disrupted (Stetler-Stevenson et al., 1989). One of the serine proteases, urokinase plasminogen activator (uPA) has been closely linked to the metastatic phenotype (Dano et al., 1985), but was found by *in situ* hybridisation to be expressed by stromal cells; its receptor (uPAR) however, was detected in cancer cells at invasive foci in colon carcinoma (Pyke et al., 1991).

1.6.4.1 Matrilysin

One member of the matrix metalloproteinase family, matrilysin (alternatively known as PUMP-1 or MMP-7), is of particular interest as a marker of carcinoma cells in blood as it is expressed by tumour cells rather than by surrounding stromal cells. Matrilysin has been included in the stromelysin subclass of matrix metalloproteinases due to their common ECM substrate specificities, in particular, fibronectin, collagen IV and laminin (MacDougall and Matrisian, 1995). Matrilysin protein differs from other members in that it has a truncated structure which excludes the carboxy-terminus. This is important in that the C-terminus contains the binding site for TIMP (tissue inhibitor of metalloproteinases) so that matrilysin may escape such inhibition (Baragi et al., 1994). Localisation of matrilysin mRNA to the tumour cells in carcinoma tissue is unusual since most of the other MMPs are produced in the tumour stromal cells (Pyke et al., 1993; Wilson and Matrisian, 1996).

1.6.5 Criteria for markers for carcinoma cells in blood

A prerequisite for a suitable expression marker for carcinoma cells in blood is that it must be expressed by the tumour cells themselves rather than by the surrounding tumour stromal cells, and, ideally have markedly increased or unique expression in metastasising cells compared to non-metastatic tumour cells or normal epithelial cells. It is for this reason that we have excluded urokinase plasminogen activator (uPA) (Pyke et al., 1991) and many of the metalloproteinases (Heppner et al., 1996) as they are found localised to stromal cells at the invading front of the tumour, matrilysin (MMP-7) being a notable exception (Itoh et al., 1996). The uPA receptor is expressed by tumour cells but is also expressed by monocytes (Fazioli and Blasi, 1994) and is thus unsuitable to use as a marker of carcinoma cells in blood.

Northern analysis of CEA mRNA expression in colon adenocarcinomas and matched normal mucosa showed widely varying expression ratios from equal to 10 fold more in the tumour samples (Boucher et al., 1989). More importantly, the significant expression of CEA in normal mucosa, and benign conditions (diverticulitis) (Boucher et al., 1989) suggested to us that CEA expression measured by RT-PCR would not be a discriminating marker of colon carcinoma cells. Other molecules, which are important in the progression and metastasis of colon cancers because of their down-regulation or loss of function e.g. E-cadherin and DCC, are also not suitable as expression markers for cancer cells in blood.

1.7 METHODS FOR THE DETECTION OF CARCINOMA CELLS IN BLOOD

1.7.1 Immunohistochemical techniques

Previously, investigators have relied on cytological assessment of bone marrow or blood smears, a technique that suffered from false positive (and negative) identification of tumour cells (reviewed in Christopherson, 1965). More recently, immunohistochemical (IHC) methods have been used to detect tumour cells in the bone marrow and lymph nodes of patients with a variety of cancers. Tumour cells in bone marrow aspirates are concentrated by centrifugation of the samples through a density gradient, typically Ficoll-Hypaque, the tumour cells migrating with the mononuclear cell (MNC) layer. A smear of the MNCs is then made and incubated with monoclonal antibodies directed against epithelial antigens. The epithelial (tumour) cells are identified by IHC or immunofluorescence techniques and counted under a light microscope. Similarly, lymph node micro-metastases may be detected by IHC of paraffin-embedded sections. In either case, the sensitivity of detection of tumour cells depends on the number of cells (or fields) counted and has been reported as $1/10^4$ – $1/10^6$ (reviewed in Pelkey et al., 1996).

Schlimok et al. (1990) used IHC detection of cytokeratin (CK) 18 to detect colon cancer cells in 42/156 bone marrow aspirates at presentation. Clinical follow-up of these patients revealed a significantly higher relapse rate in those positive for tumour cells in bone marrow at presentation. Lindemann et al. (1992) also used IHC to detect CK 18 in bone marrow aspirates from patients with CRC and found 32% positive. Follow-up studies showed that a positive result correlated with reduced disease-free survival. Pantel et al. (1993) used IHC to detect both CK 18 and ErbB2 expression in bone marrow of patients with breast and gastrointestinal cancers. The incidence of ErbB2+/CK 18+ cells was positively correlated with the stage of tumour progression.

Several studies using IHC for the detection of breast cancer cells in bone marrow have shown an increased risk of recurrence in patients in whom micro-metastatic tumour cells were detected (reviewed in Pelkey et al., 1996). In a review of a large cohort of 2,400 breast cancer patients, serial sectioning and IHC of lymph nodes increased the rate of detection of micrometastases compared to conventional histology by 13% and 16% respectively (Munro-Neville, 1990). Diel et al. (1992) studied 260 breast cancer patients for the presence of breast cancer micrometastases in bone marrow by IHC. They found 22 relapses occurred among 81 patients with positive cells in bone marrow compared with 4 relapses among 130 patients without tumour cells in bone marrow and this was independent of other staging factors ($p=0.0005$).

Although IHC of bone marrow and lymph nodes has been used to yield additional prognostic information regarding long-term outcome, the technique has not been widely used to detect tumour cells in blood samples. The reason for this, presumably, is due to the perceived lower frequency (10-100 fold) of tumour cells that may be present, so that to obtain the same level of sensitivity of detection as in bone marrow, at least 10 fold more cells must be counted making the technique a tedious, time consuming methodology.

1.7.2 Flow cytometry

Flow cytometry techniques, in particular fluorescence-activated cell sorting has been used to detect breast cancer cells in bone marrow aspirates. The reported sensitivity was 1 tumour cell in 10,000 nucleated marrow cells and was more often able to detect tumour cells than cytology alone (Leslie et al., 1990). Gross et al. (1995) used 4 colour immuno-fluorescence and flow cytometry to identify 1 breast cancer cell per 10^7

nucleated blood cells in a model system, but this technique has not been taken up by others. Recently, immunomagnetic enrichment with multiparameter flow cytometric and immunocytochemical analysis has been used to detect and quantify carcinoma cells in the blood from breast and prostate cancer patients. There was a highly significant difference in the number of circulating epithelial cells between cancer patients and normals, however, the presence of cancer cells had still to be confirmed by immunocytochemical staining (Racila et al., 1998).

1.7.3 Molecular techniques: detection of K-ras mutations

Since the introduction of the polymerase chain reaction (PCR) technique (Saiki et al. 1985, 1988) it is now possible to detect the presence of a molecular tumour marker, such as a mutation, with a high degree of sensitivity (Lee et al., 1987). Once identified in the tumour population, the marker can then be used to detect tumour cells in blood.

A mutation in codon 12 of the *K-ras* gene involving a transition from GGT-GTT is particularly useful as colorectal tumour marker as this mutation is found in about 40% of cases (reviewed in Bos 1989) and appear to be stable throughout the course of the disease (Losi et al., 1992). There are several techniques employing PCR for detection of point mutations of the *ras* genes (reviewed in van Mansfeld and Bos, 1992):

1.7.3.1 ASO (allele specific oligonucleotide) hybridisation.

In this system, a pair of oligonucleotide probes must be made for each mutation i.e. a wild type probe and a probe specific for the mutation - these probes vary by only a single base. Differentiation of the wild-type and mutant allele is based on imperfect hybridisation of the mismatched radiolabelled probe and relies on stringent washing conditions at a temperature just below the dissociation temperature (T_m) of the perfectly

matched probe. Hybridisation is detected by autoradiography. The sensitivity, even when using amplified DNA as the target material, is no greater than 5% and therefore is not suitable for detecting minimal numbers of tumour cells in blood. This technique has been used to detect *ras* mutations in the stool in 8/15 patients with curable colorectal tumours, however cloning of the PCR products was necessary to obtain hybridisation signals (Sidransky et al., 1992).

1.7.3.2 PCR 3' Mismatch technique.

The premise of this technique, also referred to as ARMS (amplification refractory mutation system) or PASA (PCR amplification of specific alleles) is that a mismatch at the 3' end of a primer greatly reduces amplification efficiency; Taq polymerase lacks a 3'-5' exonuclease activity and cannot repair a mismatch at the 3' end. It is possible therefore to detect a point mutation by selective amplification of the mutated allele, using a primer complementary to the mutant sequence (reviewed in Lo, 1998). However, the PCR conditions must be very stringent otherwise amplification of the non-mutated alleles proceeds as well. The stringent conditions reduces the efficiency and hence sensitivity of the PCR (Kwok et al., 1990). Sommer and colleagues (1992) suggest that with proper optimisation, this technique can be applied to the detection of any known single base change and is useful for carrier testing and population studies.

Ehlen and Dubeau (1989) have successfully used this technique to detect point mutations in codon 12 of *ras*. Their primers incorporated inosine at positions of non-homology within the 3 *ras* genes so that all three genes could be analysed in the same PCR. The sensitivity, using autoradiography, was such that a mutation could be detected in DNA mixed with a 10^7 excess of normal DNA. Stork et al. (1991), using ethidium

bromide fluorescence, detected a mutant *K-ras* allele in a 100 fold excess of normal cells in paraffin sections of hepatic and pancreatic tumours.

1.7.3.3 Primer-mediated restriction fragment length analysis

Several groups described this technique during 1989-91 which was referred to as restriction fragment length polymorphism (RFLP) analysis (Haliassos et al., 1989, Jiang et al., 1989, Kumar et al., 1990) or allele specific restriction analysis (ASRA) (Todd et al., 1991). Modified primers were designed to introduce a base substitution adjacent to the codon of interest in order to create an artificial restriction site with only one allelic form e.g. wild type. The PCR products were digested with the appropriate restriction enzyme and the fragments visualised on ethidium bromide stained gels. Digestion produced two fragments if the sequence was normal or left the fragment undigested if it was mutated.

The problem with this technique is that there may be another reason for lack of digestion e.g. the presence of an inhibitor and hence normal could be mistaken for mutant. This problem was addressed by Jiang et al. (1989). They designed the second (3') primer to introduce a restriction site into both normal and mutant products, to act as a control for digestion. The advantage of this technique is that the bands can be visualised on ethidium bromide stained gels without the need for radioactive hybridisation and is much more sensitive (up to 1 in 25,000) than any previously described technique. Another advantage of this technique is that it detects a mutation at either position 1 or 2 of codon 12.

Subsequently, Kahn et al. (1991) reported a modification of the technique which increased the sensitivity of detection of mutant alleles to 1 in 10,000. This was achieved by selective PCR amplification of mutant *K-ras* gene sequences employing a two stage

procedure. The first stage entails amplification of both mutant and wild type codon 12 sequences. The second stage involves amplification of undigested amplified fragments, enriched in mutant sequences i.e. after the first round of PCR, the products are digested with the enzyme BstN1 - normal sequences are cut, leaving mutant sequences undigested and available for amplification in the second round of PCR. These products are then subjected to BstN1 digestion and gel electrophoresis.

1.7.4.4 Single-strand conformation polymorphism (SSCP) analysis.

This technique is based on the principle that single-stranded DNA molecules take on specific sequence-determined secondary structures (conformers) under non-denaturing conditions. Molecules differing by as little as a single base may form different conformers and migrate at a different rate through a non-denaturing polyacrylamide gel (Orita et al., 1989 a,b). The secondary structure is difficult to predict theoretically and is highly dependent on temperature and ionic concentrations (Hayashi and Yandell, 1993). The sensitivity of SSCP may be influenced by many factors and optimisation of the electrophoretic conditions is essential. The technique is referred to as PCR-SSCP and has been used to detect *ras* mutations in human lung cancer (Suzuki et al., 1990) and p53 mutations in a variety of malignancies including colorectal and breast cancer (reviewed in Chang et al., 1995).

1.7.4 Detection of p53 mutations

P53 gene mutations tend to be clustered within exons 5 to 8, which correspond with the most highly conserved regions of the gene. However, the mutations can occur at many different positions of the gene, making sensitive detection more difficult than for a defined mutation such as in codon 12 of K-*ras*.

1.7.4.1 Sequencing

Nigro and colleagues (1989) used PCR to amplify a 2.9 kilobase fragment from genomic DNA, or a 1.3 kb fragment from cDNA, encompassing exons 5-8. They identified 21 missense mutations from 19 colorectal tumours by sequencing individual clones of the PCR products.

1.7.4.2 PCR-SSCP analysis

This technique has been used to simultaneously detect loss of one 17p allele and a point mutation in the remaining allele. Murakami et al. (1991) used 7 primer pairs to amplify overlapping fragments of the p53 coding region, which were then subjected to SSCP analysis (see above) and direct sequencing. They detected aberrations of the p53 gene and its transcript in 4/10 human tumour cell lines (2 colon carcinomas, 1 pancreatic, and 1 hepatocellular carcinoma). More recently, Eguchi et al. (1996) used PCR-SSCP to detect p53 mutations in 11/25 tumour samples from patients with colorectal cancer; 7 of these 11 had evidence of the same p53 mutation in stool samples which were negative by occult blood testing.

1.7.5 Detection of epithelial-specific gene expression

Expression of epithelial-specific genes, detected by reverse transcription PCR (RT-PCR), has since been used as markers of epithelial cells. Datta et al. (1994) used expression of cytokeratin (CK) 19 to detect breast cancer cells in peripheral blood and bone marrow. They found that 4/19 patients with stage IV breast cancer were positive for CK 19 expression in blood, and 5/6 histologically negative bone marrow biopsies were positive. Burchill et al. (1995) used RT-PCR for CKs 8, 19 and 20 to detect bladder, breast and colon cancer cell line cells in peripheral blood. They concluded that CK 20 was a useful

marker for colon cancer, but CKs 8 and 19 were expressed in a high proportion of normal blood samples making them unsuitable as markers of cancer cells in blood. This was in contrast to the findings of Datta et al. (1994) who had addressed the problem of non-specific amplification of CK 19 by designing primers that did not amplify the CK 19 pseudogene. Burchill et al., however, had used primers able to amplify the pseudogene and this could have accounted for expression of CK 19 being found in a high proportion of normal blood samples. More recently there have been numerous reports in the literature on the use of these markers to detect carcinoma cells in blood, bone marrow, lymph nodes and peripheral blood stem cell harvests and these are referred to in Chapter 8 of this thesis. To date there has only been one report on the use of MUC1 and RT-PCR to determine the presence of micrometastases in 50 axillary lymph nodes from 15 primary breast cancer patients. RT-PCR was able to detect mucin expression in 6 additional nodes which were determined to be free of metastases by immunohistochemistry (Noguchi et al., 1994).

1.7.6 Immunomagnetic bead isolation of epithelial cells

The sensitivity and specificity of the molecular detection method may be further enhanced by prior enrichment of the target cell population, in this case epithelial (carcinoma) cells, by the use of immunomagnetic bead technology. Immunomagnetic beads are super-paramagnetic polymer particles of uniform size and are available now from a number of commercial suppliers. Dynal manufacture 2 sizes of beads: one 4.5 μm in diameter, the other being 2.8 μm . Both contain approximately 20% magnetite (ferric and ferrous oxide) by weight. They are supplied with affinity-purified rat anti-mouse immunoglobulin covalently bound to the surface to enable labelling with a mouse monoclonal antibody of choice. Miltenyi Biotech manufacture a much smaller (100 nm)

magnetic polymer bead, however due to its size, separation requires purchase of a much more powerful magnetic device (Miltenyi et al., 1990). There are two ways in which to use magnetic beads to isolate cells:

1) the direct technique in which the beads are first labelled with a monoclonal antibody (or cocktail of antibodies) specific for the cell type of interest and then allowed to bind to the target cells in a mixture of other cells.

2) the indirect technique in which the cells of interest are labelled with the specific antibody (or cocktail of antibodies) and then allowed to react with the immunomagnetic beads. In either technique the cells bound to the beads are isolated using a permanent rare earth magnet.

The direct technique has been used to isolate foetal trophoblast cells from the blood of pregnant women (Mueller et al., 1990) and to purge T lymphocytes (Vartdal et al., 1987), B lymphocytes (Kvalheim et al., 1989) or neuroblastoma cells (Treleaven et al., 1984) from bone marrow prior to autologous transplantation. The indirect technique has been used to remove B-lymphoma cells (Kvalheim et al., 1988), breast cancer cells (Shpall et al., 1991; Vredenburgh et al., 1991), neuroblastoma cells (Kemshead et al., 1986) and small cell lung cancer cells (Ball et al., 1991) from human bone marrow prior to autologous transplantation. Our approach of using the beads labelled with an epithelial-specific antibody to isolate epithelial (and hence carcinoma) cells from blood, followed by molecular detection of tumour-specific or tissue-specific markers, had not been done before.

1.8 HYPOTHESES

The hypotheses relating to this thesis are as follows:

1. Haematogenous spread of the tumour may occur from an early stage of the disease, hence micrometastatic disease may be detectable in peripheral blood, prior to colorectal resection, in patients who might otherwise be considered “good prognosis”.
2. Tumour cells as well as normal epithelial cells may be released into the blood stream as a result of surgical resection of the primary tumour, but only those tumour cells with metastatic potential will be successful in establishing metastatic deposits.

1.9 AIMS

1. To determine the presence of metastatic carcinoma cells in the blood of patients with colorectal cancer perioperatively, using the combined approach of immuno-magnetic bead isolation and PCR based techniques.
2. To determine whether detection of such cells is a prognostic factor independent of the established prognostic factors of tumour size and differentiation, degree of tissue invasion, and presence of lymph node involvement which will stratify patients with early stage disease into high and low risk for early disease recurrence or development of metastases.

Specifically the aims are:

- 1) to use the colon carcinoma cell line SW480 diluted in normal blood, to optimise the technique of immunomagnetic bead isolation of epithelial cells from blood

- 2) to assess reactivity of different monoclonal antibodies against several carcinoma cell lines to establish a suitable antibody or cocktail of antibodies with which to isolate epithelial cells.
- 3) to optimise PCR analysis of specific mutations (e.g. in the *K-ras* and p53 genes), and to assess the sensitivity of the technique.
- 4) to assess markers of malignant epithelial cells using tissue- and malignancy-specific expression markers detected by reverse-transcription-PCR.
- 5) to compare the outcome of patients, with and without circulating malignant cells at the time of resection of the primary tumour, using Kaplan-Meier survival analysis.

In conclusion, this non-invasive technique may be applied not only to the detection of circulating colorectal cancer cells, but breast and other carcinoma cells as well, and may prove to be a useful prognostic indicator of survival independent of tumour stage.

CHAPTER 2

MATERIALS

AND

METHODS

2.1 DNA EXTRACTION

2.1.1 Reagents

Proteinase K stock solution 10 mg/mL.

To the 100 mg bottle of proteinase K (Boehringer Mannheim) add 10 mL sterile *HPW.

Once dissolved, aliquot into 1.5 mL eppendorf tubes and store at -20°C.

Phenol

Before use, phenol (Sigma P1037) is equilibrated with Tris buffer as follows:

1. In a fume hood, melt 100 mL phenol by incubating in hot water (70°C), tip into a separating funnel and add 0.1% 8 hydroxyquinoline (2-3 grains) and 100 mL 0.5M Tris pH 8.0
2. Mix by inverting several times. Allow mixture to settle then draw off the aqueous phase and add 100 mL 0.1M Tris pH 8.0. Mix again.
3. Repeat this step until the pH of the phenolic phase is > 7.8 then add 0.2% v/v β -mercaptoethanol and store in a dark airtight bottle.

Chloroform reagent

Chloroform/isoamyl alcohol (24:1 v/v): Mix 480 mL chloroform and 20 mL isoamyl alcohol (Sigma 405-7). Store in an airtight container at room temperature.

* Abbreviations: HPW, autoclaved high pure water from in-house Millipore filtration system; UPW, sterile ultra pure water from Fisher Biotech.

Proteinase K reaction buffer

10 mM Tris pH 7.5, 5 mM EDTA, 0.5% SDS.

In a sterile 50 mL tube add :

0.25 mL of stock 2 M Tris pH 8.0

0.5 mL of stock 0.5 M EDTA pH 8.0

0.25 mL of stock 20% SDS

Make up to 50 mL with sterile HPW.

2M Tris chloride pH 7.5

Dissolve 242.2g Tris base (Sigma) in 800 mL HPW. Adjust to pH 7.5 by the dropwise addition of concentrated HCL. Make up to 1 L with HPW in a volumetric flask and autoclave.

0.5M EDTA pH 8.0.

Dissolve 186.1g disodium ethylenediaminetetraacetate.2H₂O in 800 mL HPW. Stir vigorously on magnetic stirrer. Adjust to pH 8.0 with NaOH (approximately 20g NaOH pellets) and make up to 1 L with HPW in a volumetric flask and autoclave.

TE Buffer

10 mM Tris pH 7.5, 1 mM EDTA.

In a sterile 50 mL tube add:

0.25 mL 2 M Tris pH 7.5

0.1 mL 0.5 M EDTA pH 8.0

Make up to 50 mL with sterile HPW. Aliquot and store at 4°C.

5M sodium chloride.

Dissolve 292.2g NaCl (AR) in 1 L HPW in a volumetric flask. Store at RT.

20% Sodium dodecyl sulphate (SDS).

Dissolve 40g SDS (also called sodium lauryl sulphate Sigma L5750), in 180 mL HPW by gently heating on a magnetic stirrer. Adjust volume to 200 mL with HPW. Do not autoclave.

2.1.2 Specimen collection

1. Tumour specimens were obtained either as fresh or formalin fixed tissue pieces or from paraffin sections.
2. Blood samples of 10-20 mL in EDTA anticoagulant were collected pre-, intra- and post operatively from a peripheral vein and, where possible during the operation, from the portal or mesenteric veins.

2.1.3 Method 1: DNA extraction using phenol and chloroform

Proteinase K digestion

1. Fresh or formalin fixed tumour pieces were minced through a wire mesh into phosphate buffered saline (PBS) to give a single cell suspension.
2. The cell suspension was centrifuged once at 600g (1800 rpm on Beckman TJ6), and the pellet resuspended in 1-2 mL of Proteinase K reaction mix with 100 µl of Proteinase K stock solution in a polypropylene 10 mL tube.
3. This tube was incubated at 37°C overnight or at 50°C for 2 hours or until all the protein clumps had disappeared.

DNA extraction

1. Add an equal volume of phenol (see above) and mix thoroughly by inverting tube for 1 minute.
2. Centrifuge at 850 RCF (2000 RPM) for 10 minutes.
3. Carefully take off the upper phase (DNA) using a wide bore sterile transfer pipette into another polypropylene tube. (If the DNA solution is very viscous, dilute with TE buffer).
4. Add an equal volume of a 1:1 mixture of phenol and chloroform reagents. Mix thoroughly and repeat steps 2 and 3. (If there is still excessive protein precipitating at the interface, repeat this step).
5. Add an equal volume of chloroform reagent, mix thoroughly and repeat steps 2 and 3 (This step may be repeated once again to ensure complete removal of phenol.)

Ethanol precipitation of high molecular weight DNA.

1. Add a 1/10 volume of 3M sodium acetate to the DNA solution and mix well.
2. Add exactly two volumes of ice cold absolute ethanol and mix well.
3. Observe precipitation of the DNA coil that forms almost immediately. Pipette this coil into an eppendorf tube.
4. Fill the tube with 70% ethanol to remove traces of solutes trapped in the DNA. Spin in eppendorf for 30 seconds. Tip off supernatant carefully, drain tube and dry the pellet in a vacuum desiccator for 15 minutes.
5. Dissolve pellet in an appropriate volume of TE (20 μ l-1.0 mL depending on size of pellet). Place tube in 37°C heating block overnight to assist solution of DNA, then store at 4°C.

Estimation of DNA Concentration

1. Add 5 μ l DNA solution to 1 mL HPW (1/200 dilution). Mix well.
2. Read the absorbance (OD) using the Gene Quant spectrophotometer at 260 nm and 280 nm against a HPW blank using the 1.0 mL quartz cuvettes (1 cm light path).
3. Calculate the DNA concentration in μ g/mL by assuming that for double-stranded DNA 1 OD = 50 μ g/mL. (The Gene Quant spectrophotometer calculates this figure). Multiply by the dilution factor to obtain the final DNA concentration.
4. Calculate the ratio of A260:A280 for the DNA solution. The A260:280 ratio should be > 1.8 . If less than this, there is contamination with protein and/or phenol, and an accurate quantification of the DNA will not be possible. (If this occurs, repeat the procedure of phenol chloroform extraction)

2.1.4 Method 2: DNA extraction using high salt protein precipitation

This rapid method for DNA extraction (Miller, 1988) was subsequently adopted which avoids the use of phenol and chloroform and results in DNA of suitable quality for PCR.

Additional reagent

TES: 10 mM Tris pH 8, 1 mM EDTA, 0.1 M NaCl (salt)

for 100 mL mix 0.5 mL of 2M stock Tris, 0.2 mL of 0.5 M stock EDTA and 2mL of 5M stock NaCl, make up to 100 mL with HPW.

Method

1. Add 0.5-2 mL of TES to cell pellet depending on size i.e. cell count.
2. Add 50-100 μ l of proteinase K (10 mg/mL) and 1 drop of 20% SDS. Incubate at 37°C overnight or at 50°C for 2 hours.

3. Add an equal volume of 3M NaCl and place tube in ice for 10 minutes to allow protein to precipitate out.
4. Spin for 5 minutes in microfuge at full speed (13,000xg) or for 15 minutes in Beckman TJ6 at 3,500 rpm (3,000xg).
5. Take off supernatant with a sterile transfer pipette. If protein precipitate remains, repeat NaCl precipitation and centrifugation. To supernatant add 2 volumes of 100% ethanol to precipitate DNA. Spin down in an eppendorf tube and rinse pellet in 70% ethanol.
6. Dry under vacuum and resuspend DNA pellet in TE buffer.

2.1.5 Method 3: DNA extraction from paraffin-embedded tumour tissue

2. To three 10 µm sections, add 150 µl UPW and boil for 20 minutes (adopted from the method of Jackson et al., 1990).
3. Spin for 1 minute at 13,500 rpm in a microfuge to sediment paraffin and cell debris and use 25 µL of the supernatant in the PCR.

2.2 COLLECTION OF TISSUE SAMPLES FOR RNA

Materials

1. Liquid nitrogen or dry ice
2. Cryovials
3. Scalpels
4. Sterile forceps

5. In theatre, dissect the specimen on a sterile surface taking a slice of tumour longitudinally through the bowel wall (to get an entire cross section of the tumour). Cut into small pieces and place into a cryovial. Immediately place tube in dry ice (or into liquid nitrogen). Note: do not pack the tube full as it will be difficult to remove the tissue for processing without thawing it. It is better to freeze several vials with less material in them.
2. Collect a sample of normal mucosa as well. This must be taken at the end of the specimen furthest from the tumour. Cut open the bowel tube and, using a scalpel, scrape the internal surface. This is the 'normal' mucosa. Place into 2 or more cryovials and freeze immediately.
3. Tissue may be stored in liquid nitrogen or processed immediately for RNA extraction.
4. Tissue must not be allowed to thaw before the denaturing solution is added. The tissue is then minced through a fine steel wire mesh using scalpels. The tissue homogenate is then pipetted into 2 mL eppendorf tubes (0.5 mL per tube)

2.3 RNA EXTRACTION

RNA was extracted from $1-2 \times 10^6$ freshly cultured cell-line cells or colon tissue using TRIzol® reagent (Life Technologies, Gaithersburg, MD, USA) or TRI reagent (Sigma) according to the manufacturer's instructions. Briefly, cultured cells were lysed directly in the flask (if adherent cells) or following centrifugation in the case of suspension cultures, by addition of 1 mL of TRIzol® per $5-10 \times 10^6$ cells. Tissue samples stored

frozen in liquid nitrogen were thawed in 1mL TRIzol® per 50-100 mg tissue and minced through a fine wire mesh. Following addition of chloroform, the upper aqueous phase was recovered and RNA was precipitated by mixing with isopropanol. Following centrifugation at 4°C, the pellet was washed in 75% ethanol then air dried for 10-15 minutes to evaporate the ethanol. The RNA pellet was redissolved in UPW at a concentration of approximately 1 ug/μL.

2.4 POLYMERASE CHAIN REACTION (PCR)

2.4.1 Oligonucleotide Synthesis and Isolation

Oligonucleotides (primers) were designed with the aid of the programs "*Primer*", and "*Amplify*". Using the program "*Primer*" (Whitehead Institute for Biomedical Research), criteria as requested by the program were selected as follows:

Optimal primer length (number of bases, range)	23 (20-25)
Optimal primer melting temperature	65°C
Minimum acceptable melting temperature	55°C
Maximum acceptable melting temperature	75°C
Minimum acceptable primer GC%	20
Maximum acceptable primer GC%	80
Number of unknown bases allowed in primer	0
Maximum acceptable primer self-complementarity	4
Maximum acceptable 3'end primer self-complementarity	0
Product length range (number of bases)	150-750

The program will select the most suitable pair of primers. This pair (or any other pair, for example from a paper) can be checked using the program "Amplify". This program allows the user to determine the suitability of the primer pair with respect to primer dimer formation, other binding sites (if any), their primability and stability, and amplification efficiency.

Synthesis of oligonucleotides was initially carried out in our laboratory using the *Gene Assembler Plus* (Pharmacia LKB, Uppsala, Sweden) according to the manufacturer's instructions or, more recently obtained from a commercial supplier e.g. Bresatec, Adelaide, S.A.

2.4.2 PCR Reagents

Ultra pure water (UPW)

Purchased from Fisher-Biotech, Bentley, WA.

40 mM dNTPs:

1. Dissolve each 50 mg bottle of deoxynucleotide triphosphates i.e. dATP, dGTP, dCTP, dTTP, (Boehringer Mannheim) in approximately 1.5 mL of UPW, pooling each into one sterile 10 mL tube.
2. Adjust the pH to 7.0 by the addition of sterile 1M Tris base, monitoring the pH by spotting the solution onto pH papers.
3. Make up volume to 9 mL and sterilise by filtering through a 22µm millipore filter unit. Aliquot into 200 µL volumes and store at -20°C.

Taq polymerase

Thermostable DNA polymerase was obtained from Boehringer Mannheim, Mannheim, Germany, or more recently from Fisher-Biotech, Bentley, Western Australia.

10xPCR buffer

As supplied by the manufacturer: containing 100 mM Tris-HCl, 500 mM KCl pH 8.3, 15 mM MgCl₂ (Boehringer) or 670 mM Tris-HCl pH 8.8, 166 mM (NH₄)₂SO₄, 4.5% Triton X-100, 2mg/mL gelatin, 15mM MgCl₂ (Biotech).

2.4.3 PCR Method

The standard protocol for performing PCR was as follows, unless otherwise stated in specific methods: target DNA (100ng) was added to a master mix containing 1x PCR buffer, 0.8 mM dNTPs, 0.2 mM primers and 0.75 units *Taq* polymerase made up to a total volume of 50 uL with ultra pure water. The reaction was carried out in a water cooled thermal cycler (ARN Electronics, Adelaide, South Australia) at 94°C initial denaturation for 5 minutes, then 45 cycles of 94°C 1 minute, 55°C 1 minute, 72°C 1 minute, with a final extension of 72°C for 7 minutes.

2.4.4 Reverse transcription-PCR (RT-PCR) Reagents

1. Superscript IITM reverse transcriptase or M-MLV reverse transcriptase
2. 5x First Strand Buffer (FSB): 250 mM Tris pH 8.3, 375 mM KCl, 15 mM MgCl₂
3. Dithiothreitol (DTT) 0.1 M

All the above obtained from Life Technologies, Gaithersburg, MD, USA.

4. Random hexamers Pd(N)₆ 5'PO₄, Na⁺ salt (Pharmacia, Uppsala, Sweden)
5. 40 mM dNTPs (as for PCR)
6. Lysis mix to contain per reaction:
 - 3 µL 0.1 M DTT
 - 3 µL 1% Nonidet P-40 (Sigma, now known as IGEPAL CA-630)
 - 8 µL UPW
 - 1.0 µL RNasin (Promega).

2.4.5 RT-PCR Method

1. The pellet of cells and immunobeads from the enrichment step is resuspended in 15 µL of lysis mix to lyse the cells and release mRNA. (This mix can be stored at -80°C to batch samples before proceeding to the reverse transcription step.)
2. Following heat denaturation at 70°C for 3 minutes, reverse transcription (RT) is carried out in the same 0.5 mL eppendorf tube by adding 15 µL of a master mix containing:

random hexamers (250 ng/µL)	3 µL
5x FSB	6 µL
dNTPs	2 µL
UPW	3 µL
reverse transcriptase 200 units	1 µL

Sterile plugged tips are used to avoid carryover from sample to sample.

3. The tubes are mixed well, pulse spun and incubated at 37°C for 1 hour, followed by 3 minutes at 70°C to inactivate the enzyme. PCR is performed on aliquots of the RT mix preferably on the same day, or the samples may be stored for a short time at 4°C.

2.5 GEL ELECTROPHORESIS

2.5.1 Reagents

5xTBE buffer

To make 1L, dissolve 54g Tris base and 27.5g boric acid in 800 mL HPW, add 20 mL, 0.5 M EDTA (pH 8.0) and make up volume to 1 L with HPW. Use at 0.5x concentration to prepare agarose gels and as running buffer.

6x loading buffer

0.25g bromophenol blue

40g sucrose

Dissolve in 80 mL HPW. Adjust pH to approximately 10 with 1N NaOH and make up volume to 100 mL. Store at 4°C.

Ethidium bromide

1mg/mL stock solution: 10 mg ethidium bromide (tablet) was dissolved in 10 mL HPW and stored protected from light.

2.5.2 Preparation of agarose gels

For a 75 mL volume 1.5% gel, dissolve 1.2 g DNA grade agarose (Progen, Australia) in 100 mL 0.5x TBE by boiling in a microwave oven for 90 seconds. Cool to about 50°C under running water before pouring into the perspex gel tray. Allow to set at RT for at least 30 minutes before loading samples.

2.5.3 Electrophoresis and UV photography

Gels are run at 100 volts in a submarine apparatus (BRL) in 0.5xTBE buffer containing 200 μ L of ethidium bromide (1 mg/mL) until bromophenol blue dye front is near the end of the gel. The gel can then be viewed and photographed using a UV transilluminator (Photodyne, WI, USA) and a Polaroid camera system with "instant" film either with a negative (type 665) or without (type 667).

2.5.4 Preparation of polyacrylamide gels

1. The gel mould is prepared by clamping together 2 glass plates of the correct size for the vertical gel electrophoresis system (BRL model V-16, Gaithersburg, MD, USA).

The plates are separated by 0.4 μ m spacers. A sheet of gel bond film (FMC Bioproducts, Rockland USA), to which the gel will adhere, is included making it easier to handle the gel during silver staining. Gel bond is not used for ethidium bromide staining as it is not UV transparent. The other glass plate is treated with a silicone solution (e.g. Rain Vision®) to prevent adherence of the gel.

2. Preparation of gel solution: For a 10% polyacrylamide gel (PAG), make a total volume of 22 mL containing:

5.5 mL of 40% acrylamide/*bis*acrylamide (19:1) (Gradipore, Sydney, Australia).

2.2 mL of 5x TBE

0.4 mL 10% ammonium persulphate

14 mL HPW

10 μ L TEMED

Mix well and pipette into the mould. Place the comb in position in the top of the gel and allow to set for at least 30 minutes.

3. Loading the samples and electrophoresis: Clamp the gel assembly onto the vertical gel apparatus and fill the upper and lower buffer chambers with 0.5x TBE, ensuring that there are no air bubbles beneath the gel. Load the samples containing 1x loading buffer into the wells using 0.4 mm flat pipette tips (Sorenson MultiFlex[®], BioScience Inc., Utah, USA). Connect to the power pack and run at the appropriate power setting.
4. Staining: the gel is placed in a tray containing 500 mL of ethidium bromide (4 µg/mL) for 5-10 minutes, then destained in water for 15-30 minutes. The gel may then be photographed as before.

2.5.5 Silver staining protocol

Silver staining is used to detect band shifts on SSCP gels mounted on gel bond. To achieve high quality staining without background, chemicals must be of high purity, analytical grade, solutions prepared with deionized water, and the staining trays cleaned of all previous silver stain by wiping out with ethanol. Gloves should be worn when handling gels to avoid staining artifacts from finger prints.

Method

1. Fix the gel for 10 minutes in 10% ethanol.
2. Pour off ethanol and add 500 mL 1% nitric acid. Leave to soak 10 minutes.
3. Discard nitric acid solution and wash twice in deionized water.
4. Stain the gel in silver solution (1.5 g/L AgNO₃, 0.056% formaldehyde) for 30 minutes at RT with gentle agitation.
5. Rinse briefly in deionized water then develop image in developer solution (30 g/l Na₂CO₃, 0.056% formaldehyde, 400µg/L sodium thiosulphate) for 2-10 minutes.
6. Stop the reaction by rinsing in 7.5% acetic acid.

2.6 SOUTHERN BLOTTING AND HYBRIDISATION

2.6.1 Reagents

Salmon sperm DNA. 10mg/mL

Weigh out 100mg salmon sperm DNA (Deoxyribonucleic acid sodium salt type III from Sigma) using aseptic technique. Dissolve in 10 mL sterile HPW by leaving in 37°C heating block for several hours or overnight. Shear the DNA by sonication, using constant cycle and an output of between 2 and 3, or by drawing up and down through an 18 gauge needle. Boil for 10 minutes to denature the DNA and aliquot into 2 mL eppendorfs. Store at 4°C.

20 x SSC stock solution.

Dissolve 175.3 g NaCl and 88.2 g sodium citrate in 800 mL HPW. Check to see if pH is near 7.0 (use 10N NaOH if necessary.) Adjust volume to 1L with HPW.

Nasmyth's hybridisation solution

For 200 mL of solution (Nasmyth, 1982)

1.1 M NaCl	12.86g
0.33 M Na ₂ HPO ₄ (AR)	9.45g
0.01 M EDTA Na ₂ ·2H ₂ O	0.83g
1.85% sodium lauroyl sarcosinate	3.7g
18.5% dextran sulphate (AR)	37g

Dissolve the first 3 chemicals in about 100 mL HPW and adjust the pH to 6.2 with conc. HCl. Add dextran sulphate and allow to dissolve with gentle heating on magnetic stirrer. Sterilize by autoclaving in a 200 mL Schott bottle. When cool add

the sodium lauroyl sarcosinate with constant stirring until dissolved. Make up volume to 200 mL with sterile HPW and store at RT or 4°C.

Alternative hybridisation solution (Blotto)

For 50 mL volume:

20 x SSC	10 mL
Blotto (5% Powdered skim milk, 0.02% sodium azide)	2.5 mL
salmon sperm DNA (10 mg/mL)	0.5 mL
20% SDS	1.25 mL
HPW	35.75 mL

Transfer Solution: 0.4N NaOH, 0.6M NaCl.

26.7 mL of 60% w/v NaOH (as supplied by Univar)

120.5 mL of stock 5M NaCl

Make up to 1L with HPW.

CAUTION: 60% NaOH is caustic. Avoid contact with skin and eyes.

2.6.2 Southern blotting

1. Pour the transfer solution into a tray and place a glass plate support over it.
2. Cut 2 lengths of 3MM paper for wicks. Lay over the glass plate with ends dipping into the solution. Smooth out air bubbles with a glass rod.
3. Carefully lay the gel on to the 3MM paper (slide off the gel tray slowly) and lay membrane on to the gel, smoothing out air bubbles.
4. Cut the top left hand corner off with a scalpel to orientate the membrane.

5. Cover this assembly with plastic wrap. Using a scalpel cut out a "window", the area of the gel to be transferred.
6. Place a sheet of 3M filter paper over gel then a 10 cm wad of paper towels carefully on top of this assembly. Keep flat using a glass plate and leave to transfer overnight.
7. Next morning, peel off membrane from gel and place in neutralising solution for 2 minutes. If not proceeding directly to hybridisation, blot on clean 3M paper and allow to air dry. Wrap in plastic wrap, label and store at room temperature.

2.6.3 Prehybridisation

Prepare pre-hybridisation solution as follows:

1. Into a 50 mL sterile tube, pipette 13.6 mL Nasmyth's solution, 0.5 mL Salmon Sperm DNA and make up to 25 mL with sterile HPW. (Alternatively use Blotto).
2. Place membrane in a hybridisation oven bottle and pour in the pre-hybridisation solution, close lid firmly and place into pre-warmed oven (42°C). Turn the rotor on and leave for 4 hours or overnight.

2.6.4 End labelling

Radiolabelling of internal oligonucleotides for use as probes was performed using the Terminal Kinasing Kit (Bresatec, Adelaide, SA) according to the manufacturer's instructions. The mix contains, in a total volume of 20 μ L, 50 ng of oligonucleotide, 1x buffer, 10 mM dithiothreitol (DTT), 1 μ L polynucleotide kinase, and 5 μ L (50 μ Ci) γ P³²dCTP (Bresatec). The mix is incubated at 37°C for 20 minutes and used as a probe without further processing.

2.6.5 Hybridisation and autoradiography

The radiolabelled oligonucleotide (probe) is added to the pre-hybridisation solution in the bottle and the incubation continued at 42°C overnight. The hybridisation solution is poured off into a tube and stored behind perspex for subsequent use. (This solution may be used 4-5 times within a 4 week period). The excess radioactivity and unbound probe is washed off the membrane using several changes of a solution of 0.1x SSC, 0.1% SDS at 42°C. The membrane is then wrapped in plastic wrap, and in the darkroom, placed in an X-ray cassette with X-ray film (Hyperfilm, Amersham, UK). Autoradiography is carried out at -80°C for several hours to overnight to expose the film to the emitted β radiation. The film is developed using an automatic processor (Radiology Department).

2.7 NORTHERN BLOTTING AND HYBRIDISATION

2.7.1. Reagents

DEPC-treated water

Mix 1 mL of diethyl pyrocarbonate (DEPC) (Sigma) in 1 L HPW. Allow to stand for 24 hours at 37°C then autoclave.

10X MOPs buffer, pH 7.0

Dissolve 41.8 g MOPs free acid and 4.1 g sodium acetate in 900 mL DEPC-treated water. Adjust to pH7.0 with 6M NaOH (DEPC-treated). Add 20 mL 0.5M EDTA. Make up to 1 litre with DEPC-treated water and store at 4°C in a foil-wrapped bottle.

1X RNA loading buffer

Mix 500 μ L deionised formamide with 100 μ L 10X MOPS buffer, 167 μ L 37% formaldehyde, 100 μ L glycerol, a few grains of bromophenol blue and 3 μ L 10 mg/mL ethidium bromide to make up 1 mL of RNA loading buffer.

2.7.2 Gel electrophoresis and Northern blotting

1. To make a large agarose gel add 3g agarose (1%), 30 mL 10X MOPS, 218 mL DEPC-water and 52 mL formaldehyde.
2. To a volume of RNA containing 10 μ g RNA (approximately 20 μ L) add an equal volume of RNA loading buffer. Heat the mixture to 68 °C for at least 3 minutes prior to loading on gel to remove secondary structure. Run gel overnight in 1x MOPS at 40 volts.
3. Place gel in distilled water for 10-20 minutes to remove excess formaldehyde. Photograph gel and place in transfer buffer, 5x SSC, 10mM NaOH, as recommended for use with Ambion membrane.
4. Set up transfer as follows: place a wad of paper towels on a clean surface, then 3 sheets of Whatman paper soaked in transfer buffer, the pre-soaked membrane, the gel, 3 sheets of Whatman soaked in transfer buffer and approximately 4 sheets of soaked ROAR paper towels.
5. Transfer for 2-3 hours. Keep transfer moist by adding transfer buffer to the top of the paper towels with a pasteur pipette. Briefly rinse membrane in 2X SSC. Take a photograph of the membrane under UV, with RNA face down on transilluminator and mark the 28S and 18S bands with pencil.

6. UV-crosslink RNA to the membrane using a UV crosslinker. Place membrane face-up in crosslinker and press auto-crosslink. Store blot between two sheets of dry Whatman filter papers and seal in a bag.

2.7.3 Probe labelling and hybridisation of Northern blots

1. The prehybridisation solution is made up as follows: 7.5 mL deionised formamide (50%), 3 mL 5M NaCl (1M), 3ml 50% dextran sulphate (10%), 0.75ml 20% SDS (1%) and 0.75 mL sterile water. Also add 400 ~~μ~~L 10mg/mL salmon sperm, which has been denatured for 5 minutes at 95°C and chilled on ice. Prehybridise for a minimum of 1 hour at 42°C.
2. The appropriate PCR products were gel-purified using mini-columns (Wizard PCR Preps, Promega) according to the manufacturer's instructions and used as probes for Northern hybridisation. Denature 100-200 ng of probe at 95°C for 5 minutes and chill on ice. Add 6 μL decanucleotides, 6 μL "C" nucleotide/buffer mix, 5 μL α³²P-dCTP and 1 μL Klenow enzyme (Gigaprime Kit, Bresatec). Incubate at 37°C for 15-30 minutes. Denature at 95 °C for 5 minutes and chill on ice. Add to membrane in hybridisation bottle and incubate at 37°C overnight.
3. Wash as required to achieve approximately 50 cps on the membrane without excessive background (monitor counts with hand-held Geiger counter). Start with 2X SSC at 42°C for 30 minutes, then 1 X SSC, 0.1% SDS at 60°C for 30 minutes, and if needed, 0.1X SSC, 0.1% SDS at 65°C for 10 minutes.
4. Expose the membrane to X-ray film for several hours to overnight and develop in the automatic processor.

2.8 CELL CULTURE

2.8.1 Reagents

Trypsin-versene

For 500 mL weigh out: 1.25 g Trypsin (Sigma), 0.1 g Na₂EDTA, 4.0 g NaCl, 0.2 g KCl, 30 mg Na₂HPO₄, 30 mg KH₂PO₄, 0.25 g NaHCO₃, 0.5 g glucose, 0.1 g Phenol red
Dissolve in HPW, sterile filter using a 0.22µm filter (Millipore) and store frozen in aliquots.

Antibiotic solution 500x stock

To make 200 mL of 500x stock: 6 g Penicillin G, 10 g Streptomycin sulphate (Sigma) dissolve in 200 mL UPW and sterile filter. The stock solution is then aliquoted into 10 mL lots and stored frozen.

Glutamine stock solution (200mM, 200x)

Weigh out 0.292g of L-Glutamine (Sigma) and dissolve in 10 mL HPW. Sterile filter and store frozen.

Culture media

Media (DMEM or RPMI 1640; Gibco-BRL) is made up in HPW according to the manufacturer's instructions, with 3.7g/l NaHCO₃ (Sigma).

To 450 mL of media add:

50 mL FCS (final concentration 10%)

1 mL of antibiotic solution (5,000 IU penicillin/mL, 5000 µg/mL streptomycin)

2.5 mL of stock glutamine (1 mM final).

Store at 4°C. Add additional glutamine if media older than 2 weeks.

Phosphate Buffered Saline (PBS)

(Dulbecco's calcium and magnesium free).

To make 1L dissolve the following in HPW:

8.0 g NaCl

0.2 g KCl

1.15 g Na₂HP0₄ (anhydrous)

0.2 g KH₂P0₄

Sterilise by autoclaving at 103 KPa, 121°C for 20 minutes. Store at room temperature or 4°C.

2.8.2 Culture method

Cryopreserved cell line cells are thawed rapidly at 37-40°C and washed twice in media. The cells are resuspended in media and plated out into tissue culture flasks at a concentration of 2×10^5 cells/mL. Depending on the growth rate, cells are fed daily with fresh media until almost confluent, at which time they are harvested using trypsin to loosen adherent cells.

ANTIBODY LABELLING OF IMMUNOBEADS

1. To 60 µL immunobeads (Dynal, Melbourne) add 20 µL of monoclonal antibody Ber-EP4 (Dakopatts, Gestrop, Denmark) and 500µL of sterile PBS
2. Mix on rotating mixer for 2 hours or overnight at RT.

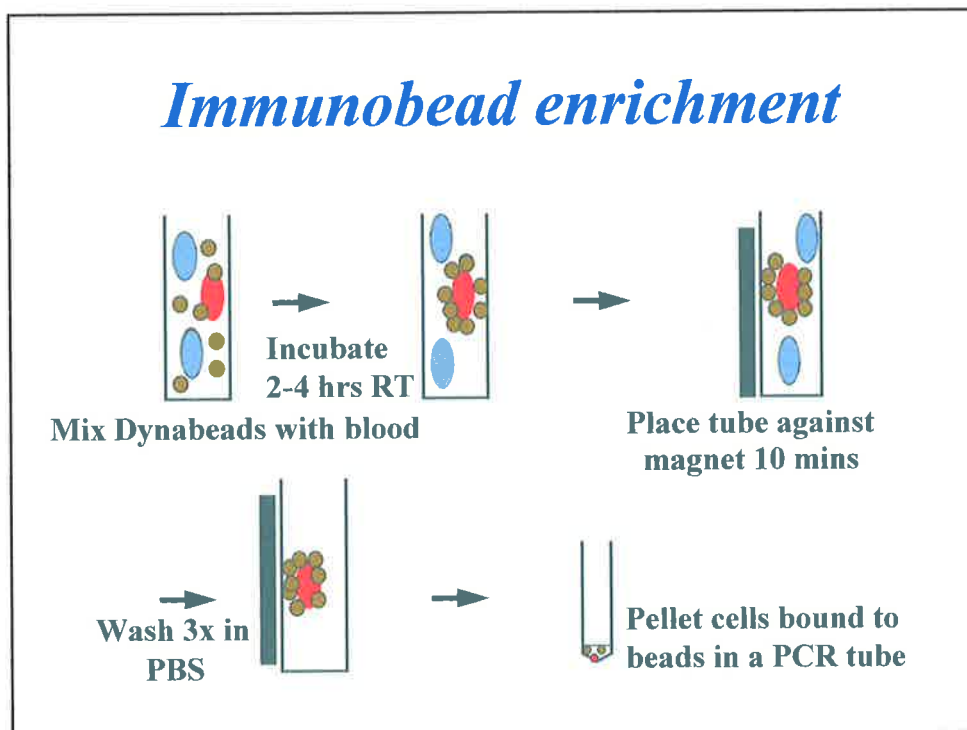
3. Wash beads 1x in PBS, resuspend in 1 mL (approx) PBS and count a 1/10 dilution (5 μ L in 45 μ L) under microscope.
4. Check labelling of beads by flow cytometry. Using the same dilution above, add 5 μ L of Phycoerythrin (PE) conjugated anti-mouse antibody F(ab') fragment (Silenus Laboratories, Hawthorn, Australia) and incubate 15-30 minutes on mixer. Wash 1x in PBS, resuspend and analyse on flow cytometer.

IMMUNOBEAD ENRICHMENT OF EPITHELIAL CELLS

1. Add 4 million beads to each 10 mL blood sample in polystyrene tubes (Disposable Products, Adelaide, SA).
2. Incubate on mixer slowly rotating for 2-4 hours or overnight if required (Figure 2.1).
3. Tape tubes to an array of cobalt samarium disc magnets and leave to settle for 10 minutes.
4. Wash 3 times in PBS with tubes attached to the magnetic array.
5. After the last wash add 2-3 mL of PBS to each tube, take tubes off magnets, mix well and allow to stand upright on magnetic array for a few minutes. Change gloves to avoid possibility of cross contamination.
6. Take off supernatant using sterile transfer pipette, leaving approximately 200 μ L.
7. Using Gilson P200 pipette and plugged tips, transfer resuspended beads into labelled PCR tube in magnetic tube holder (DynaL MPC-P-12). Change gloves before next patient sample. Take off supernatant leaving bead/cell pellet.
8. For PCR, resuspend bead pellet in 10 μ L UPW and store at -20°C.

9. For RT-PCR, add 15 μL of lysis (resuspension) mix: per tube, 3 μL 1% Nonidet P-40 (NP-40), 0.5 μL RNAsin (Promega, Madison, WI, USA), 3 μL 0.1M DTT , and 8.5 μL UPW. Include a positive and negative control tube and store at -80°C to batch samples for RT-PCR.

Figure 2.1 Immunobead enrichment of tumour cells from blood



CHAPTER 3

DETECTION OF MUTATIONS IN *K-RAS* AS A TUMOUR CELL MARKER

3.1 INTRODUCTION

The most common point mutation in the *ras* genes in colorectal cancer occurs in exon 1, codon 12 of the K-*ras* gene, such that the normal amino acid glycine in the p21 *ras* protein is substituted by another. The mutations found most commonly in colorectal tumours are, in order of frequency, GGT-GAT (aspartate), GGT-GTT (valine), GGT-TGT (cysteine), GGT-AGT (serine) and GGT-GCT (alanine) (Vogelstein et al., 1988). The amino acid substitution induces a conformational change and results in reduced GTPase activity so that GTP remains bound to p21, instead of being reduced to GDP, and downstream growth signalling continues inappropriately (reviewed in Chapter 1). In the study by Bos et al. (1987), 11/27 colorectal cancer patients had a mutation in *ras*: 9/11 were in K-*ras* at codon 12, 1 at codon 61, and 1 in codon 12 of N-*ras*. In a landmark paper, Vogelstein et al. (1988) studied 172 colorectal tumours for *ras* mutations and found that 48/172 (30%) contained a mutation in codon 12 of K-*ras*, 12/172 in codon 13 of K-*ras*, with 5/172 in N-*ras* (1 in codon 12, 2 in codon 13 and 2 in codon 61). I therefore decided to perform our preliminary study based on detection of mutations in K-*ras* codon 12.

Several techniques have been used to determine mutations in the K-*ras* gene, namely ASO (allele specific oligonucleotide) hybridisation, the 3' mismatch technique, PCR and direct sequencing of product, primer-mediated allele specific restriction analysis (ASRA), and single-strand conformation polymorphism (SSCP) analysis (reviewed in Chapter 1). From a critical review of the methods it was decided that only the 3' mismatch technique and the ASRA technique would be evaluated since only these two satisfied the criteria of being both sufficiently sensitive and simple enough to be used to screen large numbers of patients.

3.2 EVALUATION OF THE PCR 3' MISMATCH TECHNIQUE

PCR can be adapted for the rapid detection of single base changes in DNA by using specially designed oligonucleotides (primers) which bind to and amplify one allele in preference to the other, by virtue of a mismatch at the 3' end. (Ehlen and Dubeau, 1989; Sommer et al., 1992). Specific amplification can be obtained since the primer perfectly matches one allele e.g. the mutant allele, while the normal (mismatched) allele is poorly amplified or not at all. Amplification efficiency of the mismatched allele is reduced since a mismatch at the 3' end interferes with both extension of the DNA strand by *Taq* polymerase and the stability of the primer-template duplex (Kwok et al., 1990).

Ehlen and Dubeau's technique allowed for the rapid detection of mutations in any of the 3 *ras* genes viz. H-*ras*, K-*ras* and N-*ras*. They used primers with 3' ends complementary to specific point mutations and incorporating inosine residues at positions of non-homology between the 3 genes. Both variations of the mismatch technique were considered for use in detection of tumour cells in blood.

3.2.1 Synthesis and purification of oligonucleotides

The sense primer was designed with the 3' base complementary to the point mutation of interest i.e GGT→GTT in codon 12 of the K-*ras* gene (Figure 3.1). The antisense primer was adopted from Jiang et al., 1989. The primers were synthesised in our laboratory using the Gene Assembler Plus (Pharmacia) and purified as outlined in Chapter 2. The sequences are shown in Table 3.1.

Table 3.1: Primer sequences

Primer	Sequence	T _m °C
K- <i>ras</i> T sense (KT)	5' TAAACTTGTGGTAGTTGGAGCTGT <u>T</u>	68
K- <i>ras</i> antisense (K3'wt)	5' TCAAAGAATGGTCCTGCACC	60
K- <i>ras</i> sense (K5')	5' ACTGAATATAAACTTGTGGTAGTTGGAC <u>C</u> T	82
K- <i>ras</i> antisense (K3')	5' TCAAAGAATGGTCCTGG <u>A</u> CC	60

Primer sequences used in the 3' mismatch (KT, K3'wt) and ASRA (K5', K3') techniques.

3.2.2 Optimisation of the PCR

The aim of the mismatch PCR was to specifically amplify mutant alleles but not normal alleles so that detection of a product of the expected size (155 bp) indicated the presence of the mutation. The cell line SW480, which carries the homozygous mutation GGT→GTT, was used as the positive control.

As a starting point, the PCR was performed using the standard conditions i.e. the PCR master mix contained the following, in a 50ul reaction volume: 1x*Taq* buffer (Boehringer Mannheim), MgCl₂ (1.5mM), dNTPs (0.8 mM), 0.2 mM of each primer, 0.5 units *Taq* polymerase (Boehringer Mannheim), and sterile UPW to 50 µL. DNA (100-500ng) was added and amplification carried out with an initial denaturation of 5 minutes at 94°C, then 45 cycles of 1 minute at 94°C, annealing for 1 minute at 55°C, and extension for 1 minute at 72°C. A final extension at 72°C for 7 minutes was included to ensure complete extension of the product. The PCR products were electrophoresed through a 2% agarose gel, in 0.5xTBE buffer and photographed as described in Chapter 2.

events (Saiki et al., 1988), and the annealing temperature increased to 60°C, then in a subsequent experiment 62°C, using 30 cycles.

It was found that only reducing the Mg^{++} or primer concentrations had no effect on the PCR i.e. both normal and SW480 DNA samples still amplified. The first correct result i.e. a product with the cell line DNA and not the normals, was obtained using the annealing temperature of 60°C with 30 cycles of PCR and using the reduced levels of primers (0.1 μ M) and $MgCl_2$ (1.2 mM). However, the amount of product generated was very little, hence the next series of experiments were designed to increase the efficiency of amplification while still retaining specificity.

Firstly, the number of cycles was increased to 35, however this resulted in loss of specificity i.e. the normal alleles from the normal control samples were again being amplified. The Mg^{++} was kept at 1.2 mM, which favours more specific amplification but tends to reduce the yield. The next set of conditions were such that the annealing temperature was increased to 62°C in an attempt to increase specificity of primer annealing, and the number of cycles increased to 45. These parameters gave a reasonable amount of specific product without amplification from the normal template (Figure 3.2).

3.2.3 Sensitivity experiments

To determine the sensitivity of detection of the mutant sequence, SW480 DNA was diluted into normal DNA of the same concentration in ratios of 1:10 (100 ng SW 480), 1:100 (10 ng) and 1:1000 (1 ng). These dilutions were run together with undiluted SW480 DNA and DNA from normals. The PCR conditions were as above i.e. 1.2 mM $MgCl_2$, 0.8 mM dNTPs, 0.1 μ M primers, annealing temperature of 62°C for 1 min, and 45 cycles. Southern blotting and autoradiography were performed using a γP^{32} end

labelled internal oligonucleotide to enhance the sensitivity of detection. The ethidium bromide stained gel is shown in Figure 3.2. A band was detected for 1 µg and 100 ng SW480 DNA but not for 10 ng (or 1 ng). Autoradiography did not result in detection of these bands either (data not shown) indicating that the 3' mismatch PCR was not very sensitive. The overwhelming concentration of normal DNA present in these dilutions possibly sequestered most of the primers, hence amplification efficiency of SW480 sequences was severely reduced.

Figure 3.2 Mismatch technique



Ethidium bromide stained gel: Lane 1, pUC 19 MW markers; lane 2, negative control; lanes 3, 4 normal DNA; lane 5, SW480 DNA (1 µg); lane 6, blank; lane 7, SW480 DNA (100 ng); lane 8, blank; lane 9, 10 ng SW480 DNA.

3.3 EVALUATION OF THE PRIMER-MEDIATED ASRA TECHNIQUE

In this technique, also known as primer-mediated restriction fragment length polymorphism (RFLP) analysis, PCR is used to introduce restriction enzyme cutting sites into the amplified sequence by means of primers mismatched at a single base. The sense primer was designed to contain a mismatch at position 1 of codon 11 (Figure 3.3) so that C is incorporated into the sequence rather than G. This change introduces a cutting site for the restriction enzyme BstN1 at CCTGG (*Bst* N1 recognition site is CC↓^{T/A}GG, either T or A being acceptable). A second *Bst* N1 site was introduced via

the antisense primer i.e. at CCAGG (Figure 3.3) as a control site for *Bst* N1 digestion so that the product is cut at the 3' site regardless of whether or not the tumour has a mutation in codon 12 (Jiang et al., 1989).

3.3.1 Design of primers

The primers were based on those of Jiang et al., 1989 (Table 3.1), the sense primer termed K5', the antisense K3'. The sequences and mismatched sites of the primers are shown in Figure 3.3.

3.3.2 PCR optimisation

The PCR was optimised in order to obtain the maximum amount of product thus enhancing the detection rate of minimal numbers of mutant *K-ras* alleles. The parameters that were assessed as being important in amplification efficiency were: the concentration of primers and magnesium, the presence of spermidine, and the PCR cycle parameters. Each of these were tested sequentially.

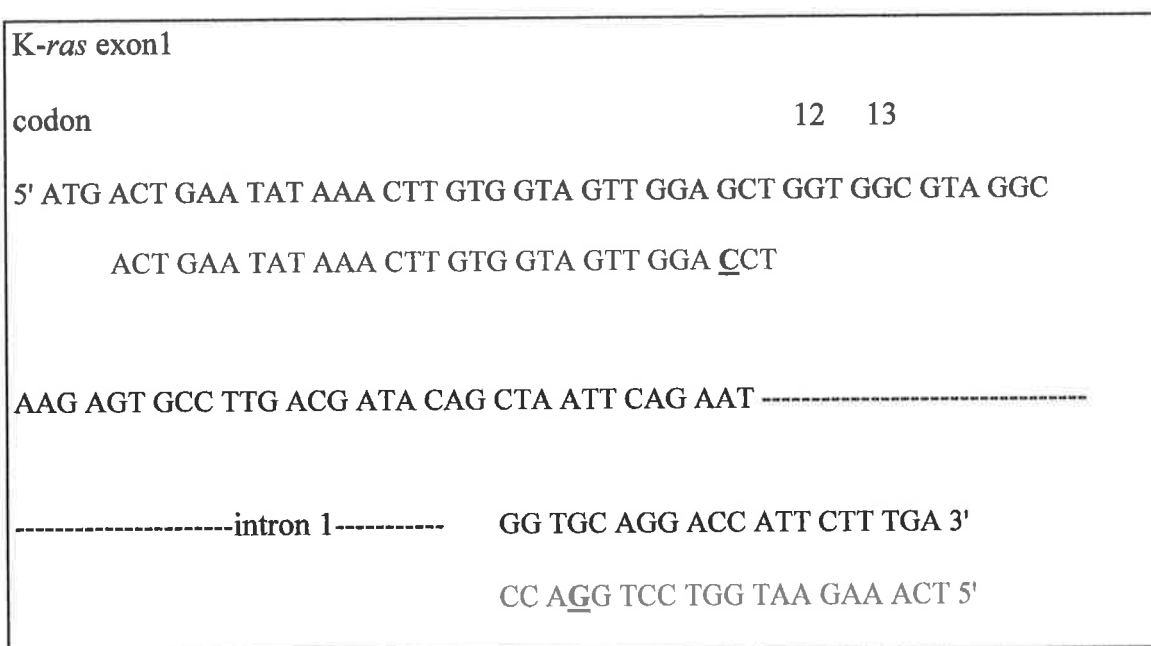
3.3.2.1 Effect of primer concentration

Sensitivity experiments were set up using dilutions of SW480 DNA in HPW to compare 0.2 mM primers (standard) and 0.4 mM primers. PCR was performed on 2 sets of dilutions of SW480 DNA, equivalent to 1 µg (undiluted), 100 ng, 10 ng, 1 ng, 100 pg, 10 pg and 1 pg (1/10 to 1/10⁶). The PCR mixes varied only in the primer concentration i.e. 1xPCR buffer (1.5 mM Mg⁺⁺), 0.25 mM spermidine, 0.8 mM dNTPS, 0.5 units *Taq* polymerase and either 0.2 or 0.4 µM primers. The cycling parameters were: 5 minutes denaturation at 94°C, then 45 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1

minute at 72°C. The products were electrophoresed through a 2% agarose gel. The results from the ethidium bromide stained gel (Figure 3.4A and Table 3.2) showed that product was obtained in the dilutions down to $1/10^3$ (1 ng) using 0.2 μM primers and down to $1/10^5$ (10 pg) using 0.4 μM primers.

The next step was to test 0.6 μM primer concentration in the same way. The results showed that 0.6 μM primers amplified product only down to the 1/100 (10 ng) dilution of target DNA (Figure 3.4B and Table 3.2). Therefore it was concluded that 0.4 μM primers resulted in a 100 fold higher sensitivity than 0.2 μM primers and 1000 fold higher than 0.6 μM primers, and were used at that concentration in all further analyses.

Figure 3.3. Partial sequence of exon 1 and intron 1 of the *K-ras* gene and primer sequences (in red).



The sense primer shows the mismatched C (bold) in codon 11 to introduce a *Bst* N1 cutting site. The antisense primer also contains a mismatch to introduce a control cutting site regardless of the sequence at codon 12.

3.3.2.2 *Effect of Mg^{++} concentration*

Four PCRs were set up simultaneously to compare different concentrations of Mg^{++} i.e. 1.5, 2.0, 2.5, and 3.0 mM, by adding appropriate volumes of 100 mM $MgCl_2$, and using 0.4 μ M primers. Dilutions of SW480 DNA were used to give target DNA amounts of 10 ng, 1 ng, 100 pg and 10 pg.

Results from the ethidium bromide stained gel (Table 3.3 and Figure 3.5) showed that 1.5 mM $MgCl_2$ gave the most sensitive results: product was detected down to 10 pg DNA (approximately 2 cells) with this concentration compared to the detection of 100 pg minimum with 2 and 2.5 mM Mg^{++} , and 1000 pg for 3 mM Mg^{++} . Therefore 1.5 mM Mg^{++} was used in all subsequent experiments as it resulted in at least a 10 fold higher sensitivity. (This was the concentration recommended by Boehringer and contained in their 10x buffer.)

3.3.2.3 *Effect of spermidine concentration*

Spermidine is a polyamine which has been reported, on the one hand, to stabilise *Taq* polymerase-DNA interactions and enhance amplification (Wan and Wilkens, 1993) and on the other hand, to result in interference of PCR (Ahokas and Erkkila, 1993).

To determine the effect of spermidine in our protocol, 4 PCRs were set up simultaneously varying only in spermidine content, i.e. 0, 0.25, 0.5 and 0.75 mM and using target DNA amounts as before from 10 ng to 10 pg. The results from an ethidium bromide stained agarose gel show that zero spermidine gave the best amplification, product being detected in the 10 pg lane, compared to 100 pg for 0.25 mM, barely detectable at 100 pg target for 0.5 mM and only detectable at higher target levels of 1 ng and 10 ng for 0.75 mM spermidine (Figure 3.6 and Table 3.4).

3.3.2.4 Effect of PCR cycle parameters

The 3 stage cycle used for the experiments above (i.e. 5 minutes denaturation at 94°C, then 45 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C) were tested using 60°C annealing with little success. Sensitivity only reached the level of 1-2+ intensity on an ethidium bromide stained gel using 10 ng target DNA. The next variation I tried was the "bounce" technique where the temperature bounces between 94°C for 30 seconds and 55°C for 30 seconds for 45 cycles. Again, the results were not as good as for the 3 stage protocol, the limit of detection being at 1ng rather than 10 pg DNA, and since optimisation was considered essential, the standard 3 stage protocol was adopted for all subsequent analyses (Figure 3.7).

3.4 DETECTION OF K-RAS MUTATIONS IN TUMOUR SPECIMENS

3.4.1 Patient samples

Following establishment of optimal PCR conditions for the ASRA technique, I proceeded to analyse carcinoma tissue from 223 consecutive patients undergoing surgery for colorectal cancer. The tissue was obtained either fresh at the time of surgery or fixed in formalin, or DNA was isolated from paraffin-embedded fixed tissue (45 cases).

3.4.2 K-ras PCR

Ten to 100 ng of tumour DNA was subjected to PCR in a total volume of 50 µl containing: 1x PCR buffer, 0.8 mM dNTPs, 0.4 µM each primer (K5' and K3'), and 0.75 units *Taq* polymerase. The cycle parameters were: 94°C for 5 minutes then 94°C 1

minute, 55°C 1 minute, 72°C 1 minute for 45 cycles, followed by a 7 minute extension step at 72°C to ensure full extension of all product.

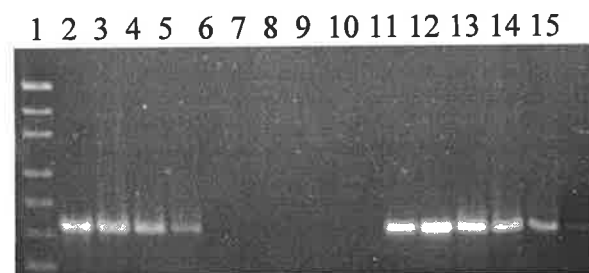
3.4.3 ASRA gel electrophoresis

Ten µl of product was run on a 2% gel to check that the PCR was working properly before continuing on to the restriction enzyme digestion step. Ten µl of product was then digested with 4 units of *Bst* N1 (New England Biolabs) according to the manufacturer's instructions i.e. 10 µl PCR product, 2 µl 10x buffer 2, 0.2 µl of 100xBSA (both supplied with *Bst*N1), 4 units *Bst*N1 and UPW to 20 µl. The digest mix was then incubated at 60°C for 2 hours to allow digestion of the amplified DNA.

The entire digest plus 3 µl of 6x loading buffer was loaded onto a 10% polyacrylamide gel prepared using gel bond (as described in Chapter 2). An undigested product control was also loaded to indicate the 157 bp undigested fragment position on the gel. The sizes of digested bands are shown in Figure 3.8. Electrophoresis was carried out at 150 volts for 3 hours or until the bromophenol dye front was near the bottom of the gel. Following ethidium bromide staining the gel was photographed under UV light (Figure 3.7). Some gels were also silver stained (Figure 3. 9), as described in Chapter 2 (2.5.5). This was not essential for the analysis of tumour samples but would be needed for the detection of rare mutant alleles in blood (Chapter 6).

Figure 3.4. Primer concentration versus target DNA concentration

A: 0.2 μ M versus 0.4 μ M primers



Lane 1, MW markers; lanes 2-10, serial dilutions of SW480 DNA 1/10-1/10⁸ (100ng-0.01 pg) with 0.2 μ M primers; lanes 11-15, SW480 DNA 1/10-1/10⁶ with 0.4 μ M primers.

B: 0.4 μ M versus 0.6 μ M primers

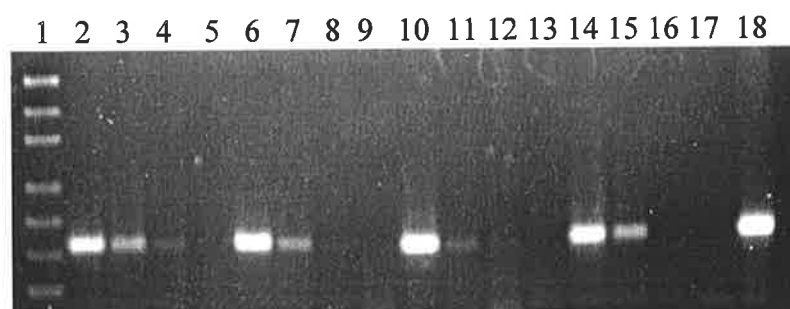


Lane 1, MW markers; lanes 2, 3 blank; lanes 4-11, serial dilutions of SW480 DNA 1/10-1/10⁸ with 0.4 μ M primers; lane 12, blank; lanes 13-16, SW480 1/10-1/10⁴ with 0.6 μ M primers. Interpretation is shown in the table following.

Table 3.2. Primer concentration versus target DNA concentration

Target DNA	Primer concentration (μ M)		
	0.2	0.4	0.6
1 μ g	3+	3+	3+
100 ng	3+	3+	3+
10 ng	2+	3+	1+
1 ng	1+	3+	-
100 pg	-	2+	-
10 pg	-	1+	-

Figure 3.5: Sensitivity of PCR: Mg^{++} versus target DNA concentration

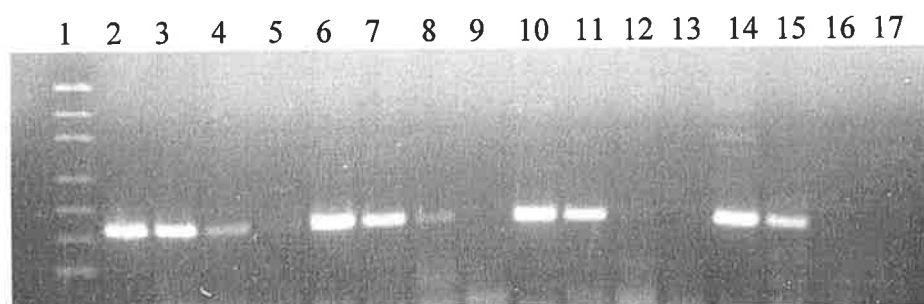


Lane 1, MW markers; lanes 2-5, 10 ng-10 pg (as per Table 3.3), 1.5 mM Mg^{++} ; lanes 6-9, 2.0 mM Mg^{++} ; lanes 10-13, 2.5 mM Mg^{++} ; lanes 14-17, 3.0 mM Mg^{++} ; lane 18, positive control. Interpretation is shown in the table below.

Table 3.3. Mg^{++} versus target DNA concentration

Target DNA	Magnesium concentration (mM)			
	1.5	2.0	2.5	3.0
10 ng	3+	3+	3+	3+
1 ng	3+	3+	2+	3+
100 pg	2+	1+	1+	-
10 pg	1+	-	-	-

Figure 3.6: Sensitivity of PCR: varying spermidine concentration.

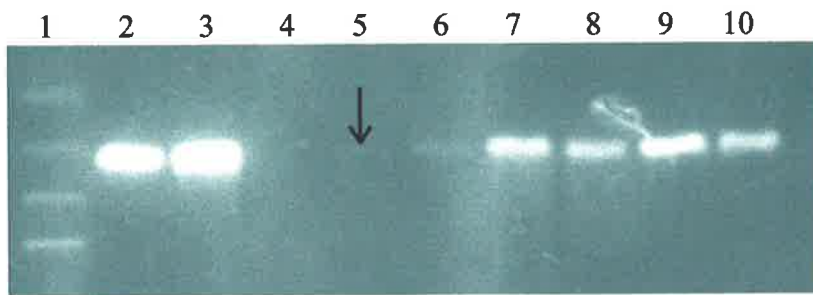


Lane 1 MW markers; lanes 2-5, (10 ng DNA) no spermidine; lanes 6-9, (1 ng DNA) 0.25 mM spermidine; lanes 10-13, (100 pg DNA) 0.5 mM spermidine; lanes 14-17, (10 pg DNA) 0.75 mM spermidine. Interpretation shown in the table below.

Table 3.4. Sensitivity of PCR: varying spermidine concentration

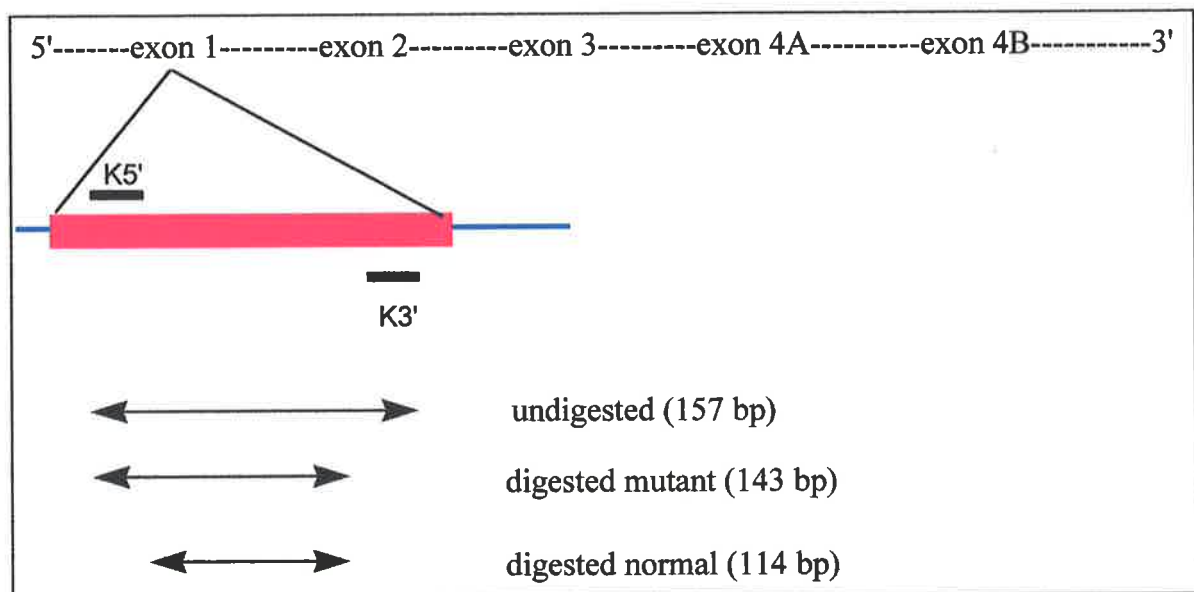
Target DNA	Spermidine concentration (mM)			
	0	0.25	0.5	0.75
10 ng	3+	3+	3+	3+
1 ng	3+	3+	3+	3+
100 pg	3+	3+	2+	-
10 pg	2+	-	-	-

Figure 3.7 Sensitivity of K-ras PCR



Ethidium bromide gel: Lane 1, MW markers; lanes 2, 3 positive controls; lane 4, 1 pg SW480 DNA; lane 5, 10 pg; lane 6, 100 pg; lane 7, 1 ng; lane 8, 10 ng; lane 9, 100 ng; lane 10, 1 μ g. A signal is present (arrowed) at the 10 pg level.

Figure 3.8: Diagram of the K-ras gene and *Bst* N1 digestion products.



Arrowheads denote product sizes in base pairs before and after digestion with *Bst* N1.

3.5. RESULTS AND DISCUSSION

3.5.1 *Mismatch technique*

From the sensitivity experiments the limit of detection of the mutant sequence was at 100 ng SW480 DNA. No product was obtained at 10 ng or 1 ng amounts. Assuming that the DNA content of a human diploid cell is 7 pg (Ausubel, 1990), 100 ng DNA represents approximately 14,000 cells. This was deemed to be not sufficiently sensitive for this application since the expected number of tumour cells isolated from a blood sample was likely to be as low as 1-100 cells. Furthermore, this technique involved an "all or nothing" end point, where a null result could mean either that no mutation was present or simply that it was not detected because of the lack of sensitivity. Due to these findings, inosine containing primers (Ehlen and Dubeau, 1989), which also incorporated a 3' mismatch, were not assessed. In their study, Ehlen and Dubeau (1989) reported a sensitivity of detection of mutant alleles in up to a 10^7 excess of normal alleles, however this level of sensitivity has not been reproduced by others. Sommer et al. (1992) used the mismatch technique as a method for detecting known single base changes in various genes for the purposes of population screening rather than as a method for the sensitive detection of rare mutant alleles.

3.5.2 *ASRA technique*

In the original ASRA technique (Haliassos et al., 1989) there was a potential problem of over-estimating the proportion of mutant alleles present due to the formation of heteroduplexes between mutant and normal alleles which are resistant to restriction enzyme digestion. In this study, they claimed a sensitivity of detection following Southern blotting and autoradiography of 1 mutant allele in 25,000 normal alleles.

Subsequently, Jiang et al. (1989) designed their 3' primer with a mismatch to introduce a BstN1 cutting site so that the 157 bp product was cut to produce a smaller product of 143 bp as a control for digestion, while the mismatch in the 5' primer introduced a cutting site if the K-ras codon 12 sequence was normal but not if there was a mutation present (Figure 3.8). Using this technique they were able to distinguish mutant alleles from 1 ng of target DNA.

In this chapter I have described the application of this technique with careful optimisation of PCR conditions and have shown that a signal could be consistently detected on ethidium bromide stained gels following amplification of 10 pg but not 1 pg (<1 cell) of SW480 DNA (Figure 3.7). Digestion of the K-ras product with BstN1 produces 2 alternative bands depending on whether or not the sequence has a mutation in codon 12. A band of 114 bp is obtained if the sample is normal (cutting has occurred at both the 3' control site and the 5' codon 12 site), bands of 114 and 143 bp if the sample is heterozygous for the mutation at codon 12 or is a heterogeneous sample of tumour and normal tissue, and a single band of 143 bp if the tumour is homozygous for the mutation (Figure 3.9). Of 221 tumour samples, 78 (35%) were positive for a K-ras codon 12 mutation (Appendix A).

The PCR-based ASRA detection system for K-ras codon 12 mutations was established in our laboratory at a very high level of sensitivity (10 pg target DNA, equivalent to 1-2 cells containing mutant alleles). The detection rate of 35% of tumours being positive in our cohort of colorectal cancer patients was similar to that reported by others using earlier techniques. Forrester et al., (1987) found 26/66 patients (39%) positive for K-ras 12 mutations using an RNase A mismatch cleavage technique, and Bos et al., (1987) found 9/27 (33%) positive using PCR and dot-blot hybridisation. Vogelstein et al., (1988) analysed 92 carcinomas and 80 adenomas, also by PCR and

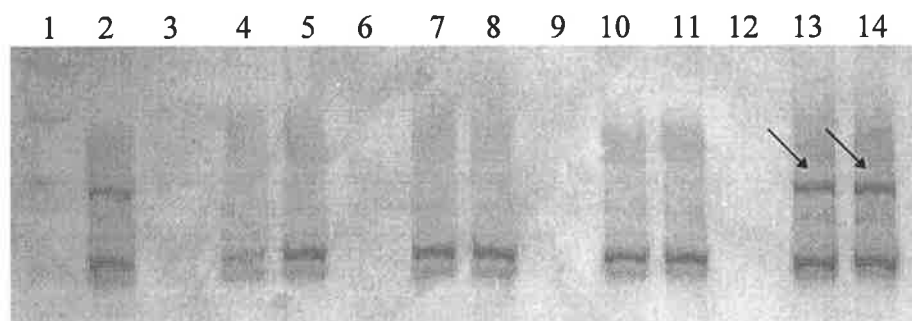
dot-blot hybridisation and found 30/92 carcinomas (33%) positive, but only 17/80 (21%) adenomas positive. The conclusion was that the ASRA technique was the one of choice to use in detection of minimal numbers of circulating tumour cells in those patients whose tumours carried a mutation in codon 12 of the *K-ras* gene.

Figure 3.9 BstN1 digested *K-ras* PCR products from tumour samples



Ethidium bromide stained polyacrylamide gel: Lane 1, MW markers, pUC 19; lane 2, positive control (SW480); lane 3, blank; lane 4, normal control; lane 5, undigested control; lane 6, tumour, no mutation; lane 7, blank; lanes 8, 10, 11, tumours with a *K-ras* codon 12 mutation; lanes 13, 14, 16, 17, tumours without a mutation; lane 19, undigested DNA.

Figure 3.10. BstN1 digested *K-ras* PCR products from tumour samples



Silver stained gel: Lane 1, MW markers; lane 2, positive control tumour DNA (heterozygous for *K-ras* codon 12 mutation); lane 3, negative (no DNA) control; lanes 4, 5 patient EW tumour (no mutation); lane 6, blank; lanes 7, 8, patient FIC tumour (no mutation); lane 9, blank; lanes 10, 11, patient EVR tumour (no mutation); lane 12, blank; lanes 13, 14, patient PHI tumour (arrows show the mutant 143 bp band).

CHAPTER 4

IMMUNOBEAD LABELLING WITH EPITHELIAL- SPECIFIC ANTIBODIES

4.1 INTRODUCTION

Since tumour cells circulating in blood were likely to be present in very low numbers in some cases, it was necessary to develop a detection system with very high sensitivity. To this end, an enrichment step for epithelial (carcinoma) cells was incorporated, prior to the PCR-based detection method, to remove unwanted blood cells. The enrichment step involves the use of magnetic microbeads (immunobeads) that are supplied with anti-mouse immunoglobulin covalently bound to their surface. A mouse monoclonal antibody, directed against epithelial membrane antigens, or tumour-specific surface antigens could then be bound to the immunobeads.

As with all immunological based isolation techniques, the efficiency of the isolation relies on the specificity and avidity of the selected antibody to its target antigen. A second equally important criteria for the antibody in this application was that it reacts with epitopes on the cell surface membrane, not just within the cytoplasm, since the tumour cells would be largely intact in a freshly drawn blood sample, and recovery of intact cells is important for subsequent detection using molecular techniques.

This chapter describes the selection of the most appropriate antibody with which to label the immunobeads, optimisation of antibody binding and assessment using flow cytometry and immunohistochemistry.

4.2 SELECTION OF EPITHELIAL-SPECIFIC ANTIBODIES

4.2.1 Target cells and tissues

The cell lines used to determine the most suitable antibody to bind to the target epithelial (carcinoma) cells were the colorectal carcinoma cell lines SW480, SW48, LIM-1863, LIM-2463, LIM-2405, LIM-1215, LIM-2412, LIM-1899, LIM-2099 and HT29. SW480 was provided by Dr E.J. Stanbridge, University of California, Irvine, USA. The LIM cell lines were provided by Dr R. Whitehead, Ludwig Institute for Cancer Research, Melbourne, Australia. HT29 and SW48 were obtained from the American Type Culture Collection, USA. The cells were maintained in culture in RPMI 1640 or DMEM (Gibco BRL, Grand Island, NY) with 5% foetal calf serum as described in Chapter 2. Normal peripheral blood mononuclear cells were used as negative controls for flow cytometry to ensure the antibodies being tested were in fact epithelial specific.

Sections for immunohistochemical assessment of antibody labelling were obtained from formalin-fixed paraffin-embedded tumours from 20 colorectal cancer patients. The sections were stained using a standard immunoperoxidase technique and the results assessed by light microscopy.

4.2.2 Monoclonal antibodies

Monoclonal antibodies directed against epithelial surface antigens were obtained commercially from Dakopatts, Denmark, viz. Dako-Ber-EP4 (Latza et al., 1990), Dako-CEA (Harwood et al., 1986) and Dako-EMA (Heyderman et al., 1985). BC-2, directed against mucin, was kindly provided by Professor IFC McKenzie, Austin Research Institute, Melbourne. All antibodies were used in labelling experiments on cell lines at

concentrations of 20 ng-4.0 ug/ 1×10^6 cells and the results assessed by flow cytometry on a Coulter Epics Profile II. The antibody concentrations used in immunohistochemistry were 250 ng/mL (Ber-EP4) and 500 ng/mL (EMA and CEA) determined by Ms V. Pascoe (Immunopathology laboratory, The Queen Elizabeth Hospital). BC-2 was not assessed by immunohistochemistry.

4.2.3 Labelling cells for flow cytometry

1. Cell-line cells were harvested after 24-48 hours in culture, using 1 ml of trypsin-versene per flask to dissociate the cells. Warming to 37°C was sometimes necessary to release strongly adherent cells.
2. The cells were washed once in media and resuspended in same. A cell count was performed using a haemocytometer counting chamber.
3. Approximately 250,000 cells were dispensed into 1.5 ml eppendorf tubes in a volume of 300 μ l, to which was added an appropriate volume of the monoclonal antibody, and incubated on ice for 20 minutes. Negative controls consisted of cells alone (autofluorescence) and cells incubated with a negative control antibody of the same immunoglobulin subclass as the antibody under test.
4. The cells were washed once in PBS, resuspended in 300 μ l PBS and incubated with 5 μ L of phycoerythrin conjugated goat antimouse IgG (Caltag Laboratories, San Francisco, Ca.) for 20 minutes.
5. The cells were washed once with PBS and resuspended in same for analysis on the flow cytometer.

4.2.4 Immunohistochemistry

The antibodies Ber-EP4, CEA and EMA were assessed. Five micron thick sections were stained using a standard immunoperoxidase technique i.e. following incubation with primary antibody, a peroxidase conjugated second antibody is added which binds at sites of primary antibody binding. Subsequent incubation with DAB (3,3' diamino benzidine tetrahydrochloride) as substrate produces an intense brown product after reacting with peroxidase.

4.3 ANTIBODY LABELLING OF IMMUNOMAGNETIC BEADS

The immunomagnetic beads (Dynabeads® M-450, Dynal, Melbourne) are polystyrene beads of uniform size, 4.5 microns in diameter, containing ferrous and ferric oxides thus making them paramagnetic. They were originally supplied with affinity purified sheep anti-mouse immunoglobulin covalently bound to the surface. These secondary antibodies are able to bind all mouse IgG and IgM antibodies thus allowing binding of a mouse monoclonal antibody of choice. More recently, beads coated with rat anti-mouse antibodies of the IgG1 or IgG2a subclasses have become available for binding antibodies of specific light chain subclass.

4.3.1 Determination of optimal antibody concentration

To assess the amount of antibody required to saturate the beads, one million Dynabeads® coated with sheep anti-mouse IgG, were incubated with the mouse monoclonal antibody Ber-EP4 at concentrations of 50, 100, 150, 200 and 250 ng per 1×10^6 beads according to the manufacturer's recommendations. Following incubation for 30-120 minutes, the bead-

antibody suspensions were washed to remove unbound antibody, and resuspended in PBS. The results were assessed by flow cytometry.

4.4 RESULTS

4.4.1 Labelling cells with monoclonal antibodies

Using flow cytometry, the monoclonal antibody Ber-EP4 showed at least a 100 fold increase in median fluorescence compared to the same concentration (200 ng/10⁵ cells) of CEA or EMA monoclonal antibodies for 9 of the 10 cell lines tested (Figure 4.1a); the remaining cell line, LIM-2463, showed a 10 fold increase (Figure 4.1b). All 10 cell lines were also >98% positive with Ber-EP4 (Table 4.1). On the other hand, the antibodies against CEA and EMA only reacted with a proportion of the cells, even at the highest antibody concentration used (2 ug/10⁶ cells). CEA reacted with 6 of 10 cell lines while EMA reacted with only 5 of 10 cell lines. Similarly BC-2 reacted with only a proportion of cells and was effectively negative for 6 of the 10 cell lines (Table 4.1).

Figure 4.1A Comparison of antibody labelling

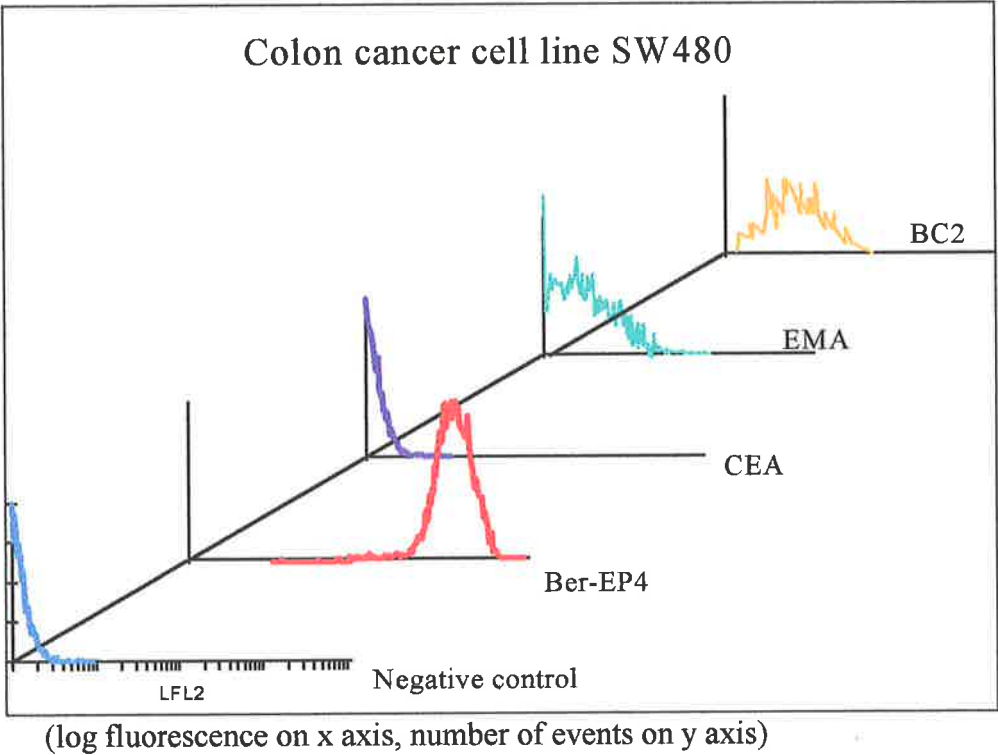
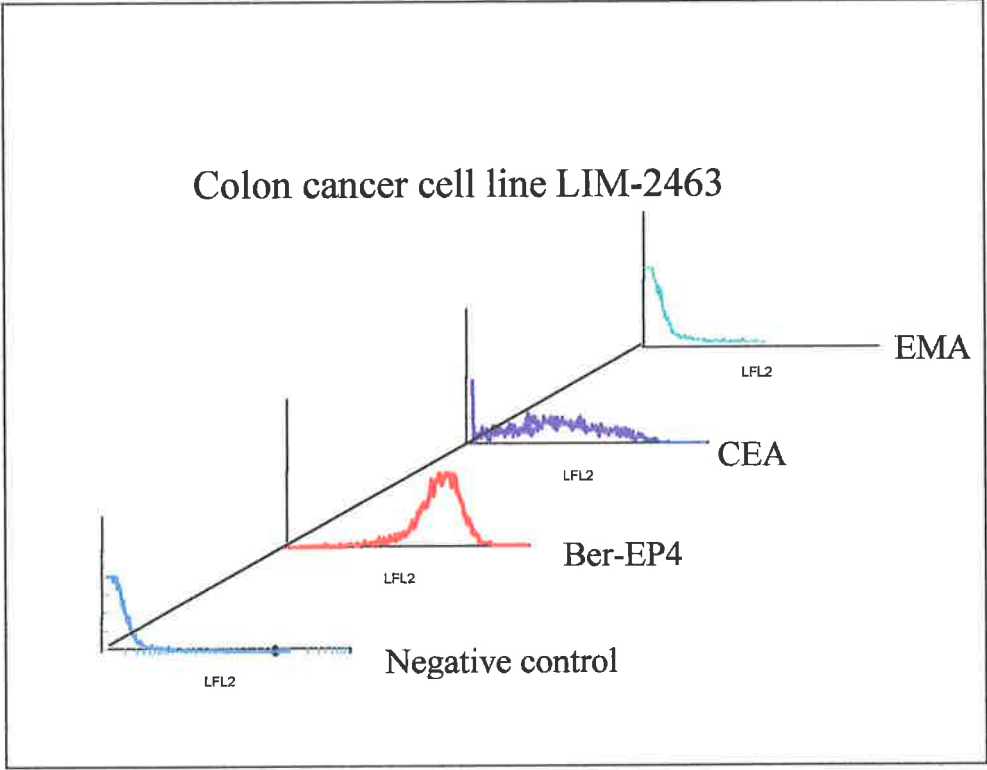


Figure 4.1B Comparison of antibody labelling using cell line LIM-2463



4.4.2 Labelling immunobeads with Ber-EP4

The amount of Ber-EP4 required for labelling the immunobeads was assessed by flow cytometry. Saturation labelling, measured by percent fluorescence, was obtained at a concentration of 150-250 ng Ber-EP4/ 1×10^6 beads (Figure 4.2a). The concentration chosen for subsequent experiments was 200 ng/ 1×10^6 beads (Figure 4.2b)

Table 4.1 Flow cytometry: antibody reactivity with colon carcinoma cell lines.

Target cells	Monoclonal antibodies			
	Ber-EP4	CEA	EMA	BC2
SW48	99.9	2	0	4
SW480	99.9	0	19	45
LIM-2405	99.5	8	33	6.5
LIM-1899	100	29	0	0
LIM-2099	98.9	0	12	7
LIM-1215	99.8	87	0	21
LIM-2412	99.5	51	8	2
LIM-1863	99.9	85	0	10
LIM-2463	99.5	65	<1	<1
HT29	100	41	3	44
Normal PB	<1	3	-	-

4.4.3 Immunohistochemistry results

Tumour sections stained with the antibody Ber-EP4 gave superior results compared to either CEA or EMA (Figure 4.4). Ber-EP4 stained all the epithelial cells uniformly and intensely, whereas CEA and EMA staining was variable in extent and patchy in intensity.

The negative control slides (blocked with normal human serum) were negative in all cases, staining only with the counterstain.

Figure 4.2 Labelling of immunobeads with Ber-EP4

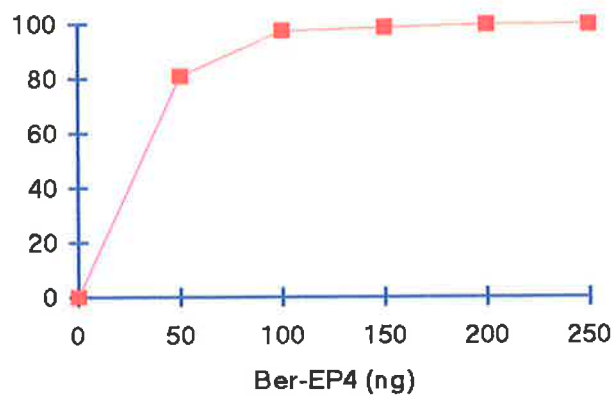
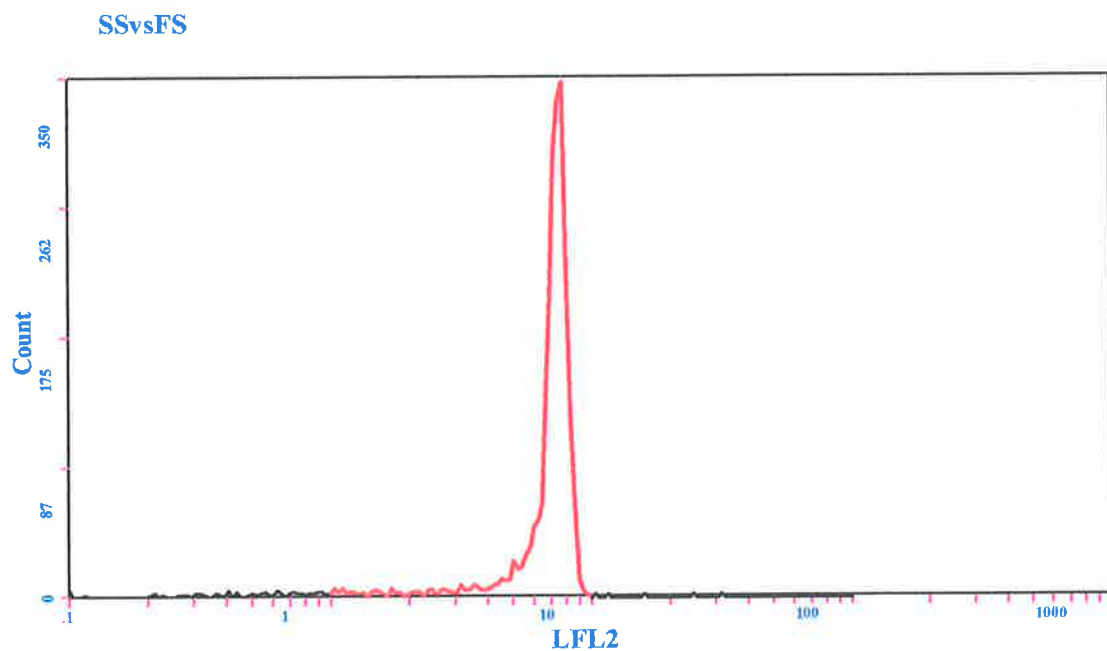


Figure 4.3 Flow cytometry - 1×10^6 immunobeads labelled with 200 ng Ber-EP4



4.5 DISCUSSION

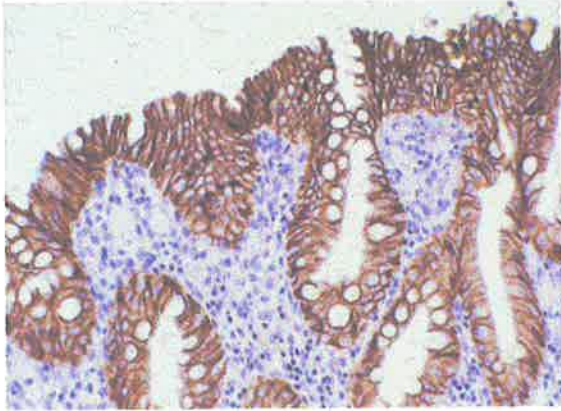
The monoclonal antibody Ber-EP4 (Dakopatts) was derived from hybridoma supernatant from mice immunized with MCF-7 cells and is directed against an epitope on the protein moiety of 2 glycoproteins (34 and 39 kilodaltons) present in the membrane and cytoplasm of epithelial cells (Latza et al., 1990). Immunostaining of a wide range of normal and neoplastic human tissues and cell lines showed that all carcinomas and most non-neoplastic epithelial cells homogeneously expressed Ber-EP4 antigen, the exceptions being hepatocytes, parietal cells and apical cell layers in squamous epithelia. Importantly for our application of labelling epithelial cells in a haemopoietic environment, Ber-EP4 is not expressed by haemopoietic cells (Latza et al., 1990). Ber-EP4 has also been used as the antibody of choice to enrich for epithelial cells in a suspension of leucocytes using a magnetic activated cell sorter (Wong et al., 1995).

The antibodies against CEA and EMA on the other hand are not expressed in all epithelia and their expression within a given tumour may be heterogeneous as shown by Edwards (1985) and by our own data from flow cytometry (Table 4.1). Furthermore, EMA has been shown to be expressed by human lymphoid cells (Delsol et al., 1984). The monoclonal antibody BC-2 was raised against human milk fat globule membranes and reacts with an epitope of the mucin 1 core protein (Xing et al., 1989). Colon cancer cell lines have been shown to produce both mucin 1 and 2, albeit in variable amounts (Devine et al., 1992).

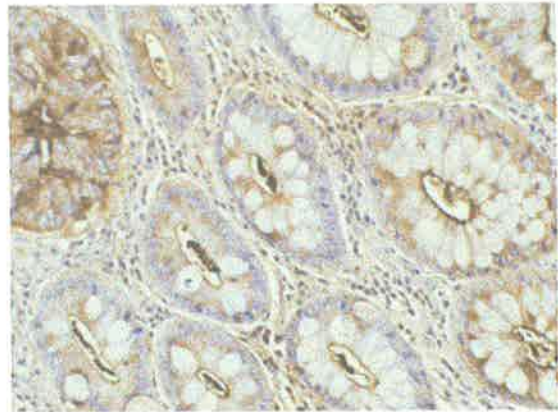
The monoclonal antibody Ber-EP4 was chosen to label the immunomagnetic beads at a concentration of 200 ng per 1×10^6 beads since it was superior to the others both by flow cytometric analysis of 10 colon tumour cell lines and by immuno-histochemistry of paraffin-embedded sections of 20 human colon tumours.

Figure 4. 4 Immunohistochemical staining of colon carcinoma

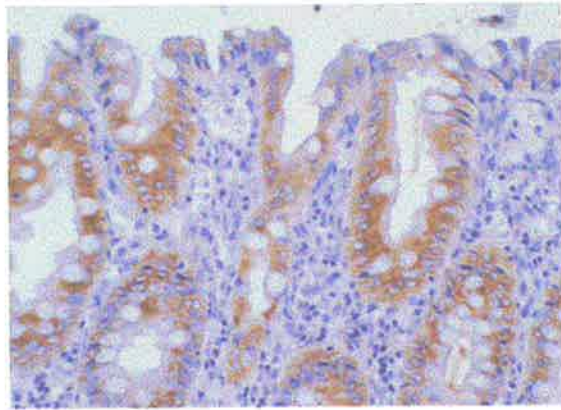
A



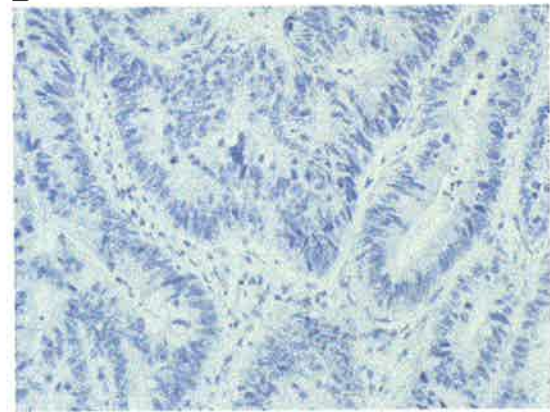
C



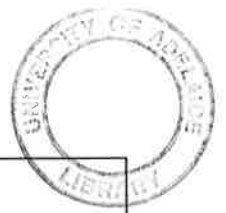
B



D



Ber-EP4 (A), EMA (B), CEA (C), Negative control (D)



CHAPTER 5

IMMUNOBEAD-PCR: OPTIMISATION OF THE IMMUNOBEAD STEP

5.1 INTRODUCTION

The combined approach of isolation of epithelial cells from blood using immunomagnetic beads followed by PCR identification of a molecular marker was termed "immunobead-PCR". The sensitivity of the first step of the immunobead-PCR, the immunobead isolation of tumour (epithelial) cells from blood, was dependent on optimisation of all the variables associated with the procedure i.e. the number of beads used per 10 mL blood sample, the time and temperature of incubation of the labelled immunobeads with the sample, whether gradient separated cells were better than whole blood for recovery of tumour cells, and whether the direct or indirect technique was more sensitive. The following sections describe the experiments performed to test the effect of these variables on the final sensitivity of immunobead-PCR in detecting the mutant *K-ras* band from small numbers of SW480 tumour cells diluted into normal whole blood.

5.2 OPTIMISATION EXPERIMENTS

5.2.1 Ratio of immunomagnetic beads and tumour cells

Ber-EP4 labelled Dynabeads® were incubated with SW480 cells at bead-cell ratios of 5:1, 10:1, 20:1 and 30:1 for 3 hours with mixing at room temperature. The suspensions were cytospun onto glass slides (500 rpm for 5 minutes), stained with May Grunwald and Giemsa stains in an automated slide staining machine and assessed by light microscopy to determine the optimal ratio.

5.2.2 Effect of prior cell separation on immunobead-PCR

Experiments were performed initially using the *indirect* technique, in which the cells are first incubated with the specific primary antibody and then incubated with Dynabeads®, as opposed to the *direct* technique in which the primary antibody is bound directly onto the beads prior to incubation with the cell suspension. According to Dynal product information, the chosen antibody may not bind as well to Dynabeads® as might be expected from cell labelling experiments, so that the indirect technique may in theory produce better results. Both techniques were compared in the following experiments.

5.2.2.1 Comparison of buffy coat versus whole blood-indirect technique

To determine the most appropriate medium from which to isolate the tumour cell/bead complexes, duplicates of 10 mL samples of blood from the same donor (large volume venesections from polycythaemic patients) were used for each dilution within the one experiment. SW480 cells were diluted into 10 mL whole blood at $1/10^4$ (10,000 cells), $1/10^5$ (1,000 cells) and $1/10^6$ (100 cells) relative to the white cell count. To each set of dilutions, Ber-EP4 was added at a concentration of 200 ng/mL and the tubes incubated for 45 minutes at 4°C with gentle mixing to allow the antibody to bind to the SW480 cells. The tubes were centrifuged at 1800 rpm (Beckman TJ6, 500xg) for 10 minutes, the buffy coat, or whole blood pellet were collected and washed twice in PBS with 0.1% bovine serum albumen (BSA) to remove unbound antibody. Finally the cells were resuspended in 5 mL of PBS/BSA and 2×10^6 Dynabeads® were added and incubated for 1 hour at room temperature with mixing. Isolation of cells bound to beads was performed as described in Chapter 2 and the pellets stored at -20°C until assayed using the *K-ras* PCR.

5.2.2.2 Comparison of whole blood versus Histopaque or Ficoll-hypaque separation-direct technique

Three sets of SW480 seeded blood samples were set up as before. Since clumping of cells taken from the density gradient interface can be a problem in subsequent procedures, Na₂EDTA (4.5%) was first added to the blood in order to minimise this. Ber-EP4-labelled immunobeads (2×10^6) were added to one set (whole blood), while the other two sets were first separated on either a Histopaque™ density gradient 1.119 g/mL (Sigma) or a Ficoll-hypaque density gradient 1.077 g/mL (Lympho-paque™, Nycomed Pharma AS, Oslo). Cells recovered from the interface were incubated, without washing, with 2×10^6 Ber-EP4 labelled immunobeads. PCR was performed on the bead/cell pellets as before.

5.2.2.3 Comparison of whole blood versus hetastarch separation

A 6% solution of hetastarch (Hespan™, Dupont, Washington, USA) in normal saline was used as a means of sedimenting out red cells while leaving leucocytes in the plasma fraction, presuming that tumour cells would also remain in the plasma fraction. Hetastarch, a high molecular weight sugar, acts by coating red cells thereby making them much denser which accelerates the rate at which they sediment. Dilutions of SW480 cells in 10 mL blood samples were prepared as before. To one set of dilutions a 1/10 volume of the hetastarch solution was added, mixed and the red cells allowed to sediment for 1 hour at room temperature. The supernatant was collected and incubated with labelled immunobeads. The other set of dilutions were incubated, without prior manipulation, with labelled beads.

5.2.2.4 Comparison of Histopaque gradient separation versus hetastarch separation

A duplicate set of dilutions of SW480 cells in blood were set up to compare recovery of tumour cells from the density gradient interface with tumour cells recovered after the red cells were sedimented out using hetastarch, as assessed by immunobead-PCR.

5.2.3 Effect of temperature of incubation

The indirect technique was used to determine the effect of temperature of incubation on immunobeads binding to tumour cells. Dilutions of SW480 in whole blood were incubated with 2×10^6 immunobeads at either room temperature or 4°C, with gentle mixing. PCR was performed as before.

5.2.4 Direct versus indirect binding of tumour cells in blood

There are 2 ways in which to use Dynabeads® to isolate tumour cells from blood or other body fluids:

1. The beads are first labelled with the antibody, washed and then incubated with the blood sample (direct technique), or
2. The antibody is added to the blood sample, incubated so that the antibody attaches to any epithelial (tumour cells) that may be present, then Dynabeads® (coated with sheep anti-mouse IgG) are added and allowed to bind to any cell that has bound the primary antibody (indirect technique).

5.2.4.1 Indirect technique

To one set of dilutions Ber-EP4 was added at a concentration of 200 ng/mL* and the tubes incubated for 45 minutes at 4°C with gentle mixing. The tubes were centrifuged at 1800 rpm (Beckman TJ6, 500xg) for 10 minutes, the buffy coat and supernatant were collected and washed twice in PBS with 0.1% BSA. Finally the cells were resuspended in 5 mL of PBS/BSA and 2×10^6 Dynabeads® were added and incubated for 1 hour at room temperature with mixing. The K-ras PCR was performed as previously described.

5.2.4.2 Direct technique

To another set of dilutions, 2×10^6 Dynabeads®, labelled with Ber-EP4 as described in Chapter 2, were added, incubated as above and immunobead-PCR performed.

5.2.5 Comparison of incubation conditions: standing versus mixing for 4 hours or overnight

Dilutions were incubated with 2×10^6 labelled beads at room temperature with the tubes either mixing on the rotor or standing upright on the bench, for 4 hours or overnight.

5.2.6 Effect of the number of immunobeads on the sensitivity of immunobead-PCR

Initial experiments were performed using $1-2 \times 10^6$ beads per 10 mL as recommended by Dynal and as suggested from our own mixing experiments on the optimum ratio of beads to tumour cells 20:1 (results this chapter), such that $1-2 \times 10^6$ would be in excess. However, I decided to check the optimum number of beads required for 10 mL samples of blood

* The concentration of Ber-EP4 200 ng/mL of blood was calculated from tumour cell labelling experiments described in Chapter 4.

seeded with SW480 cells. Tubes were set up in duplicate to contain 1,000 ($1/10^5$) and 100 ($1/10^6$) SW480 cells and 1, 2, 3, and 4×10^6 immunobeads.

5.2.7 Effect of the presence of beads in the PCR

Immunobead-PCR was performed on two sets of dilutions. One set was used in the PCR as usual i.e with the beads still in the tube. In the other set, the supernatant (DNA) was transferred to clean PCR tubes, leaving the beads behind, prior to PCR.

5.2.8 Immunobead-PCR versus PCR alone

An experiment was performed to test whether or not in fact it was necessary to first select for epithelial cells in blood. Two sets of dilutions of SW480 cells in blood were made as before. Immunobead-PCR was performed on one set, while DNA was made from the other set and used in the *K-ras* PCR.

5.3 RESULTS AND DISCUSSION

A ratio of 20:1 gave optimal binding of antibody labelled beads to SW480 cells (Figure 5.1), optimal binding being defined as saturation binding of beads to target cells. No binding of beads to haematopoietic cells was seen. In practice, the addition of an excess of beads (in the order of 3-4 million beads per 10 ml blood sample) was used as the number of tumour cells present in a patient's blood sample was unknown. In sensitivity experiments, this strategy resulted in detection of 10,000 down to as few as 10 SW480 cells per mL of blood (Figure 5.2).

Results of *K-ras* immunobead-PCRs are shown in Table 5.1; 3+ represents a strong signal for the mutant band, 2+ a moderate signal, and 1+ a weak signal. No signal is represented by a minus sign. In comparing recovery of tumour cells from whole blood versus buffy coat, the only positive signal i.e. a mutant band, was obtained at the $1/10^4$ dilution for the whole blood experiment suggesting that tumour cells were recovered from the whole blood pellet more efficiently than from the buffy coat. From these experiments it was shown that tumour cells did not accumulate in the buffy coat during centrifugation, presumably as they are of higher density than leucocytes, and so were lost when the buffy coat was harvested.

Results of the *K-ras* PCRs (Table 5.1) also show that using gradient separation prior to addition of the immunobeads resulted in a 10-100 fold loss of sensitivity compared to the recovery from whole blood. The loss of sensitivity using Ficoll-Hypaque™ was worse than using Histopaque presumably due to the density of the tumour cells being greater than 1.077 g/mL, so that they pass through a 1.077 g/mL density interface readily. More tumour cells were recovered at the 1.119 g/mL interface (Histopaque™), however additional loss of tumour cells may occur through the centrifugation and washing steps involved in gradient separation of cells. Taken together these factors would account for the loss of sensitivity using gradient separation compared to whole blood.

Similarly, the results (Table 5.1) show that by using hetastarch, not only are the red cells sedimented out but also other cell types including SW480 cells. This resulted in a 10 fold loss of sensitivity compared to whole blood. The comparison of immunobead-PCR results obtained from hetastarch separation versus Histopaque™ gradient separation shows that the two techniques are equivalent, being detected at $1/10^4$ in both, and very weakly (1+) at $1/10^5$ after hetastarch sedimentation. Neither method is as sensitive as using unmanipulated whole blood.

Comparison of incubation temperatures shows that incubating at room temperature was superior to incubating at 4°C and resulted in a 1 log increase in sensitivity of detection of the mutant K-*ras* band.

The results of a representative immunobead-PCR comparing the direct with the indirect technique is shown in Table 5.1. The conclusion from 6 experiments comparing the two techniques was that the direct technique resulted in at least 1 log greater sensitivity and used much less Ber-EP4 per sample (400-600 ng compared to 2 µg). The loss of sensitivity in the indirect technique was likely to be due to loss of cells from the centrifugation and washing steps and the less efficient labelling of tumour cells in a large volume (10 mL) compared to the labelling of the immunobeads which is performed in a 1 mL volume.

It was found that continuous mixing of the beads in the blood for at least 4 hours resulted in greater sensitivity of detection than if the tubes were left to stand (Table 5.1). The number of immunobeads used to recover tumour cells also had a marked effect on the sensitivity as shown in Table 5.2.

As can be seen from Table 5.1, no difference in results was observed between tubes in which beads were present compared to tubes from which the beads had been removed i.e the presence of beads was not detrimental to subsequent amplification efficiency. In comparing immunobead-PCR to PCR alone, a positive result was obtained down to the $1/10^6$ dilution for immunobead-PCR while no signal was obtained from the DNA preparations, even at a high tumour cell ratio of $1/10^4$. This shows that prior selection of tumour cells is essential in attaining the high degree of sensitivity.

In conclusion, the representative results of many experiments have been described as evidence for choosing one set of conditions over another. Finally, the optimal sensitivity in detecting a mutant band after immunobead-PCR was obtained using the *direct* technique,

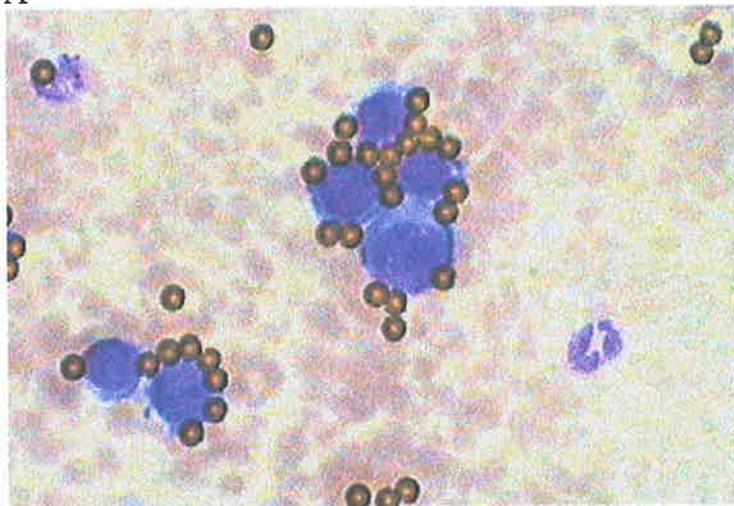
with 3×10^6 Ber-EP4-labelled beads per 10 mL whole blood. The incubations were carried out at room temperature with mixing for at least 4 hours. The highest sensitivity obtained in detecting the mutant band in the *K-ras* PCR was $1/10^6$, equivalent to 100 SW480 cells diluted into 10 mL normal blood i.e. 10 tumour cells per mL of blood (Figure 5.2). Hybridisation to a radio-labelled internal oligoprobe and autoradiography could not be employed with the *K-ras* mutation detection technique due to two reasons: firstly the digested PCR products are separated on a polyacrylamide gel which does not easily permit Southern transfer of bands, and secondly the separation of the bands is not of sufficient resolution to allow radioactive labelling. Subsequently, the technique of silver staining was developed which allowed much better visualisation of weak bands.

Table 5.1 Comparison of isolation techniques: effect on immunobead-PCR.

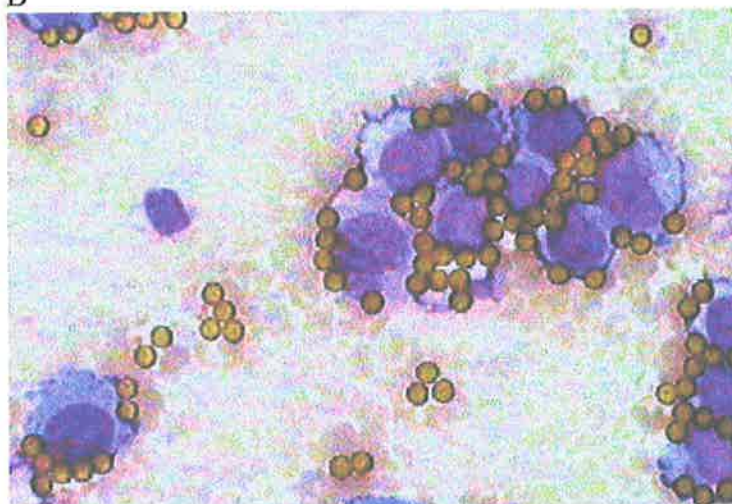
Technique	Dilution tumour cells: white cells		
	1/10 ⁴	1/10 ⁵	1/10 ⁶
<i>Indirect technique</i>			
whole blood	2+	-	-
buffy coat cells	-	-	-
<i>Direct technique</i>			
whole blood	3+	2+	1+
Histopaque™ gradient	2+	-	nd
Ficoll-Hypaque™ gradient	1+	-	nd
hetastarch sedimentation	2+	(1+)	-
<i>Using whole blood</i>			
Direct	3+	2+	1+
Indirect	2+	1+	-
<i>Direct, whole blood</i>			
Mixing 4 hours RT	3+	3+	1+
Standing 4 hours RT	3+	2+	-
Mixing overnight RT	3+	3+	1+
Standing overnight RT	1+	1+	-
Beads present in PCR	3+	3+	1+
No beads in PCR	3+	3+	1+
Incubation at 4°C	1+	-	nd
Incubation at RT	3+	2+	nd
Immunobead-PCR	3+	2+	1+
PCR alone	-	-	-

Figure 5.1 Comparison of ratio of immunobeads to SW480 cells
A 5:1, B 10:1, C 20:1, D 30:1

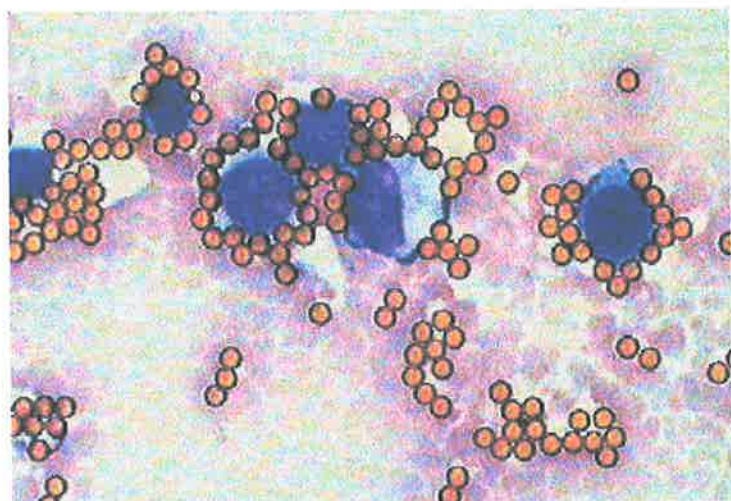
A



B



C



D

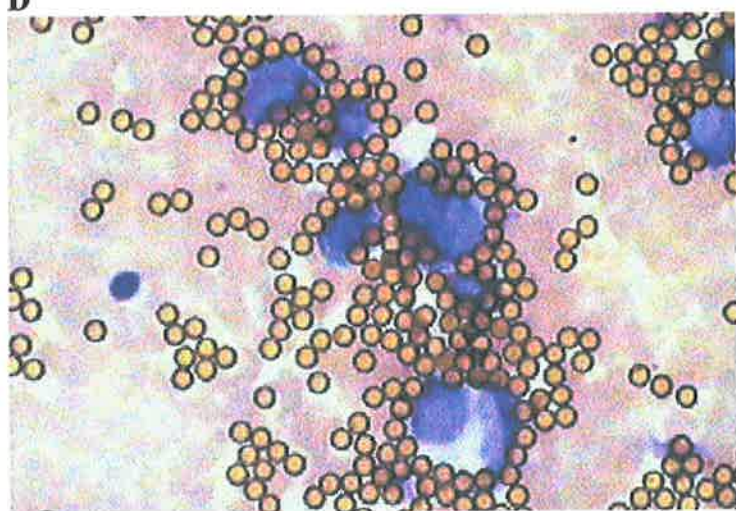
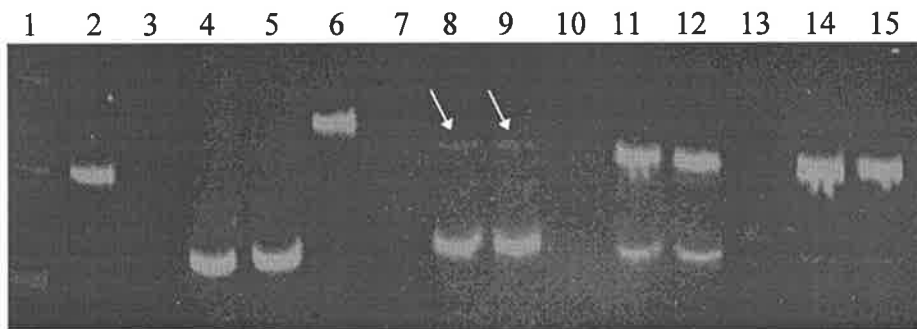


Table 5.2 Effect of number of immunobeads on sensitivity

Ratio tumour cells: white cells	No. of immunobeads			
	1×10^6	2×10^6	3×10^6	4×10^6
$1/10^5$	1+	2+	3+	3+
$1/10^6$	-	-	1+	1+

3+, strong positive; 2+, intermediate; 1+, weak positive; -, negative

Figure 5.2 Immunobead-PCR sensitivity experiment.



Dilutions of SW480 cells in normal PB (ethidium bromide stained gel). Lane 1, MW markers; lane 2, positive control (SW480 DNA); lane 3, blank; lanes 4, 5, normal controls; lane 6, undigested control (157 bp); lane 7, blank; lane 8, 9, dilution $1/10^6$; lane 10, blank; lane 11, 12, dilution $1/10^5$; lane 13, blank; lane 14, 15, dilution $1/10^4$ (arrows show 143 bp band equivalent to the mutant allele).

CHAPTER 6

IMMUNOBEAD-PCR DETECTION OF TUMOUR CELLS IN BLOOD

6.1 INTRODUCTION

Mutations in codon 12 of the *K-ras* gene have been identified as an early event in the development of up to 35% of colorectal tumours (Bos et al., 1987; Vogelstein et al., 1988). Identification of this mutation in a tumour thus provides a marker for tumour cells that may be present in the blood. Having developed a sensitive method, immunobead-PCR, for the detection of tumour cells bearing this mutation in blood (Chapters 3, 4 and 5) I have used this technique to address the question of whether or not the presence of *K-ras* positive cells in the peripheral circulation is associated with development of metastatic disease or recurrence and whether tumour cells are released during surgical resection for malignancy.

The process of metastasis necessarily involves transition of tumour cells through the blood (and lymph) circulatory systems. In animal model studies of metastasis, most of the tumour cells that entered the circulation died and only a very small proportion succeeded in establishing metastases (Fidler, 1991; Liotta et al., 1991). However, the detection of tumour cells in the peripheral circulation may be a marker of disseminated disease. The aims of this study were to detect the presence of circulating tumour cells peri-operatively in patients whose tumour carried the *K-ras* mutation and to determine the relationship of tumour cells in blood to disease-free survival following 'curative' resection for colorectal cancer.

6.2 PATIENTS AND METHODS

Ninety consecutive patients were enrolled into the study to detect tumour cells in peripheral blood taken pre- and post resection for colorectal malignancy. The study was approved by the institutional Ethics of Research Committee and informed consent was obtained from each patient. Tumour specimens were obtained either fresh, or from formalin fixed, paraffin-embedded sections and analysed for *K-ras* codon 12 mutations, using the RFLP technique described in Chapter 3. The tumour was positive for a *K-ras* codon 12 mutation in 27/90 patients and these 27 patients were included in the study to detect tumour cells in peripheral blood peri-operatively. The disease was staged according to the Dukes' classification. The median age was 74 years (range 56-91) with 13 males and 14 females. Peripheral venous blood samples (20 mL) were collected before surgery from 24 cases, immediately after mobilisation of the bowel in 13 cases, and the following morning in 24 cases. Normal controls consisted of 20 mL blood samples from 11 non-surgical patients without cancer and from 2 patients undergoing colonic surgery for benign disease.

6.2.1 Immunobead-PCR analysis

To detect tumour cells in blood, the immunobead-PCR technique was performed. Firstly, epithelial cells were isolated from blood using immunomagnetic beads labelled with the epithelial specific antibody Ber-EP4 (as described in Chapter 4). This antibody was found to react strongly with all 27 tumour samples, either by flow cytometry (for fresh tumour samples) or immunohistochemistry (formalin fixed paraffin-embedded tumours). Secondly, the PCR analysis was performed as described in Chapter 3, on the

isolated epithelial cell/bead aggregates, followed by digestion of the products with *Bst* N1 to determine the presence of a *K-ras* codon 12 mutation. The fragments were run on a 10% polyacrylamide gel and stained with ethidium bromide (Figure 6.1) or silver (Figure 6.2) as described in Chapter 2. The sensitivity of detection of *K-ras* mutations using immunobead-PCR was in the order of 1 tumour cell per million white blood cells, i.e. 10 tumour cells per ml of whole blood (Chapter 5, Figure 5.1). A univariate Kaplan-Meier analysis (BMDP Statistical Software Inc., Los Angeles, CA) was used to assess the significance of circulating *K-ras* mutant cells with respect to disease-free survival in our patient cohort.

6.3 RESULTS

K-ras mutant cells were detected in peri-operative peripheral blood samples in 9 of 27 (33%) patients (Table 6.1). Results from blood taken post-mobilisation of the bowel and day 1 post-surgery were combined since no additional information was obtained from those 11 cases (3 positive, 8 negative) in which I tested both samples. In 6 of 9 patients, mutant cells were detected in blood samples before surgery indicating that tumour dissemination had already occurred. Five of the 6 patients (Table 6.1 nos. 17, 18, 20, 21 and 25) have relapsed and died from recurrent or metastatic disease at a median of 14 months (range 7-23 months) post surgery. The other patient (no. 5) had been staged as B (tumour contained within the muscularis propria and no nodal metastases) and is presently disease-free at 15 months post surgery. Tumour cells were also present in post-operative blood in all but one of these 6 patients (in this patient I

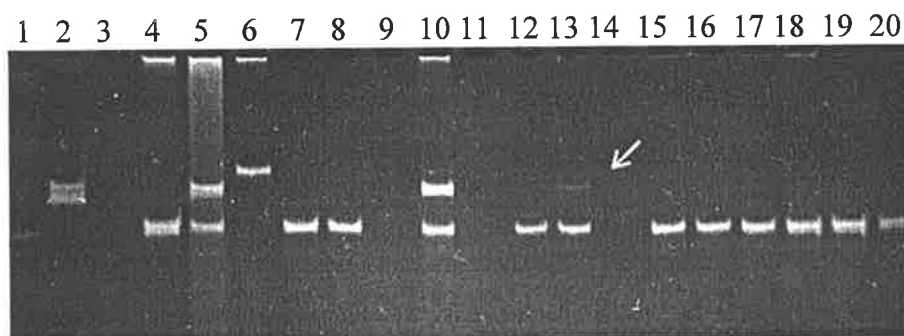
did not receive a post surgery specimen). *K-ras* positive cells were not detected in any of the normal control samples.

There were 2 cases in which tumour cells were detected in the post-operative but not the pre-operative blood specimen suggesting that tumour cells were shed into the circulation during surgical resection. Alternatively, the discrepancy in results between blood samples may reflect a sampling error as a result of sporadic shedding of tumour cells. One of these patients (no. 3) is alive at 17.5 months post surgery, the other (no. 22) suffered a local recurrence and died 9.5 months post surgery. In the remaining case (no. 13) tumour cells were found in post-operative blood, but I did not receive a pre-surgery blood sample; this patient, who was staged as B, developed a metastatic deposit in the bladder and died 10 months post surgery.

Overall, 9 of 27 patients subsequently relapsed with either local recurrence, metastatic disease or both, and 7 of the 9 had circulating tumour cells detected in peri-operative blood specimens. Of 18 patients who have remained disease-free post surgery, 16 of 18 were negative for circulating tumour cells at the time of surgery with a median follow-up of 24 months (range 12.5-42 months). Kaplan-Meier analysis (Figure 6.3) showed that disease-free survival was significantly reduced ($p=0.0001$) in patients in whom circulating tumour cells were detected (mean survival time 11.2 versus 30.4 months, SE 2.88).

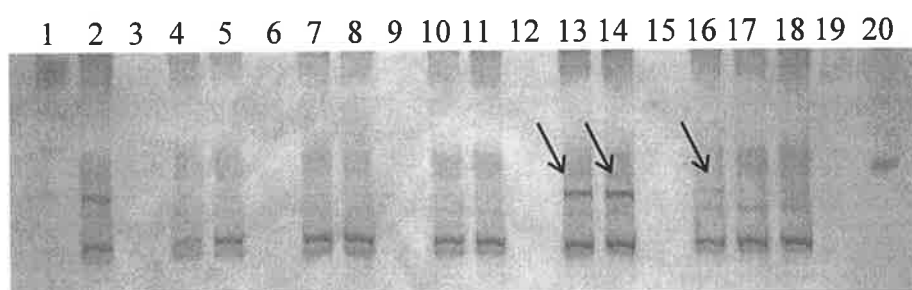
Tumour cells were detected in peri-operative peripheral blood samples in a third of the patients and this finding showed a strong association with shortened disease-free survival (Figure 6.3). Due to the small number of patients in this study it was not possible to show (statistically) whether or not detection of tumour cells in blood was independent of stage as a prognostic indicator.

Figure 6.1 Immunobead-PCR to detect *K-ras* mutations



Products digested with *Bst* N1 and electrophoresed in a 10% 19:1 ethidium bromide stained PAG gel. Lane 1, MW markers; lane 2, positive control SW480; lane 3, negative control; lane 4, normal DNA control; lane 5, tumour sample ELL (mutation positive); lane 6, undigested control marker (157 bp); lane 7, 8, pre- and post-operative blood ELL (no mutation detected); lane 9, blank; lane 10, ORE tumour (mutation positive); lane 11, blank; lane 12, 13, pre- and post-operative blood ORE (post-operative blood shows *K-ras* mutation, arrowed); lane 14, blank; lanes 15-20, tumour samples without the *K-ras* mutation.

Figure 6.2 Immunobead-PCR - Silver stained gel



Lane 1, MW markers; lane 2, positive control tumour DNA (heterozygous for *K-ras* codon 12 mutation); lane 3, negative (no DNA) control; lanes 4, 5, patient EDW pre-, post-operative PB; lane 6, blank; lanes 7, 8, patient FIC pre-, post-operative PB; lane 9, blank; lanes 10, 11, patient EVR pre-, post-operative PB; lane 12, blank; lanes 13, 14, patient PHI tumour (arrows show the mutant 143 bp band); lane 15, blank; lanes 16-18, patients MAR pre-, intra- and post-operative PB (arrow shows mutant band in pre-operative sample); lane 19, blank; lane 20, undigested control (157 bp).

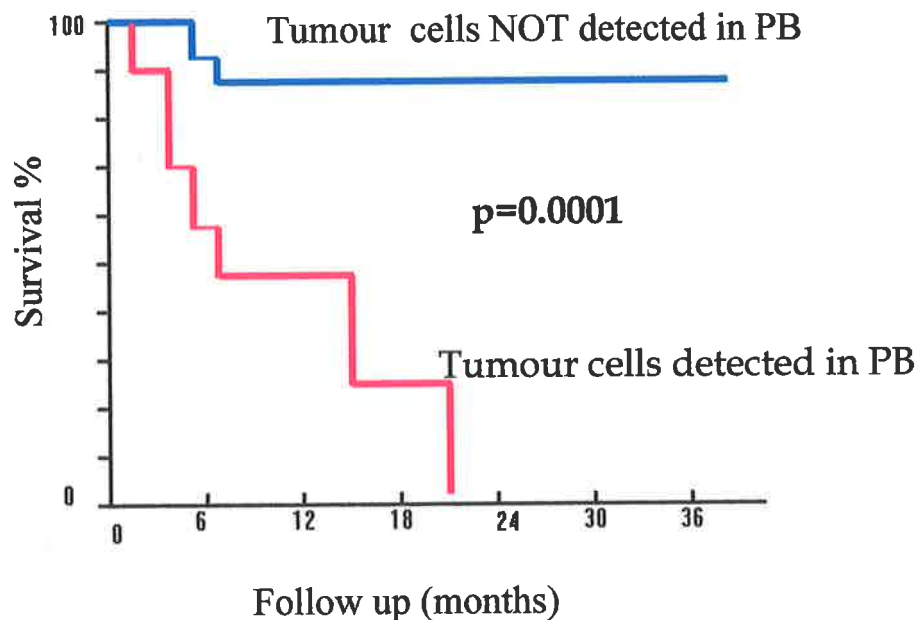
Table 6.1. Patient characteristics and detection of tumour cells in blood.

PT No.	Pt. ID	AGE	SEX	TUMOUR CELLS		STAGE	RELAPSE (months)	SURVIVAL (months)	STATUS
				PRE-OP	POST-OP				
1	535	73	M	NEG	NEG	A	nil	23.5	alive
2	425	56	F	NEG	NEG	A	nil	23	alive
3	169	64	M	NEG	POS	A	nil	17.5	alive
4	173	79	M	NEG	NEG	B	nil	38	alive
5	304	68	F	POS	POS	B	nil	15	alive
6	954	82	F	NEG	NEG	B	nil	28	alive
7	711	77	M	NEG	NEG	B	nil	15	alive
8	647	65	M	NEG	NEG	B	nil	15	alive
9	177	88	F	NEG	NEG	B	nil	16	dec*
10	966	80	M	na	NEG	B	nil	42	alive
11	988	69	M	NEG	NEG	B	nil	14	alive
12	703	72	M	NEG	NEG	B	nil	16	alive
13	356	60	F	na	POS	B	7 m	10	dec
14	712	91	F	NEG	NEG	B	nil	37.5	alive
15	831	76	M	NEG	NEG	B	nil	29	alive
16	433	76	F	NEG	NEG	B	nil	30	alive
17	840	80	F	POS	POS	B	4m	7	dec
18	352	82	M	POS	na	B	22 r	23	dec
19	925	79	F	NEG	NEG	C	nil	12.5	alive
20	606	59	M	POS	POS	C	14.5 r,m	18	dec
21	477	68	F	POS	POS	C	4 m	10	dec
22	748	75	F	NEG	POS	C	5 r	9.5	dec
23	43	73	M	NEG	NEG	C	nil	32	dec*
24	895	71	F	NEG	NEG	C	nil	33	alive
25	412	63	F	POS	POS	D	1 m	7	dec
26	195	83	F	NEG	NEG	D	6 r,m	8	dec
27	625	76	M	na	NEG	D	7r	7	dec

Abbreviations: r, local recurrence; m, metastasis; na, not available; dec, deceased;

*, died of unrelated causes.

Figure 6.3. Kaplan Meier disease-free survival analysis



6.4 DISCUSSION

Intravasation of viable malignant cells into the bloodstream is one step in the metastatic cascade leading to dissemination of the primary tumour. The presence of tumour cells in the circulation before surgery suggests that an active metastatic process is involved and in this study 5/6 patients with tumour cells detected in blood pre-operatively succumbed to recurrent or metastatic disease within 2 years of surgery. The presence of tumour cells in the circulation in 2 patients appeared to be due to direct release of tumour cells during surgical resection, as they were negative for tumour cells in blood before surgery but positive post-surgery. One of these patients (Table 6.1, no. 22) suffered a local recurrence which may have arisen from a pre-existing sub-clinical adenoma, or may have occurred as a result of "reseeding" of the mucosa by blood-borne

tumorigenic cells. The other patient (Table 6.1, no. 3) has remained disease-free at 17.5 months post surgery consistent with the theory that tumour cells gaining direct access into the circulation may not necessarily have metastatic potential; Tarin et al. (1984) demonstrated the absence of metastatic disease in some patients with ovarian cancer after months of continuous infusion of malignant ascites fluid into the bloodstream.

Previously, immunocytochemistry has been used to detect tumour cells in blood. An immunofluorescence method, using the anti-cytokeratin antibody AE3, detected carcinoma cells in blood from the renal vein in 8 of 10 patients undergoing nephrectomy for renal carcinoma; 3 of 8 patients had clinically detectable metastases prior to the assay, while 5 of 8 have remained disease-free for up to 35 months, suggesting that most of the tumour cells released into the renal vein were arrested and destroyed in the pulmonary micro-vasculature (Glaves et al., 1988). Immunocytochemistry for an epithelial marker, cytokeratin 18, was used by Lindemann et al. (1992) to determine the prognostic significance of tumour cells in bone marrow taken pre-operatively from patients with colorectal cancer; 32% of the patients were positive and these patients had a significantly reduced disease-free survival.

More recently, the molecular techniques of reverse transcription and PCR have been used to detect very small numbers of tumour cells in blood in a range of neoplasms (Smith et al., 1991; Mattano et al., 1992; Seiden et al., 1994; Datta et al., 1994; Brown et al., 1995), however results from these studies were not correlated with clinical outcome after long-term follow-up. Brown et al. (1995) detected breast carcinoma cells in pre-operative blood in one of 9 patients, in intra-operative blood in 4 of 9, but in 0 of 9 in blood collected 24 hours post surgery, suggesting that a proportion of tumours shed cells as a result of surgery and that they were removed from the

circulation within 24 hours. This is in contrast to our findings where 8 patients were positive for tumour cells in peripheral blood taken 16-24 hours post surgery.

The biological significance of very small numbers of tumour cells in the circulation remains to be elucidated. A study by Mayhew and Glaves (1984) using a murine model of metastasis for Lewis lung carcinoma determined that 1,000 Lewis carcinoma cells were needed to generate at least one pulmonary metastasis. At the limit of detection of tumour cells using our assay i.e. 10 tumour cells per ml of blood, some 50,000 tumour cells may be circulating (based on an average blood volume of 5 litres) at a given time point. If similar tumour kinetics can be applied to human carcinoma, then this implies that 50,000 circulating tumour cells would be sufficient to enable metastases to form. Taking into account the inefficiency of the metastatic process such that less than 0.01% of these cells will survive (Fidler, 1970; Weiss, 1986), there is still the potential for some 5 metastatic deposits to develop. The observed frequency is even less in our patient group with only 2 patients developing secondary deposits at more than one site, although multiple seeding at the same site can not be excluded.

In 2 patients with stage D (metastatic) disease circulating tumour cells were not detected prior to surgery and this may reflect the sporadic release of tumour cells from the primary (or secondary) tumour(s) into the blood (Glaves, 1983). Hence samples taken at any one time will necessarily contain variable numbers of cancer cells perhaps below the threshold of detection, depending on the rate of shedding at the time.

This study has identified circulating tumour cells at the time of surgery in 5 patients whose tumours did not show any evidence of metastasis by conventional staging. These patients would not have been offered adjuvant therapy since this is currently restricted to patients with more advanced disease. Perhaps patients with circulating micrometastatic disease would benefit most from adjuvant chemotherapy or

radiotherapy, or systemic treatment with monoclonal antibodies such as 17-1A, which in Dukes' C patients has been shown to reduce the overall death rate by 32% and the recurrence rate by 23% (Riethmuller et al., 1994, 1998).

In conclusion, this preliminary study shows that *K-ras* immunobead-PCR is a sensitive and specific technique for identifying tumour cells in blood in a proportion of patients with CRC*. The correlation between the presence of tumour cells in blood and shortened disease-free survival shows that the technique is useful for identifying CRC patients at diagnosis who are at increased risk of relapse.

* These results have been published (Hardingham et al., 1995).

CHAPTER 7

DETECTION OF P53 MUTATIONS BY PCR-SSCP

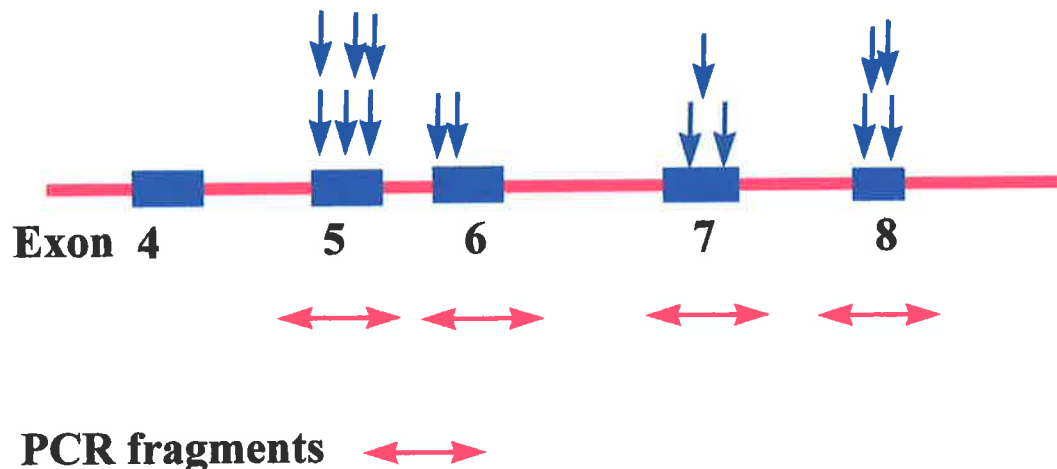
7.1 INTRODUCTION

As mutations in the *K-ras* gene were present in only a proportion (35%) of colon tumours, another candidate marker for colon tumour cells was sought. Mutations in the p53 tumour suppressor gene appear to be the most commonly occurring mutations in human cancer (Hollstein et al., 1991; Levine et al., 1991). The wild-type p53 protein functions as a sequence-specific DNA-binding protein that is active as a transcription factor, having the ability to both transactivate genes and suppress the transcription of other genes (Bargonetti et al., 1991). In response to DNA damaging agents, intracellular p53 increases dramatically. This results in increased expression of WAF1/Cip1, the protein product of which, p21, binds to cyclin-dependent kinases and inhibits their activity (Harper et al., 1993). Thus cells are arrested at G1 (the G1 checkpoint) which allows time for DNA repair or, if repair is not possible, p53 may induce programmed cell death (apoptosis) thus preventing replication of altered DNA. Mutations in p53 inactivate this G1 checkpoint function, allowing cell division to occur even in the presence of damaged DNA, so that the mutant cell continues to proliferate unchecked and apoptosis is prevented (reviewed in Chang et al., 1995).

Point mutations of the p53 gene have been reported to occur in about 50% of colorectal adenocarcinomas (reviewed in Chapter 1). The majority of the mutations are missense mutations, involving G-A and C-T transitions, clustered at CpG dinucleotide "hotspots" in the evolutionarily conserved domains of the gene, exons 5-8 (Figure 7.1) (Nigro et al., 1989, Hollstein et al., 1991). Since these mutations are single point mutations occurring over a large area of the gene, detection is not as straightforward as for the *K-ras* mutation, hence a different approach had to be adopted, namely single-strand conformation polymorphism (SSCP) analysis. This technique utilises the fact that single DNA strands,

which may differ by as little as one base, form different secondary structures which migrate at different rates through a polyacrylamide gel. A mutant strand can thus be readily identified by comparison to the gel position of normal strands.

Figure 7.1. Mutational "hot-spots" of the p53 gene.



Relative distribution of mutations is indicated by the downward arrows (adapted from Nigro et al., 1989).

The sensitivity of the SSCP technique varies depending on both size and base composition of the PCR product, on the gel composition and electrophoretic conditions. The conformation of single-stranded DNA is influenced by the balance between thermal forces and weak stabilising forces such as intra-strand base pairing and base stackings. Changes in environmental conditions such as temperature and ionic strength are likely to cause a change in conformation such that mobility is altered to a greater or lesser degree. Optimisation of these parameters is therefore necessary to achieve the best separation of normal and mutant strands (Orita et al., 1989a).

A variety of conditions for SSCP have been reported in the literature since Orita's original report. In that study, they used genomic DNA which had been digested by

restriction enzymes, denatured in alkali solution and electrophoresed through a neutral polyacrylamide gel. Several months later they reported the rapid and sensitive detection of point mutations using PCR products instead of genomic DNA (Orita et al., 1989b). They used radiolabelled PCR products which were electrophoresed through a non-denaturing 6% polyacrylamide sequencing gel containing either zero or 10% glycerol. They found that most single base changes in fragments of 200 bases or less could be detected as mobility shifts in SSCP gels. In 1991, Dean and Gerrard described a modification to Orita's technique to further enhance the sensitivity i.e. running gels at both room temperature (with glycerol) and 4°C (without glycerol) increased the rate of detection of some mutations.

Further improvements to the protocol for SSCP were investigated by Spinardi et al. (1991). They tested the effect of 4 parameters on mobility of mutant HPV sequences: temperature, percentage of acrylamide, ionic strength of the electrophoresis buffer, and glycerol concentration. Their results showed that 6% sequencing gels containing 5% glycerol and run in 0.5xTBE at room temperature resulted in sharper bands and enhanced mobility shifts. For certain mutations, a second run at 4°C without glycerol was informative.

Sheffield et al. (1993) reported that the sensitivity of SSCP in detecting mutations varied dramatically with the size of the fragment. The greatest sensitivity (97%) of detection of single base substitutions was in 155 bp fragments. The rate of detection decreased in larger fragments to 70% in fragments of 212 bp, and to only 3% in a 600 bp fragment. Similarly there was a lower limit of sensitivity so that the detection rate was reduced in fragments of 95-135 bp in size; 150 bp was recommended as being optimal. The same study also found that the relative position of the base substitution was more important than the type of mutation (transition versus transversion) in determining whether a mutation was detected by SSCP. The position of the base substitution within the PCR

fragment influenced the secondary structure of the DNA and hence the mobility of the strands through the gel to a greater or lesser degree.

7.2 MATERIALS AND METHODS

7.2.1 Design of primers

Genomic primer pairs were designed for each of the exons 5, 6, 7, and 8 using the p53 gene sequence obtained from GenBank and the "Primer" and "Amplify" programs. Since we were interested in mutations occurring in any part of the 4 coding sequences, the primers were chosen from intron sequences either side of the exons, except for the 3' antisense primer for exon 8 which has 10 of the 22 bases within the coding region. The product size was kept as short as possible while still encompassing the entire exon, as longer products have been reported to give less reliable results in SSCP analysis (Sheffield et al., 1993). Sequences of the primers and resulting PCR product sizes are shown in Table 7.1.

7.2.2 PCR optimisation

The cycling parameters of each of the 4 PCRs was optimised according to the melting temperature (T_m) of the primer pairs. For exon 5 (T_m 59.5 and 62°C, Table 7.1) the starting point was the standard PCR as described in Chapter 2, run as follows: 5 minutes at 94°C denaturation, then 45 cycles of 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C. The result of this PCR (band intensity on an ethidium bromide stained gel) was compared to other PCRs performed using different conditions i.e. 1) reducing the cycles to 30 seconds each instead of 1 minute, 2) increasing the annealing temperature to 58°C and 3) to 60°C at each cycling time i.e. 30 seconds or 1 minute. The best results were obtained using

1 minute cycles and 55°C annealing, and all subsequent exon 5 PCRs were performed using these parameters.

A similar approach was taken to optimise the other 3 PCRs, comparing annealing temperatures of 55°C and 62°C. The exon 6 and exon 7 PCRs were optimal at 1 minute at 94°C, 1 minute at 62°C, and 1 minute at 72°C for 45 cycles, exon 8 at 1 minute at 94°C, 1 minute at 55°C, 1 minute at 72°C for 45 cycles (same as exon 5).

Table 7.1. P53 primer sequences

Primer	Sequence 5'-3'	T _m (°C)	Product size (bp)
Exon 5 sense	5' TCT GTC TCC TTC CTC TTC CTA CA	59.5	247
antisense	5' AAC CAG CCC TGT CGT CTC TC	62	
Exon 6 sense	5' GTC CCC AGG CCT CTG ATT CC	65	185
antisense	5' CCT CCT CCC AGA GAC CCC CAG T	69	
Exon 7 sense	5' CCT CAT CTT GGG CCT GTG TTA TC	70	159
antisense	5' TGG CAA GTG GCT CCT GAC CTG GA	68	
Exon 8 sense	5' CCT CTT GCT TCT CTT TTC CTA TC	66	182
antisense	5' CTG CTT GCT TAC CTC GCT TAG T	63	

7.2.3 Optimisation of SSCP

The variables to be tested included the temperature at which the gel was electrophoresed, gel composition (%T, %C), presence of glycerol, ionic strength of the running buffer, and power settings. No single set of conditions proved optimal for all four p53 PCR products, hence each had to be optimised individually. Common to each gel however was that the

same amount of PCR product (10 μ l) was loaded, mixed with 10 μ l formamide loading buffer (95% formamide, 20 mM EDTA, 10 mM NaCl, 0.05% bromophenol blue) and heated at 95°C for 5 minutes to denature the DNA strands. The tubes were kept on ice while loading the gel to minimise renaturation. Finally the gels, mounted on gel bond and silver stained, were preserved by fixing in 10% acetic acid and sealing in a plastic bag.

7.2.3.1 Cell lines as positive controls

The colon carcinoma cell lines SW48, HT29, the LIM series 1215, 2463, 2405, 2412, 2099 and 1899 and the breast cancer cell lines MDA-MB-453, MDA-MB-231 and ZR75.1 were tested to determine the presence of p53 mutations for use as controls i.e. cell lines with known band shifts were used as controls in different runs to ensure constant running conditions. In addition, known mutations in 3 other cell lines were used: the human erythroid leukaemic cell line K562, which has an insertion of a cytosine between codons 135 and 136 of exon 5, the normal allele being lost, and the breast cancer cell line, T47D which has a C-T substitution in codon 194 of exon 6. SW480 with a G-A substitution in codon 273 of exon 8 was used as the positive control for exon 8 PCR-SSCP. None of the other cell lines tested by SSCP were shown to have a mutation in exon 7, so that after screening a number of patient tumour samples, one with a mutation was found and used subsequently as a positive control for exon 7 PCR-SSCP.

7.2.3.2 Effect of temperature

Gels containing 5% glycerol were run at room temperature, gels without glycerol were run in a 4°C cold room. The detection of known mutations in cell lines was deemed to be no better in the cold than at room temperature, and in several cell lines the mobility shift was more obvious in gels run at room temperature than in the cold. Subsequent gels were run at

room temperature. These results are in keeping with the findings of several other studies (Spinardi et al., 1991; Sheffield et al., 1993; Glavac and Dean, 1993).

7.2.3.3 Effect of gel composition and power settings

Single DNA strands are separated more readily using a polyacrylamide matrix containing a low percentage of crosslinker (N,N'-methylenebisacrylamide) referred to as the %C in the total acrylamide monomer, %T. The gel fibres in a low %C gel are spatially less rigid and result in more efficient resolution of subtle differences in the DNA strands (reviewed in Hayashi and Yandell, 1993).

Initially, a standard 6% sequencing gel (3%C) was used to separate "hot" PCR products (labelled with α - ^{32}P during PCR), however band shifts were not detected. Following discussions with Dr Brett Dix from the Department of Surgery, Queen Elizabeth II Hospital, Perth, I decided to use the smaller BRL electrophoresis apparatus, which they claimed gave adequate separations and which was much more economical on acrylamide and buffer solutions. Subsequently an additional advantage was that the gel could be mounted on gel bond and silver stained which alleviated the need to use P^{32} labelled PCR products, reducing the costs even further, and making the procedure much less hazardous. Gels of 8, 10, 12 and 16%T (1%C) were tried for each PCR product.

For exon 5 PCR products, using DNA from the cell line K562 as the positive control, optimal gel composition was 12%T, 1%C, run at 10 watts (W) for 5 hours or until the xylene cyanol dye front was near the bottom edge of the gel. Exon 6 products run under the same conditions showed no mobility shift between the mutation control and normals, therefore the gel composition was changed to 16%T, 1%C. Due to the increased resistance of the higher % gel, the power setting had to be reduced to 2 W to avoid 'smiling' and the electrophoresis carried out overnight, otherwise the distortion of the gel made it difficult to

discern mobility differences. Similarly for exons 7 and 8, no differences were seen in a 12%T gel, but were readily observed in a 16%T gel run under the same electrophoretic conditions as for exon 6.

7.2.3.4 Effect of ionic strength

Another variable that was tested was the ionic strength of the electrophoresis buffer i.e. 1x or 0.5x TBE, and several experiments showed that 0.5x TBE enhanced the mobility shifts between the mutant and normal sequence.

7.2.4 Sensitivity experiments

7.2.4.1 Lower limit of detection of the mutant allele

DNA from a patient with a known p53 exon 5 mutation was serially diluted in water to contain 150 ng, 15 ng, 1.5 ng, 150 pg, and 15 pg (equivalent to 2-3 cells) per PCR tube. The PCR was performed with α dCTP³² incorporation (hot PCR). The SSCP gel was silver stained then wrapped in plastic wrap and exposed to X-ray film at -80°C overnight.

7.2.4.2 Sensitivity of immunobead-PCR-SSCP

To assess the sensitivity of immunobead-PCR-SSCP in detecting rare mutant p53 sequences, SW480 cells were diluted into duplicate 10 mL blood samples at 5000 cells per 10 mL ($1/10^4$ white cells), 500 ($1/10^5$) and 50 ($1/10^6$) and incubated with 3×10^6 immunobeads. Hot PCR-SSCP was performed for exon 8, the gel was silver stained then autoradiography was carried out as before for 72 hours.

7.2.5 Detection of p53 mutations in tumour samples

Before using p53 mutation as a marker of tumour cells in blood, the particular mutation must be identified in the patient's tumour. Tumour samples were obtained from one hundred consecutive patients undergoing resection for colorectal cancer at The Queen Elizabeth Hospital. There were 58 males and 42 females with a median age of 70 years (range 37-88 years). DNA was isolated from fresh tumour or in some cases from paraffin-embedded formalin fixed tissue. PCR-SSCP for exons 5, 6, 7, and 8 was performed according to the optimised protocols described above.

7.3 RESULTS AND DISCUSSION

The results of the p53 mutation status for the 100 tumours are set out in Appendix B. Representative examples of silver stained PCR-SSCP gels for each exon are shown in Figures 7.4-7.7. Overall 56/100 tumours were positive for a mutation in exons 5-8 of the p53 gene; 15 (26%) had a mutation in exon 5, 9 (16%) in exon 6, 16 (29%) in exon 7, and 16 (29%) in exon 8. This distribution corresponds to the incidence of mutations at "hot spot" codons 175 in exon 5, 248 in exon 7 and 273 in exon 8 in 35 colon tumours studied by Levine et al. (1991).

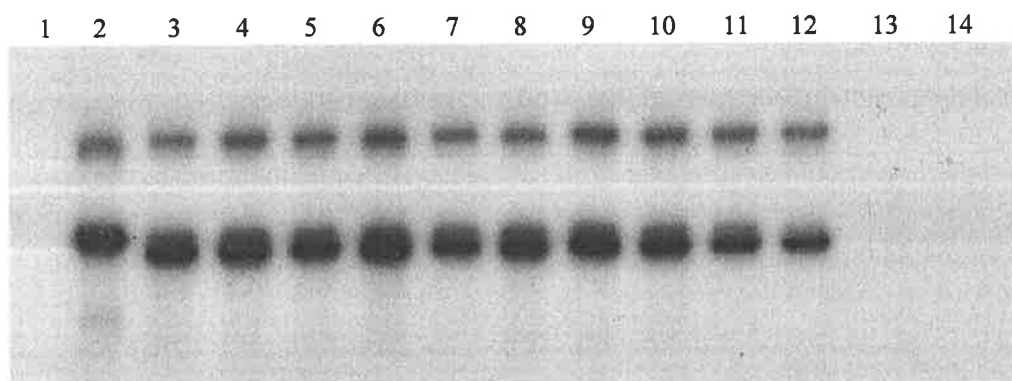
7.3.1 Sensitivity experiments

In dilution experiments in which tumour DNA, with a previously identified p53 mutation in exon 5, was serially diluted in water, a band representing the mutant allele could be detected down to the level of 150 pg DNA (equivalent to 20-30 cells) (Figure 7.2).

The detection of mobility shifts of the mutant sequence in tumour DNA could be discerned readily following silver staining of the gels (autoradiography was no more

sensitive than silver staining), however in some cases the mutant sequence stained with considerably less intensity than the normal (Figure 7.3), presumably due to the different conformation. This suggested that if the target sequence was only present at the level of 5-500 pg DNA (equivalent to 1-100 cells), as might be the case in the immunobead isolates, instead of at the level of 100 ng of tumour DNA, it would not be detected. Sensitivity experiments using positive cell line cells diluted into normal blood confirmed this theory, showing that the sensitivity was limited to the $1/10^4$ dilution equivalent to 5000 tumour cells per 10 mL blood sample (Figure 7.3). The mutant allele in the $1/10^5$ (500 cells), and $1/10^6$ (50 cells) dilutions could not be detected and this was deemed to be not sufficiently sensitive for immunobead-PCR detection of tumour cells in blood.

Figure 7.2. Sensitivity experiment-p53 PCR-SSCP for exon 5: lower limit of detection of the mutation in serial dilutions of tumour DNA

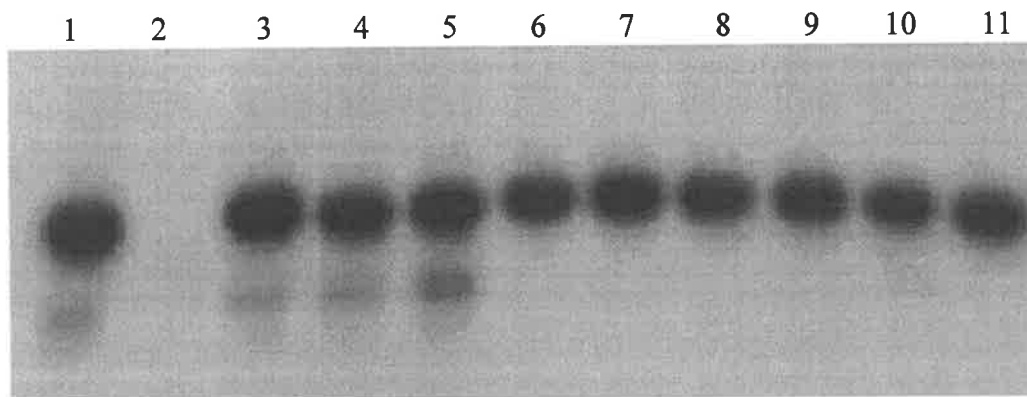


Lane 1, negative control; lane 2, normal control; lanes 3, 4, 1500 ng tumour DNA; lanes 5, 6, 150 ng; lanes 7, 8, 15 ng; lanes 9, 10, 1.5 ng; lanes 11, 12, 150 pg; lanes 13, 14, 15 pg. The 2 bands in lane 2 represent the 2 normal alleles. Note the mobility shift of the lower band in lanes 3-12 representing the mutant allele in tumour DNA (the normal allele is still visible due to presence of normal DNA from the tumour sample).

7.3.2 Detection of p53 mutations in tumour samples

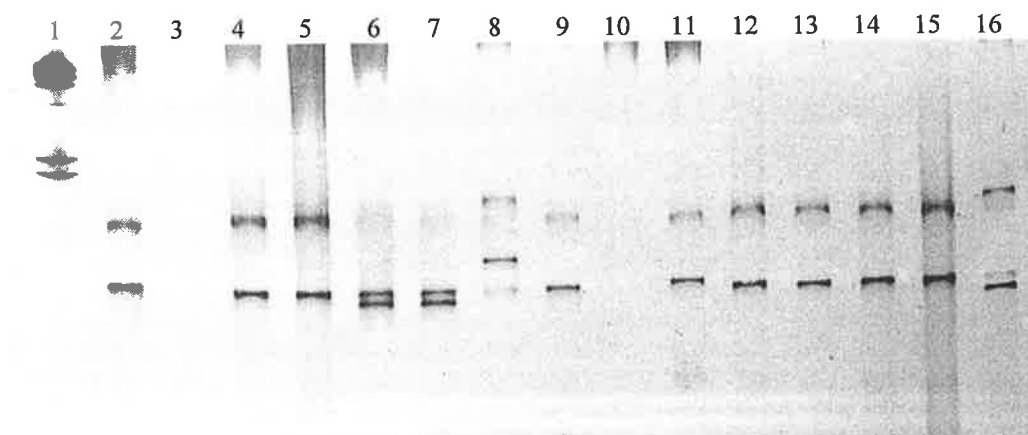
The results of the detection of p53 mutations in tumours were used in a study of colon cancer patients to determine if p53 mutations, with or without K-*ras* mutations, were prognostic markers of survival that were independent of Dukes' stage. The Kaplan-Meier survival data is shown in Table 7.2 and the survival curves from the Cox proportional hazards regression analysis are shown in Figure 7.8. The analysis showed that patients with both p53 and K-*ras* mutations had a 4.2-fold case mortality (95% CI 1.5-11.6) compared to a p53 negative reference group ($P < 0.05$) (Hardingham et al., 1998; raw results see Appendix B).

Figure 7.3. Immunobead-PCR-SSCP sensitivity



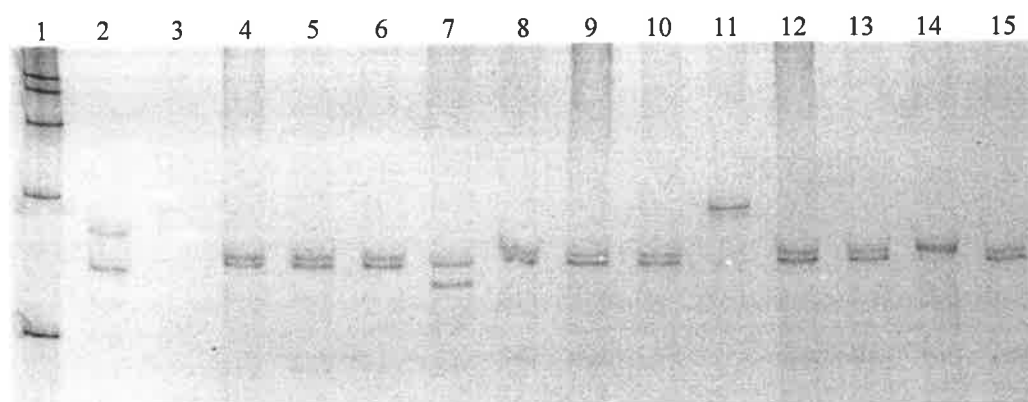
SW480 cells were diluted into duplicate 10 mL blood samples at 5000 cells per 10 mL ($1/10^4$ white cells), 500 ($1/10^5$) and 50 ($1/10^6$). Immunobead-PCR-SSCP for p53 exon 8 was performed using incorporation of P^{32} , and the products analysed after autoradiography. Lane 1, normal DNA; lane 2, negative PCR control; lanes 3, 4, 5 SW480 $1/10^4$; lanes 6, 7, 8, SW480 $1/10^5$; lanes 9, 10, 11, SW480 $1/10^6$. The signal for the mutant allele is only visible at the $1/10^4$ dilution.

Figure 7.4. PCR-SSCP for p53 exon 5



Silver-stained gel. Lane 1, MW markers SPP-1; lane 2, positive control; lane 3, negative (no DNA) control; lane 4, normal DNA control; lanes 5-16, patient tumour DNA, band shifts representing mutant sequences are evident in lanes 2, 6, 7, 8, 11 and 16. Note the subtle band shift in lane 11.

Figure 7.5. PCR-SCP for p53 exon 6



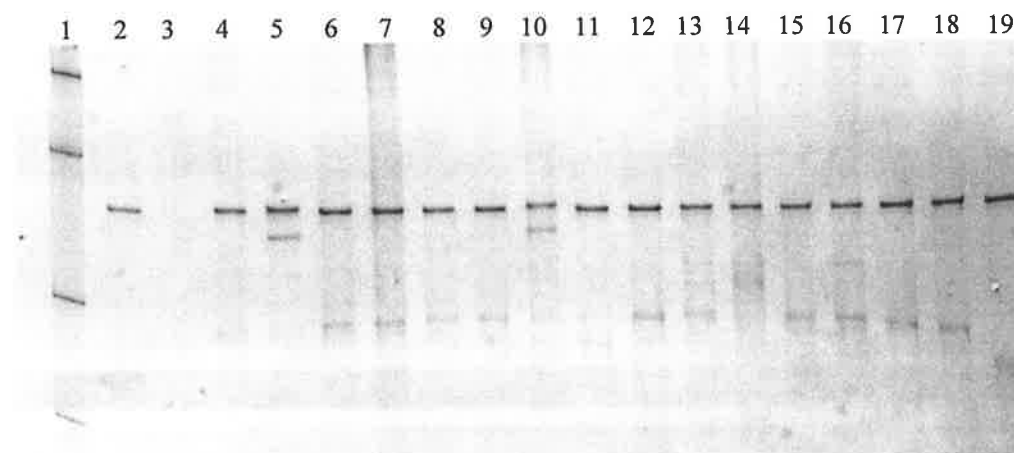
Silver-stained gel. Lane 1, MW markers SPP-1; lane 2, positive control (); lane 3, negative (no DNA) control; lane 4, normal DNA control; lanes 5-15, patient tumour DNA. Band shifts are evident in lanes 2, 7, 11 and 14.

Figure 7.6. PCR-SSCP p53 exon 7



Silver-stained gel. Lane 1, MW markers SPP-1; lane 2, positive control (known tumour DNA); lane 3, negative (no DNA) control; lane 4, normal DNA control; lanes 5-15, patient tumour DNA. Band shifts are evident in lanes 2, and 11.

Figure 7.7. PCR-SSCP p53 exon 8

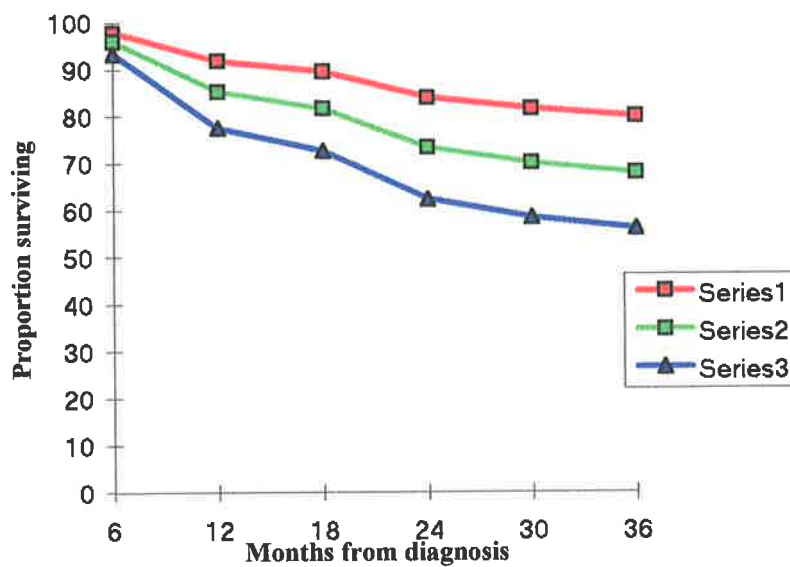


Silver-stained gel. Lane 1, MW markers SPP-1; lane 2, positive control (SW480); lane 3, negative (no DNA) control; lane 4, normal DNA control; lanes 5-19 patient tumour DNA. Band shifts are evident in lanes 2, 5, 10, 11, 13, 14, 16 and 19. Note the weak silver staining of some bands representing mutant alleles e.g. in lanes 13, 14 and 16.

Table 7.2. Survival data of patients and mutation status

months from diagnosis	% of patients surviving		
	p53 neg	p53 pos, K-ras neg	p53 pos, K-ras pos
0	100	100	100
6	97.9	96	93.3
12	91.8	85.3	77.5
18	89.5	81.6	72.6
24	83.9	73.3	62.3
30	81.5	70	58.4
36	79.9	67.8	56

Figure 7.8 Survival curves: Cox proportional hazards regression analysis.



Series 1, p53 negative reference group; series 2, p53 positive, K-ras negative; series 3, p53 positive, K-ras positive (significantly different to the reference group, $P < 0.05$).

Although p53 PCR-SSCP was a satisfactory technique for determining p53 mutations in tumour DNA, it was not sensitive enough for the detection of rare mutant alleles in patients' peripheral blood samples. In many cases, the mutant allele from undiluted tumour DNA was barely discernible on gels; once the DNA was diluted down to the equivalent of 2-3 cells, the signal was not detectable. Similarly, the dilution of positive cell line cells into 10 mL of blood showed that a signal was only detectable at the highest concentration of 5000 cells per 10 mL (equivalent to $1/10^4$ leucocytes). The use of P^{32} labelled PCR products and autoradiography made no difference to the sensitivity of detection (Figure 7.2). These findings were in marked contrast to the ASRA technique used to detect the *K-ras* codon 12 mutation, which could be detected down to $1/10^6$ tumour cells in blood.

CHAPTER 8

CYTOKERATIN GENE EXPRESSION AS A MARKER OF EPITHELIAL CELLS

8.1 INTRODUCTION

To overcome the problem of *K-ras* mutations being present in only a proportion of colorectal tumours, I investigated the use of RT-PCR assays for cytokeratin (CK) 19 and 20 expression as markers of epithelial derived cells (cancer cells). This approach has the advantage that CK expression is an appropriate marker for all of the patients undergoing surgery for colorectal carcinoma, and means that we do not need a tumour sample to determine a specific mutation. RT-PCR has another advantage in that it is potentially a more sensitive assay than PCR using single-copy genes as the target, since there are multiple CK mRNA transcripts per cell as a starting point.

Cytokeratins 19 and 20 are members of the multi-gene family of intermediate filament proteins that are a major component of the cytoskeleton. So far, 20 distinct CK polypeptides have been identified in various epithelial cell types (Moll et al., 1982, 1993) and the expression pattern correlates with different pathways of epithelial differentiation thereby allowing classification of epithelial cells into various subtypes (Tseng et al., 1982).

8.1.1 Expression of CK 19

CK 19 has been reported to be present in a broad range of epithelial tissues as a major component of the cytoskeleton in simple epithelia and as a minor component in stratified epithelia (Moll et al., 1982). In that study, CK 19 was found to be strongly expressed in normal colon and breast and their malignant counterparts. More recently, Traweek et al. (1993), using RT-PCR, studied CK gene expression in both epithelial and non-epithelial tissues. They found ubiquitous expression of CKs 8 and 18 in epithelial tissues and in a range of non-epithelial tissue i.e. peripheral blood mononuclear cells

(MNCs), endothelial cells, fibroblasts, normal lymph nodes and normal bone marrow. CK 19 expression was more restricted to epithelial tissue; it was not found in any normal haemopoietic tissue or lymph nodes, but was detected weakly in MNCs from 2/4 cases of non-Hodgkin's lymphoma.

In using CK 19 RT-PCR, a potential problem exists due to the presence of a CK 19 processed pseudogene (Savtchenko et al., 1988), such that a band of similar size to that generated from the CK 19 cDNA is produced from the processed pseudogene in DNA present in the lysed cell preparation. Processed pseudogenes are DNA segments that arose from integration of transcripts of functional genes by RNA polymerase II, or by a viral promoter, into the genome at some time in evolution (reviewed in Weiner et al., 1986). The problem of pseudogene amplification can be overcome by the design of primers with mismatches for the pseudogene sequence (Datta et al., 1994).

8.1.2 Expression of CK 20

Moll et al. (1993), using riboprobes corresponding to exon 6 of the CK 20 gene showed that the 1.75 kb mRNA encoding CK 20 was specifically produced in cells of the gastrointestinal mucosa including tumours and derived cell lines. A series of mouse monoclonal antibodies against CK 20 was used in immunostaining cells from a range of different tissues including skin epithelia, respiratory tract, gastrointestinal tract, liver, pancreas, urinary tract, endothelia, lymph nodes, smooth muscle, and brain. Specific CK 20 immunoreactivity was not seen in any of the non-epithelial tissues tested; positivity was restricted to the gastrointestinal tract, taste buds and Merkel cells (sensory cells of the palm of the hand and sole of the foot) (Moll et al., 1992).

The same group also analysed a broad spectrum of 711 carcinomas by immunocytochemistry and found that 89/93 colorectal adenocarcinomas were positive

for CK 20 including metastatic deposits, compared to breast cancer where only 1/150 cases showed any degree of reactivity. The other carcinomas to show significant reactivity to CK 20 were Merkel cell carcinoma (15/15), mucinous carcinoma of the ovary (6/6), transitional cell carcinoma of the urinary tract (18/24) and adenocarcinoma of the upper GI tract (51/96).

8.2 RT-PCR FOR CYTOKERATIN 19

8.2.1 Design of primers and RT-PCR optimisation

The primers to amplify CK 19 were modified from Datta et al. (1994). Care was taken to avoid amplification of the pseudogene by using primers incorporating differences between the gene and its pseudogene at the 3' ends as Datta et al. had done. The difference in our technique (Eaton et al., 1997) was that it was a one-stage RT-PCR, instead of a nested technique. The primer sequences are shown in Table 7.1.

The reverse transcription step was designed to be carried out directly on lysed cells isolated by the immunobead technique and stored frozen at -80°C. The detergent Nonidet P-40 was used to assist lysis of the cell membranes thus releasing mRNA (Eaton et al., 1997). The freeze-thaw cycle completed lysis. A panel of carcinoma cell lines was used in optimisation of the RT-PCR and to assess CK 19 expression. Negative controls were included in every RT-PCR to ensure that non-specific amplification was not occurring; these included an RT negative which contained all the RT reagents except target cells/RNA, a PCR negative containing all PCR reagents except cDNA target, and a genomic DNA control to ensure that the pseudogene or other genomic sequences were not being amplified. Positive controls consisted of 200 colon cancer cell-line cells (Chapter 4.2.1) in lysis mix (stored at -80°C) or a 2 µL aliquot of RNA prepared from

cell-line cells. PCR products were run on a 1.5% agarose gel and Southern blotted. Autoradiography was performed using a gamma P32 labelled oligo probe (Chapter 2.6.4), the sequence of which lies between the two PCR primers and is shown in Table 7.1.

8.2.2 Sensitivity experiments

The sensitivity of the CK 19 immunobead RT-PCR was assessed by dilution of 1000, 100 and 10 SW48 or LIM-2412 cells in duplicate 10 mL samples of donor blood. Immunobead RT-PCR was performed as described using the standard PCR master mix (Chapter 2.4.3) with the cycling parameters being 1 minute at 94°C, 1 minute at 68°C, and 1 minute at 72°C for 45 cycles. Results were obtained following Southern blot hybridisation and autoradiography (Chapter 2.6). Under these conditions no signal was obtained from the DNA (i.e. pseudogene amplification) control.

Table 7.1 Oligonucleotide sequences

Primer	Sequence 5'-3'	*T _m °C
CK 19 C2 sense	GACTACAGCCACTACTACACGACC	74
CK 19 D2 antisense	AGCCGCGACTTGATGTCCATGAGCC	80
Internal oligo probe	GATCTGCATCTCCAGGTCGGTCC	74
CK 20 sense	TCT TTG ATG ACC TAA CCC TAC	60
CK 20 antisense AS3	CTA CTT CTT GCA CGA CTG TCT TA	66
Internal oligo probe	AGC TCC GTT AGT TGA ACC TCA	62

*T_m, melting temperature

8.3 RT-PCR OF CYTOKERATIN 20

8.3.1 Design of primers and RT-PCR optimisation

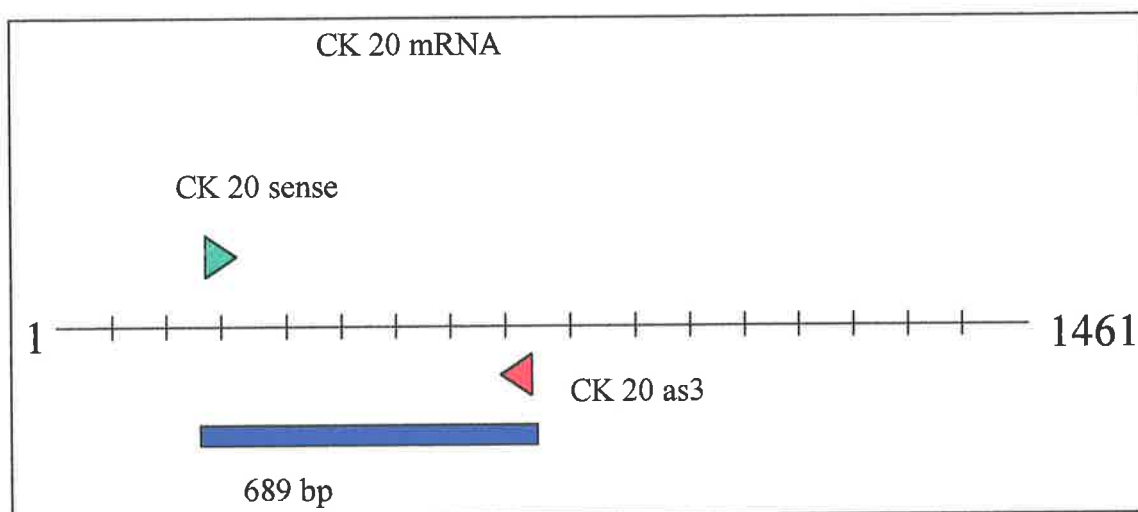
Primers to amplify CK 20 cDNA were designed with the help of the programs "Amplify" and "Primer" using cDNA and genomic sequences from GenBank. The first set of primers were designed to give a product of 333 base pairs (bp) so that CK 20 products could be readily distinguished from CK 19 products in a multiplex PCR. These primers, however, also amplified an 846 bp genomic fragment from DNA present in the cell lysis milieu, and which seemed to be amplified in preference to the 333 bp cDNA product. A new antisense primer was designed (CK 20 as3) which produced a 689 bp product from cDNA (Figure 8.1). The new primer pair also bound to genomic DNA to form a product of 4298 bp but which, in practice, was not amplified due to its length. The primer sequences are shown in Table 7.1. Since the Tms of these primers were 60°C and 66°C respectively, the annealing temperature in the PCR was tested at 55°C and 58°C, 58°C being selected as producing the highest intensity bands on ethidium bromide staining. This low temperature however precluded the possibility of performing multiplex PCR for CK 19 and 20 since the optimum annealing temperature for CK 19 was 68°C.

Experiments to optimise the CK 20 PCR also included comparing 0.25 mM spermidine with zero spermidine, increasing the extension time from 1 minute to 1.5 minutes, and comparing the standard 1.5 mM MgCl₂ with 2.0 mM MgCl₂ in the PCR master mix.

8.3.2 Sensitivity experiments

The sensitivity of immunobead RT-PCR for CK 20 was assessed by diluting 100, 10 and zero LIM-2412 cells into duplicate 10 mL blood samples. Immunobead RT-PCR was performed and results assessed after Southern blot hybridisation and autoradiography.

Figure 8.1. CK 20 RT-PCR: product and primer binding sites



Primer binding sites and product size as depicted by the program "Amplify", using CK 20 mRNA as the target sequence.

8.3.3 RT-PCR for CK 19 and CK 20 on cell lines and tumour samples

The colon cancer cell lines SW48, SW480, HT29, LIM-2412, LIM-1215, LIM-2099, LIM-2405, LIM-1899, LIM-2463, and LIM-1863 were tested by RT-PCR for expression of the two epithelial-specific markers CK 19 and CK 20. Fresh tumour, obtained from 14 patients and snap frozen in liquid nitrogen, was also tested. RNA extraction from cell lines and tumour samples was performed as described in Chapter 2.3.

8.4 RESULTS

8.4.1 *Optimisation experiments*

Most of the optimisation of the CK 19 PCR had been done separately in the laboratory by Michael Eaton (MD thesis, 1997). Final optimisation was achieved by increasing the temperature of annealing to 68°C to avoid amplification of the pseudogene from DNA. Experiments performed using 60°C annealing showed a band of the expected size from genomic DNA samples that had not been reverse transcribed, i.e. the pseudogene was being amplified due to the low stringency annealing temperature. This band was of a similar size to that obtained for CK 19 from cDNA, thus the two could not be distinguished on the gel.

In separate optimisation experiments for CK 20, comparing 2 different Mg^{++} concentrations, stronger signals were obtained using 1.5mM Mg^{++} rather than 2.0 mM Mg^{++} (Figure 8.2). No signal was obtained using spermidine in the PCR master mix compared to good signals without spermidine (Figure 8.3). Increasing the annealing temperature from 55°C to 58°C resulted in bands of higher intensity (Figure 8.4). Finally, increasing the extension time at 72°C from 1 minute to 1.5 minutes resulted in bands of slightly higher intensity (4+ instead of 3+) (data not shown). The final PCR conditions therefore were determined to be 1.5 mM Mg^{++} , without spermidine, and the cycling parameters as 1 minute 94°C, 1 minute 58°C, and 1.5 minutes at 72°C.

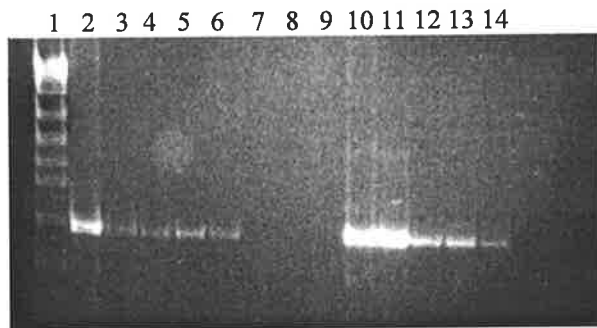
8.4.2 *Sensitivity experiments*

In sensitivity experiments for CK 19, diluting SW48 cells into duplicate 10 mL blood samples, a signal could be detected after autoradiography in all dilutions down to 10 SW48 cells per 10 mL blood sample. This is equivalent to 1 tumour cell per mL or a

sensitivity of 1 tumour cell per 5-10 million leucocytes (Figure 8.5). No signal was detected in donor blood used in these experiments without added tumour cells. Thus the CK 19 RT-PCR was sensitive down to the single cell level, while the overall sensitivity of immunobead RT-PCR was 1 tumour cell per mL of blood (5-10 million leucocytes).

Sensitivity experiments for CK 20 using LIM-2412 cells showed that a signal could be detected after autoradiography down to 10 cells per 10 mL blood sample (1 tumour cell per 5×10^6 leucocytes) (Figure 8.6).

Figure 8.2 Effect of Mg^{++} concentration on CK 20 RT-PCR



Lane 1, MW markers; lanes 2-6, CRC cell line cDNA amplified using 2mM $MgCl_2$; lanes 7-9, blank; lanes 10-14, the same cDNA amplified using 1.5mM $MgCl_2$.

Figure 8.3 Effect of spermidine on CK 20 RT-PCR



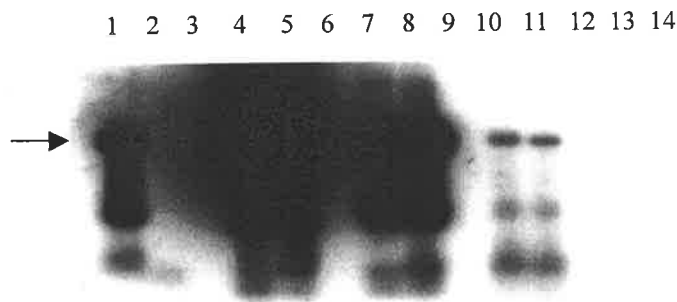
Lane 1, MW markers; lanes 2-4, no spermidine;
lanes 5-7, blank; lanes 8-10, 0.25 mM spermidine

Figure 8.4 Effect of annealing temperature on CK 20 RT-PCR



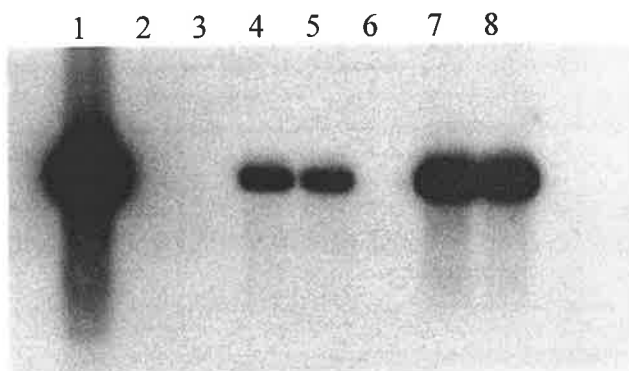
Lane 1, MW markers; lanes 2-5, CRC cell line
cDNA amplified at an annealing temperature of
58°C; lanes 6-7, blank; lanes 8-11, the same cDNA
amplified at 55°C.

Figure 8.5 Sensitivity of CK 19 immunobead RT-PCR.



Autoradiograph showing results of immunobead RT-PCR for CK 19, diluting SW48 cells into 10 ml blood samples; CK19 signal from the tumour cells was detected at the dilution of 10 cells per 10 ml sample (1 cell per ml). Lane 1, positive control (200 cells); lane 2, negative control; lane 3, blank; lanes 4, 5, 1000 SW48 cells diluted into 10 mL blood; lane 6, blank; lanes 7, 8, 100 SW48 cells per 10 mL blood; lane 9 blank; lanes 10, 11, 10 SW48 cells per 10 mL blood, lane 12, blank; lanes 13, 14, zero SW48 cells per 10 mL blood. CK 19 band is arrowed.

Figure 8.6 Sensitivity of CK 20 immunobead RT-PCR



Autoradiograph showing results of immunobead RT-PCR for CK 20 after dilution of LIM-2412 cells into duplicate 10 mL tubes of normal donor blood. Lane 1, positive control (200 LIM-2412 cells in lysis mix); lanes 2 and 3, zero cells in 10 mL blood; lanes 4 and 5, 10 cells in 10 mL blood; lane 6, blank; lanes 7 and 8, 100 cells in 10 mL blood. The filter was hybridised to a P^{32} labelled oligo-probe internal to the CK 20 primers.

8.4.2 RT-PCR for CK 19 and CK 20 on cell lines and tumour samples

In the colon cancer cell lines, all 10 were positive for CK 19 expression with 9/10 being positive for CK 20 (SW48 was negative). In tumour RNA samples, 13/14 were positive for CK 19 expression (the negative sample was positive for CK 20), and 13/14 were positive for CK 20.

8.5 DISCUSSION

In this chapter I have described the development and optimisation of one-stage RT-PCR assays for the detection of 2 markers (CK 19 and CK 20) specific for epithelial cells. Both markers were detectable at the level of 10 epithelial cells (colon cancer cell lines) diluted into 10 mL normal donor blood, equivalent to a sensitivity of 1 cancer cell per 5×10^6 leucocytes. The finding that one of the cell lines and one of the tumour RNA samples was negative for CK 20, with another tumour RNA sample being negative for CK 19, indicated that a panel including both these markers would be necessary in the detection of rare tumour cells in patients' blood samples.

Using reverse-transcription with a two-stage "nested" PCR, Datta et al. (1994) assayed CK 19 transcripts in peripheral blood, bone marrow and stem cell harvests in 34 patients with stage I-IV breast cancer and in 39 control subjects without breast cancer. Four of 19 stage IV patients had detectable CK 19 transcripts in peripheral blood, and 5/6 patients who were histologically negative for tumour cells were positive by this assay. The sensitivity of detection of their assay was 10 breast carcinoma cells per 1×10^6 normal peripheral blood mononuclear cells, a 10 fold lower sensitivity than for my immunobead RT-PCR technique. Their PCR primers were designed to incorporate differences at the 3' ends between the CK 19 cDNA and its known processed

pseudogene, thus avoiding amplification of the pseudogene from any DNA present in the sample, which would invalidate the results. In breast cancer patients, 6/8 BM samples were positive for CK 19, 4/27 peripheral blood samples were positive, while stem cell harvests from 4/4 patients were negative for CK 19 transcripts. RNA prepared from PB of 10 control patients was negative for CK 19, 28/30 RNA preparations from BM of 15 chronic myeloid leukaemia (CML) patients were also negative, as was RNA from 14 healthy BM donors. The finding of CK 19 positive BM aspirates in 2 CML patients highlights a potential problem with 'nested' RT-PCR and that is detection of illegitimate transcription. Chelly et al. (1989) determined that any gene may be expressed in any cell type albeit at very low levels. This becomes a serious problem when using a highly sensitive assay such as nested PCR which is sensitive enough to detect very low levels of mRNA, regardless of the origin, resulting in false positives. Preliminary enrichment of epithelial cells from blood, however, abolishes the need for nested PCR which also carries a very high risk of cross contamination of samples.

Schoenfeld et al. (1994), using RT-PCR for CK 19 expression in breast cancer, found that after 2 rounds of PCR, the expected CK 19 product was appearing not only in involved lymph nodes but in all 11 nodes from 11 patients without carcinoma. To determine if this was derived from the pseudogene or was in fact low level transcription of CK 19 mRNA, they exploited the presence of a *Hae* II restriction site within the sequence derived from mRNA, but which is absent from the pseudogene sequence. The results showed that the 2 stage "nested" PCR was detecting low level transcription from CK 19 and therefore they concluded that the one stage PCR with Southern hybridisation was the most sensitive and specific test for lymph node micrometastases in breast cancer.

Krisman et al. (1995) reported low specificity of CK 19 RT-PCR as an assay for the detection of haematogenous lung micrometastases. Again, using a nested RT-PCR, they found CK 19 expression in PB MNCs in 25/50 lung cancer patients. The control population of 65 healthy volunteers also showed CK 19 expression in 25/65 (38%) PB MNCs and this frequency was not significantly different from that observed in lung cancer patients. They concluded that CK 19 expression was not a useful marker for lung cancer and questioned its proposed applicability in detecting tumour cells in blood from patients with other epithelial tumours. Their results could once again be explained by detection of low level "illegitimate" transcription.

Burchill et al. (1995) also used RT-PCR to detect CK 19 and CK 20 transcripts from epithelial cells in peripheral blood. They extracted total cellular RNA from the blood samples and reported a detection sensitivity of 100 epithelial cells (HT29 colon carcinoma cell line) per mL of blood which is 10 times less sensitive than our immunobead RT-PCR technique. They also concluded that CK 19 expression was not suitable as a marker of tumour cells in blood as they found that both CK 8 and CK 19 were expressed in a high proportion of normal peripheral blood samples (88% and 40% respectively), whereas CK 20 expression was not detected in any of 15 normal blood samples or 6 normal bone marrows. The CK 19 results could be explained in their study by amplification of the pseudogene since the primers matched the pseudogene sequence at all but one base and although they had DNase treated their RNA samples prior to reverse-transcription, any remaining DNA present may have accounted for their false positive results in normal control samples.

Gunn et al. (1996) compared detection of CK 19 gene expression by RT-PCR with CK 20 to detect occult metastases in bone marrow and lymph nodes in patients with bowel cancer. CK 19 was detected in 84/109 lymph nodes and 6/15 bone marrows

versus 26/109 lymph nodes and 0/15 bone marrows using CK 20 as the marker. CK 19 however was also detected in 34/40 lymph nodes and 5/12 bone marrows of controls (patients undergoing bowel surgery for benign conditions), whereas CK 20 gene expression was undetectable in any control sample. They concluded that CK 19 lacked specificity, however they too used nested PCR.

Some investigators are prepared to accept a low rate of false positivity in the assay, which is otherwise highly sensitive and specific. Moscinski et al. (1996) used nested RT-PCR and autoradiography for CK 19 expression and found 19/19 cytologically positive bone marrow aspirates positive for CK 19, 3/11 cytologically negative aspirates positive, and 2/52 negative controls positive. Interestingly, the 2 false positive samples were from CML patients as was the case with the false positive of Datta et al. (1994). Investigators in Moscinski's laboratory used CK 19 as a marker to detect occult metastases in bone marrow aspirates from breast cancer patients. The incidence of metastases detected in this way increased from 52% in stage II patients to 82% in stage IV, and there was a significant survival advantage ($p=0.002$) among stage IV patients who were negative for CK 19 expression (Fields et al., 1996).

More recently, Denis et al. (1997) used immunomagnetic bead separation of epithelial cells (based on our published technique) and RT-PCR for CK 8, CK 19 and CK 20 to detect dissemination of tumour cells into the blood in colorectal cancer patients prior to resection. They found that 42 normal control bloods were positive for CK 8 expression whereas they were negative for CK 19 and CK 20 expression. Subsequently, results from CK 19 were used since they found that marker to be more sensitive than CK 20 in PCR experiments. Results of patient blood samples showed CK 19 expression in 12/23 patients including 2 with early stage disease (Dukes' stage A or B).

Soeth et al. (1996) showed that detection of CK 20 RT-PCR positivity in bone marrow aspirates from CRC patients was related to the stage of the disease. None of 20 marrows from control patients were positive. A subsequent study comparing detection of disseminated colon cancer cells in bone marrow (BM) or blood using RT-PCR for CK 20 showed a higher detection rate in BM (35% versus 17%). Survival analysis of 65 CRC patients showed reduced survival in the BM CK 20 positive patients compared to BM negative patients (Soeth et al., 1997). Weitz et al. (1998) reported the use of CK 20 RT-PCR to detect colorectal cancer cells in 24/58 including 6/7 patients with liver metastases. Their limit of sensitivity was determined to be 10 cancer cells per mL of blood which is the same as our immunobead RT-PCR technique. They have not done long term follow up of the patients to determine the significance of detecting cancer cells in peripheral blood.

From these studies, it was concluded that by using a one-stage PCR, rather than nested PCR, with Southern hybridisation and autoradiography, detection of CK 19 and CK 20 transcripts by RT-PCR are appropriate markers for colorectal carcinoma cells in a haematopoietic environment. The results of immunobead RT-PCR using CK 19 and 20 as markers for epithelial cells in patients' blood samples are described in Chapter 10.

CHAPTER 9

MUCIN GENE EXPRESSION

AS A MARKER OF

EPITHELIAL CELLS

9.1 INTRODUCTION

Mucins are high molecular weight glycoproteins characterised by carbohydrate sugars attached via "O-glycosidic" linkages to serine or threonine. Mucins are synthesised by a variety of epithelial tissues as membrane-bound or secreted proteins and are the major component of mucus. Mucin (MUC) 1 and 2 in the plasma membrane of colon cancer cells are characterised by over-expression of the terminal tetrasaccharide residue sialyl-Lewis x, and this has been associated with metastatic potential (Irimura et al., 1991). Excessive and abnormal mucin synthesis has been observed in a number of cancers including breast and colorectal cancer (reviewed in Verma, 1994).

The human MUC 1 gene codes for a mucin glycoprotein PEM (polymorphic epithelial mucin) which is expressed by most glandular epithelia and which shows increased expression in the lactating breast. The gene and its expressed product (also designated MUC 1, episialin and CA 15-3 antigen) show a high degree of polymorphism due to the presence of different numbers of tandem repeats. The gene was first cloned from breast by Gendler et al. (1990), who deduced the complete amino acid sequence and showed the mucin to be a trans-membrane protein with a large extracellular domain of 20 amino acid tandem repeats and a cytoplasmic tail of 69 amino acids. MUC 1 is one of a family of mucin genes, each of which contains a variable number of tandem repeats that differ in size and sequence (Verma, 1994 and references therein).

Although PEM is expressed on both normal and malignant epithelial cells, the carbohydrate side chains of the cancer-associated mucin may be shorter, resulting in the expression of new carbohydrate epitopes and in the exposure of novel epitopes in the core protein. Changes to the surface carbohydrate structures may result in altered cell adhesion, avoidance of immunological destruction, and increased invasive capability

(reviewed in Verma, 1994). Other workers demonstrated that glycopeptides of malignant cells were excessively sialylated as well as having increased branching of the oligosaccharide chains and that these features were associated with tumour progression and increased metastatic potential (Santer et al., 1989; Fernandes et al., 1991).

MUC 1 is found in the apical cell membrane of normal breast epithelial cells and is highly expressed in many breast tumours. McGuckin et al. (1995) studied MUC 1 expression using immunohistological analysis and the monoclonal antibody BC2. They found that patients whose tumours showed MUC 1 expression in >75% of tumour cells had significantly poorer disease-free and overall survival ($P<0.5$) and that a high level of MUC 1 expression was significantly correlated to the presence of axillary node metastases and oestrogen receptors.

MUC 1 mRNA is normally expressed in many glandular tissues including the bronchus, breast, uterus, prostate, salivary gland ducts, stomach and pancreatic ducts, but not in the small or large intestine. Colonic adenocarcinomas expressed mucin core peptides and mRNA of all 3 mucin genes examined (MUC 1, 2, 3). The MUC 1 epitopes, detected by monoclonal antibodies DF3 and 139H2, were strongly expressed in 86% and 71% of colon tumours respectively, while MUC 2 epitopes detected by HFB and MRP were positive in 79% and 100% of tumours respectively and at much greater staining intensity. Using Northern blot analysis, MUC 1 mRNA levels were low in both colon tumours and normal colon, while MUC 2 mRNA levels were detected in both at 10-100 fold higher levels (Ho et al., 1993).

A study aimed at defining the relationship between mucin production in colon cancer cells and metastatic ability was undertaken by Bresalier et al. (1991). They used the poorly metastatic parental cell line LS174T and its variant LS LiM 6, which has high liver metastasising ability in a nude mouse model, and showed that LS LiM 6 in culture

produced 2 fold more intracellular mucin and secreted 4-5 fold more mucin into the media than the poorly metastatic parental line. They also showed that mucin polyA+ mRNA, detected by Northern blot hybridisation, was proportionally increased in LS LiM 6 compared to the parental cell line. They concluded that these results provided further evidence that mucin expression is associated with colorectal cancer metastasis.

9.2 RT-PCR FOR MUCIN 1

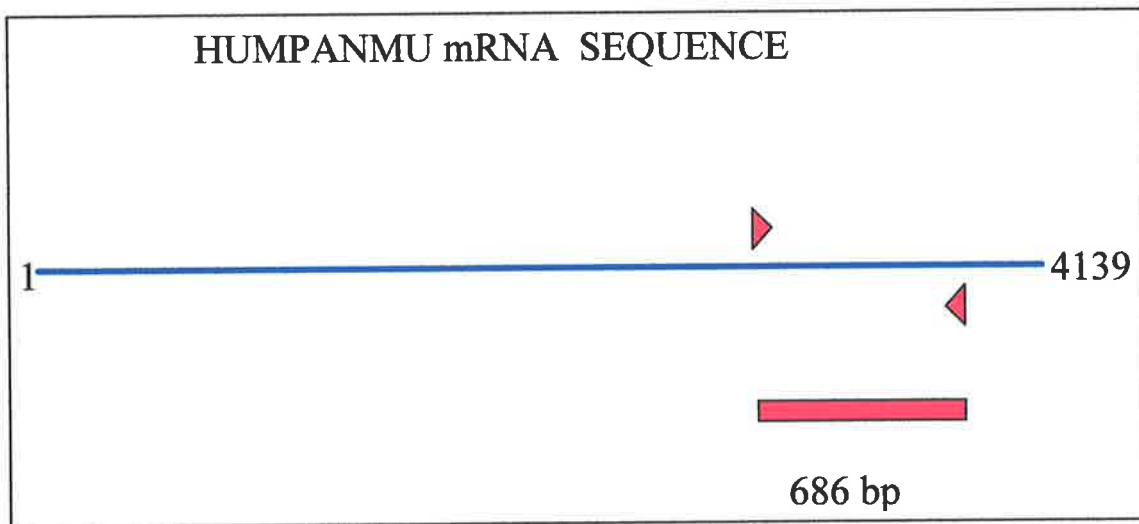
9.2.1 Design of primers and RT-PCR optimisation

The sequence for human pancreatic mucin 1 mRNA was obtained from Genbank. The sense primer was designed to span the intron junction between exons 5 and 6 (i.e. 12 bases of the primer are within exon 5, with the following 12 bases within exon 6) thus avoiding amplification of contaminating genomic DNA sequence. The primer sequences were selected using the program "*Primer*" with the criteria of a T_m of $>60^{\circ}\text{C}$ and matched as closely as possible for GC content (about 50%). The primers were then checked for the presence of other annealing sites using the program "*Amplify*".

Table 9.1 Oligonucleotide sequences

Primer	Sequence 5'-3'	T_m $^{\circ}\text{C}$
MUC 1 sense (3101)	ACCAAGACTGATGCCAGTAGCACT	64
MUC 1 antisense	ACCGTTACCTGCAGAAACCTTCT	63
MUC 1 internal probe	CTATGAGAAGGTTTCTGCAGG	60
MUC 2 sense	TGGCTGCGTGGTGGAGAAGGAA	71
MUC 2 antisense	TTGGAGCAGGTGACGCCCGTAGT	71
MUC 2 internal probe	TCAAGGTGGACTGCAATACCT	59

Figure 9.1. Mucin 1 PCR product and primer binding sites



A single band of 686 bp was obtained using the primers MUC 1 3101 sense and MUC 1 antisense (from the program *Amplify*). HUMPANMU=human pancreatic mucin

The program *Blast* (NCBI Blast network service) was also used to determine annealing sites elsewhere in the genome. All 7 sequences which were 100% homologous for the sense primer were in human epithelial mucins, including mucin 1. The antisense primer showed a probability of binding of 0.99 with exon 7 mucin 1 sequence (for which it was designed) and the genomic sequence for human polymorphic epithelial mucin (HUMPEM) as was expected. No other homologous binding sites were found where the primers could amplify a product, indicating that this primer pair were specific for MUC 1.

Initially, an annealing temperature of 55°C was used, however this resulted in some additional non-specific bands being produced. The annealing temperature was increased to 61°C, which was 2°C below the T_m of the antisense primer, but no amplification was obtained. Finally an annealing temperature of 58°C with a 2 minute extension at 72°C was used. These PCR conditions (1 min 94°C, 1 min 58°C, 2 min 72°C, 45 cycles) produced a band of the expected size (686 bp) in the positive control cell lines; the DNA controls were negative i.e. no amplification was obtained from genomic

DNA. (Figure 9.3). Products were run on a 1.5% agarose gel, transferred to Hybond N+ membrane and hybridised to radio-labelled mucin 1 internal oligonucleotide (Table 9.1).

9.2.2 Comparison of reverse transcriptase enzymes Superscript II and MMLV

Due to the expense of Superscript II, a comparison of the two was carried out to determine whether MMLV could be substituted without detriment to the sensitivity. RT-PCRs were performed on T47D breast cancer cells which strongly express mucin 1, using 200 units of each enzyme. Products were assessed on an ethidium bromide stained gel.

9.2.3 Comparison of storage conditions of the control cells

To determine the optimal storage conditions of cell-line cells for use as controls in RT-PCR, aliquots of 200 T47D cells were stored in lysis mix either at 4°C or -80°C. RT-PCR for mucin 1 expression was performed on paired aliquots.

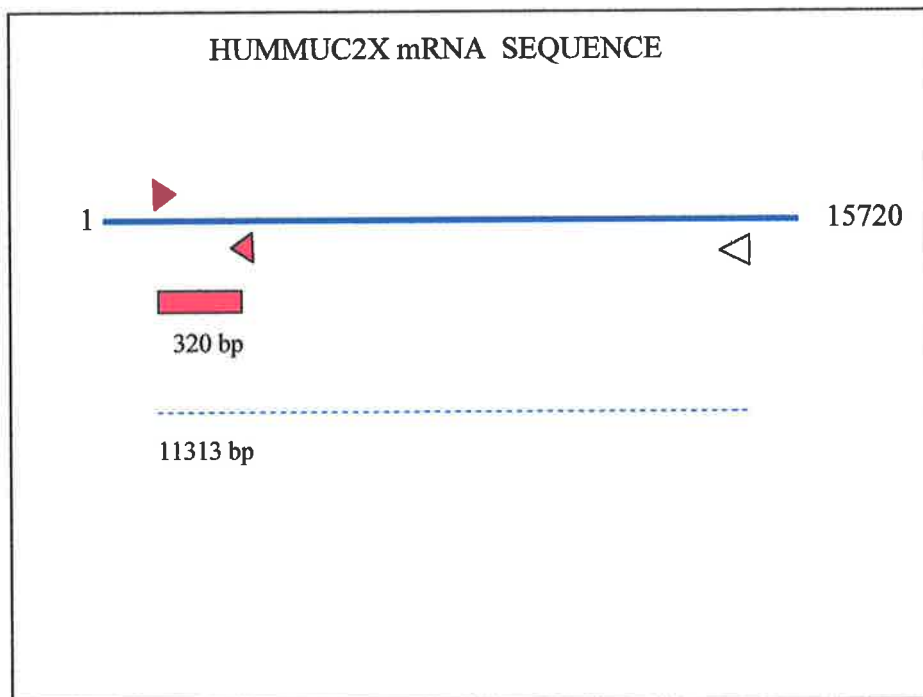
9.3 RT-PCR FOR MUCIN 2

Primers were designed using the same criteria as for mucin 1. Again the *Blast* search did not show binding to sequences other than human mucin sequences. Optimisation of the PCR showed that an annealing temperature of 68°C avoided any non-specific priming and amplification of like sequences. DNA from several different patients was tested in the PCR to ensure the primers did not amplify a product from genomic DNA.

Since the MUC 2 PCR was optimal at a 68°C annealing temperature, it was decided to perform MUC 2 and CK 19 PCRs in the one PCR tube i.e. multiplex PCR,

where both sets of primers are added to the one reaction mix and PCR performed as a means of conserving reagents.

Figure 9.2. MUC 2 PCR product and primer binding sites



The primer pair results in a strong band at 320 bp. The antisense primer also binds downstream to give a theoretical band of 11,313 bp, but which in practice would not result in a product because of its low binding stability and large size of product (from *Amplify*).

Multiplex RT-PCR

RNA extracted from cell lines LIM-1899, LIM-1215, LIM-1863, LIM-2463, and HT29 was reverse transcribed and an aliquot used in multiplex PCR for CK 19 and MUC 2. The products were transferred onto 2 membranes, one of which was hybridised to a radiolabelled CK 19 probe, the other to a radio-labelled MUC 2 probe (Chapter 2.6.4). The results of autoradiography are shown in Figure 9.5 and Table 9.2.

9.3.2 RT-PCR for MUC 1 and MUC 2 on cell lines and tumour samples

The colon cancer cell lines SW48, SW480, HT29, LIM-2412, LIM-1215, LIM-2099, LIM-2405, LIM-1899, LIM-2463, and LIM-1863 were tested by RT-PCR for expression of the two epithelial-specific markers MUC 1 and MUC 2. Fresh tumour was obtained from 14 patients and snap frozen in liquid nitrogen. RNA extraction from cell lines and tumour samples was performed as described in Chapter 2.

9.4 RESULTS

A positive result for mucin 1 RT-PCR was recorded if a band of 686 bp was observed following ethidium bromide staining for the controls. The results of the patients' samples were determined after autoradiography, a positive result being recorded for a band at the same position (686 bp) as in the control lanes (Figure. 9.3)

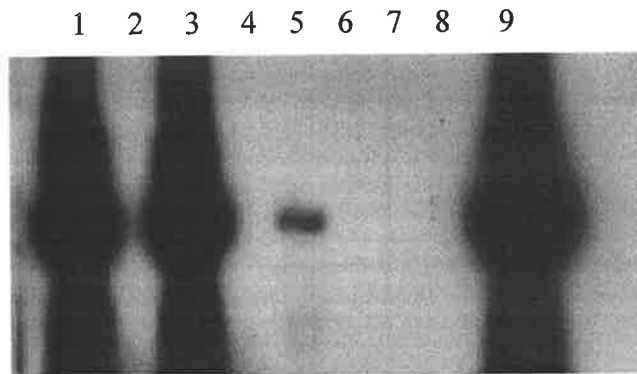
9.4.1 Comparison of reverse transcriptase enzymes Superscript II and MMLV

On comparison of ethidium bromide staining intensity, there did not appear to be a difference between the bands obtained for mucin 1 product using Superscript II or MMLV (Figure 9.4).

9.4.2 Comparison of storage conditions of the control cells

The storage conditions of the lysed cells had a marked effect on the results. Storage at -80°C produced a strong band of the appropriate size (Figure 9.4 lanes 3 and 8) whereas storage at 4°C resulted in no RT-PCR product (Figure 9.4, lanes 4 and 9).

Figure 9.3 Autoradiograph of MUC 1 RT-PCR.



Lane 1, positive control T47D cells; lane 2, negative control (no target); lane 3, positive control LIM-1215 cells; lane 4, patient MH pre-operative PB; lane 5, patient MH post-op PB; lane 6, patient NP pre-op PB; lane 7, patient NP post-op PB; lane 8, genomic DNA control; lane 9, positive control LIM-2405 cells.

Figure 9.4 Comparison of RT enzymes and storage conditions of lysed cells



Lane 1, MW markers; lanes 2-6, Superscript reverse transcriptase; lanes 7-11, MMLV reverse transcriptase; lanes 2 and 7, T47D RNA positive control; lanes 3 and 8, 200 T47D cells stored in lysis mix at -80°C; lanes 4 and 9, same cell mix stored at 4°C; lanes 5 and 10, 200 T47 D cells stored in PBS at -80°C; lanes 6 and 11, negative RT control (no RNA); lane 12, blank; lane 13, positive RT control.

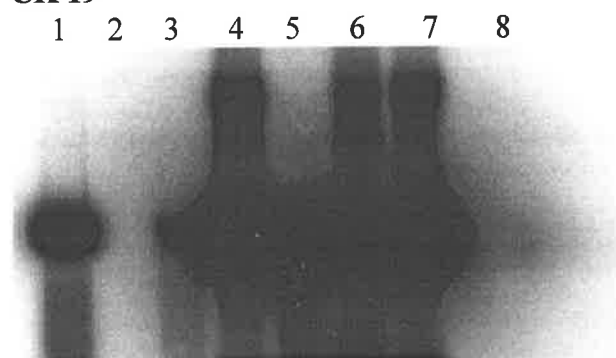
9.4.3 Multiplex RT-PCR for MUC 2 and CK 19

The comparison of the results obtained on an ethidium bromide stained gel are shown in Table 9.2 and Figure 9.5. Differences exist in detection of expression in the cell lines LIM-1899 and LIM-1863, being stronger for CK 19 expression in both cases. Due to this

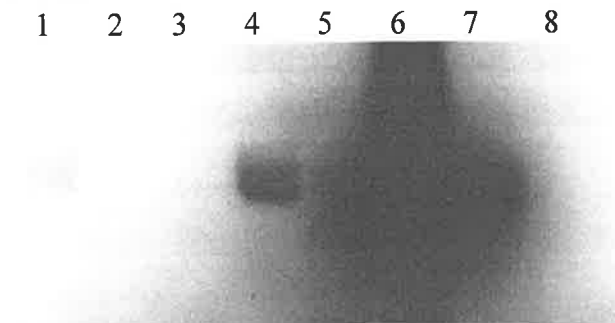
discrepancy, which may have been due to a competitive effect in the PCR tube i.e. the CK 19 PCR may have been more robust and used the Taq polymerase at a faster rate or more efficiently than the MUC 2 PCR, or a true difference in expression, we decided against multiplex PCR for this application. The over-expression of MUC 2 in the cell line LIM-2463, which was derived from adenoma tissue, has been previously reported (Devine et al., 1992).

Figure 9.5. Multiplex RT-PCR

CK 19



MUC 2



Autoradiographs of multiplex RT-PCR products. Blots were hybridised separately to probes for CK 19 or MUC 2. Lane 1, LIM-1899 29/5; lane 2, RT negative control; lane 3, LIM-1899 28/4; lane 4, LIM-1215; lane 5, LIM-1863; lane 6, LIM-2463; lane 7, HT29; lane 8, PCR negative control.

9.4.4 RT-PCR for MUC 1 and MUC 2 on cell lines and tumour samples

In the colon cancer cell lines, 8/10 were positive for MUC 1 expression (LIM 1863 and SW48 were negative), and 8/10 for MUC 2 (SW 48 and LIM 2405 were negative; SW48 was positive for CK 19 expression). In tumour RNA samples 14/14 were positive for MUC 1 and 13/14 were positive for MUC 2 expression. Results of patient blood samples (immunobead RT-PCR) are included in Chapter 10.

Table 9.2. Multiplex RT-PCR for MUC 2 and CK 19

LANE	cDNA SOURCE	CK 19	MUC 2
1	LIM-1899 29/5*	4+	1+
2	RT NEG CONTROL	neg	neg
3	LIM-1899 28/4	2+	neg
4	LIM-1215	4+	3+
5	LIM-1863	4+	1+
6	LIM-2463	4+	4+
7	HT 29	4+	4+
8	PCR NEG CONTROL	neg	neg

Abbreviations: 4+, strong positive, 3+, 2+, intermediate positive, 1+, weak positive; neg, negative. *, date code refers to RNA samples reverse-transcribed at different times.

9.5 DISCUSSION

To date, there has only been one study reported in the literature using RT-PCR for MUC 1 expression as a cancer cell marker. Noguchi et al. (1994) compared immunohistochemistry using SM-3, a monoclonal antibody against polymorphic epithelial mucin, and RT-PCR of MUC 1 to determine the presence of micrometastases in 50 axillary lymph nodes from 15 primary breast cancer patients. Both methods detected metastases in 9 nodes, however RT-PCR was able to detect mucin expression in 6 additional nodes which were determined to be free of metastases by immunohistochemistry. Importantly, MUC 1 expression by RT-PCR was not detected in 10 control lymph nodes (patients with benign disease). Their study showed that RT-PCR was much more sensitive in detecting micrometastases than immunohistochemistry.

There have been no reports of studies using RT-PCR detection of MUC 2 as a marker of carcinoma cells. Ho et al. (1993) used Northern blot analysis to compare the expression of MUC 1, 2 and 3 in many normal and malignant tissues including colon. The results showed that MUC 1 mRNA was present in the highest amount in breast cancer tissue, but MUC 2 was greatly over-expressed in colon tissue from patients with colon adenocarcinomas. MUC 2 expression was absent in both normal and carcinoma tissue from breast, lung, bronchus, gastric, stomach and gall bladder cancer patients.

Our results suggest that MUC 1 and MUC 2 expression are appropriate markers for epithelial cells in blood but due to the fact that some cell lines and tumour samples were negative, they need to be used as part of a panel of markers e.g. together with CK 19 and CK 20.

CHAPTER 10

SIGNIFICANCE OF EPITHELIAL-MARKER POSITIVE CELLS IN BLOOD

10.1 INTRODUCTION

The aim of this study was to investigate the presence of tumour cells in peripheral venous blood of patients undergoing curative resection for colorectal cancer (CRC) and to determine if detection of positive cells was correlated with shortened survival post surgery. My earlier immunobead-PCR pilot study, using a mutation in codon 12 of the *K-ras* gene as a marker of colon tumour cells, found that detection of tumour cells in peripheral blood of CRC patients at diagnosis was strongly predictive of relapse ($p=0.0001$) (Hardingham et al., 1995). This was only applicable in the 35% of our CRC patients whose tumour carried a *K-ras* mutation, so, to include all CRC patients enrolled in the study, epithelial-specific expression was used as a marker for tumour cells disseminated into the bloodstream. Immunobead RT-PCR was performed on peripheral blood samples taken before and after resection to detect epithelial-specific expression of CK 19, CK 20 (Chapter 8), MUC1 or MUC2 (Chapter 9) as a marker of epithelial-derived (carcinoma) cells in blood.

10.2 MATERIALS AND METHODS

10.2.1 Patients and specimens

Ninety six consecutive patients admitted to The Queen Elizabeth Hospital for curative resection for primary CRC were enrolled in the study. The median age of the patients was 72 years (range 32-99) with a median follow-up of 392 days (range 16-1505). Peripheral blood samples (20 mL) were collected in dipotassium EDTA from 81 patients pre-operatively and from 95 post-operatively. Fresh tumour tissue was obtained from 14 patients and snap frozen in liquid nitrogen for RNA extraction. Pre- and post-operative blood samples were also collected from 28 patients undergoing large bowel

resection for adenoma, and from 28 benign controls (patients undergoing resection for Crohn's disease or diverticulitis). The study was approved by the institutional Ethics of Research Committee and informed consent was obtained in all cases. Blood was also obtained from 11 healthy volunteers as negative controls in immunobead RT-PCR.

10.2.2 Positive controls – cell lines

The colon cancer cell lines SW48, SW480, HT29, LIM-2412, LIM-1215, LIM-2099, LIM-2405, LIM-1899, LIM-2463, and LIM-1863 were tested by RT-PCR for expression of the epithelial-specific markers. Positive cell lines were used as controls and to optimise the RT-PCR for each of the markers. Cell lines were maintained in RPMI 1640 with 10% foetal calf serum as described in Chapter 2. Aliquots of cell-line cells (200 cells in a volume of 2 uL PBS) were stored at -80°C as controls. Several aliquots were thawed and included in each RT-PCR. RNA was also extracted from cell lines and tumour samples (Chapter 2) and used as positive RT-PCR controls.

The primers to amplify CK 19, CK 20, MUC 1 and MUC 2 have been described in detail in Chapters 8 and 9. The primer sequences are shown in Table 10.1.

10.2.3 Immunobead RT-PCR

Immunobead isolation of epithelial cells from blood has been described in detail in Chapter 4, and optimisation of RT-PCR for the cytokeratin and the mucin markers was described in Chapters 8 and 9 respectively. Briefly, each 20 mL blood sample was processed as it arrived in the laboratory. The blood was transferred to polystyrene tubes as these did not pose a problem with electrostatic forces interfering in the magnetic isolation step. Following incubation with the Ber-EP4 coated-immunobeads, captured cells were isolated using a magnetic field. Cell/bead pellets were resuspended in 15uL

of lysis mix containing RNase inhibitor and stored at -80°C awaiting batch RT-PCR assays and autoradiography.

10.2.4 Statistical analysis

Survival distributions were estimated using the product-limit method of Kaplan and Meier (1958) and the log-rank test (Peto and Peto, 1972) was used to test significance.

Table 10.1. Sequences of primers and internal probes

Primer/probe	Sequence 5'-3'
CK 19 sense	GACTACAGCCACTACTACACGACC
CK 19 anti-sense	AGCCGCGACTTGATGTCCATGAGCC
CK 19 internal probe	GATCTGCATCTCCAGGTCGGTCC
CK 20 sense	TCTTTGATGACCTAACCTAC
CK 20 anti-sense	CTACTTCTTGACGACTGTCTTA
CK 20 internal probe	AGCTCCGTTAGTTGAACCTCA
MUC 1 sense	ACCAAGACTGATGCCAGTAGCACT
MUC 1 anti-sense	ACCGTTACCTGCAGAAACCTTCT
MUC 1 internal probe	CTATGAGAAGGTTTCTGCAGG
MUC 2 sense	TGGCTGCGTGGTGGAGAAGGAA
MUC 2 anti-sense	TTGGAGCAGGTGACGCCCCTAGT
MUC 2 internal probe	TCAAGGTGGACTGCAATACCT

10.3 RESULTS

10.3.1 Expression of epithelial-specific markers in tumour samples and cell lines

Tumour RNA samples were tested for expression of each of the markers by RT-PCR: 13/14 were positive for CK 19 (the negative sample was positive for CK 20), 13/14 were positive for CK 20, 14/14 were positive for MUC 1 and 13/14 were positive for MUC 2 expression. In the colon cancer cell lines, all 10 were positive for CK 19 expression, 9/10 for CK 20 (SW48 was negative), 8/10 for MUC 1 (LIM-1863 and SW48 were negative), and 8/10 for MUC 2 (SW48 and LIM-2405 were negative). The inability to amplify CK 19, CK 20 or mucin transcripts in some tumours and cell lines, despite successful amplification of other markers using the same cDNA, indicated that a panel of markers was necessary. Previous dilution experiments showed that the sensitivity of detection of cell-line cells was at the level of 10 cells per 10 mL blood sample (1 cell per mL, equivalent to 1 cell per 5-10 million white cells) (Chapter 8).

10.3.2 Immunobead RT-PCR analysis of blood samples

Overall 29/96 (30%) CRC patients were positive for epithelial RT-PCR markers in blood. Five of these were staged as Dukes' A, 8 as Dukes' B and 16 as Dukes' C (Table 10.2). All samples were tested for at least 2 markers. Fewer samples were tested for MUC 1 as this marker was added to the panel at a later date. Table 10.3 shows the frequency of positive markers in pre- and post-operative blood samples from CRC patients. The overall frequency of positive markers in blood samples is shown in Table 10.4. The observed frequency of positivity was similar for CK 19, MUC 1, and MUC 2, while being less for CK 20. Examples of the results of immunobead RT-PCR for CK 20 and MUC 2 are shown in Figures 10.2 and 10.3 respectively.

Table 10.2. Patients positive for epithelial cells in blood

Dukes' stage	Pre-operative (n=81)	Post-operative (n=95)	No. patients positive
A (n=21)	2	5	5 (24%)
B (n=42)	3	7	8 (21%)
C (n=33)	11	11	16 (48%)
Total	16	23	29 (30%)
Adenoma (n=28)	3	4	6 (21%)
Benign (n=28)	1	4	4 (14%)

In pre-operative blood samples from CRC patients, 16/81 (20%) were positive for one or more epithelial markers, including 2 patients with Dukes' A and 3 with Dukes' B disease (Table 10.2). Kaplan-Meier survival analysis was performed using disease recurrence, development of metastases or death from disease as end-points. Data from 2 patients dying from other causes or from 3 patients lost to follow-up were treated as censored observations. The log-rank test was used to compare survival curves of patients positive or negative for colon cells in blood. There was a significant difference between the two groups, those positive for epithelial markers showing reduced time to relapse or death from disease ($p=0.001$, hazard ratio 3.7, 95% CI 2.05-17.96) (Figure 10.1A). Among adenoma patients 3/28 were positive in pre-operative blood, while 1/28 benign controls was positive (Table 10.2).

Table 10.3. Frequency of positive markers in blood samples

Epithelial markers	Blood samples	
	Pre-operative	Post-operative
CK 19	*9/70 (13%)	11/86 (13%)
CK 20	5/57 (9%)	4/68 (6%)
MUC 1	2/21 (10%)	5/29 (17%)
MUC 2	4/56 (7%)	9/64 (14%)

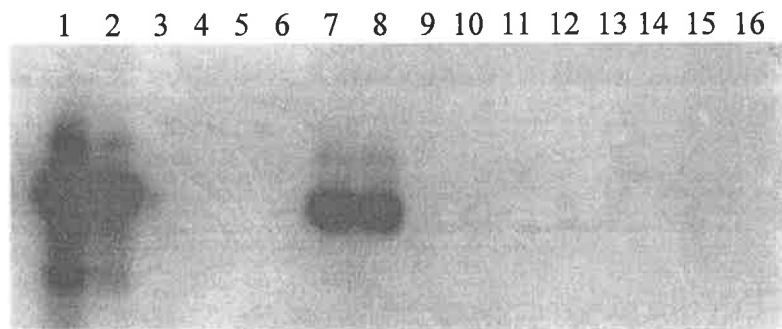
*Number of samples positive/number tested

Figure 10 2. Immunobead RT-PCR for CK 20



Lanes 1, 2, LIM-2463, 1215 (200 cells each); lane 3, RT negative control; lane 4, PCR negative control; lanes 5-16, patients' blood pre- and post-operative; lanes 17, 18 positive control cell lines LIM 2412, 1215. Note the positive signal in lane 13 from a pre-operative blood sample.

Figure 10.3. Immunobead RT-PCR for MUC 2



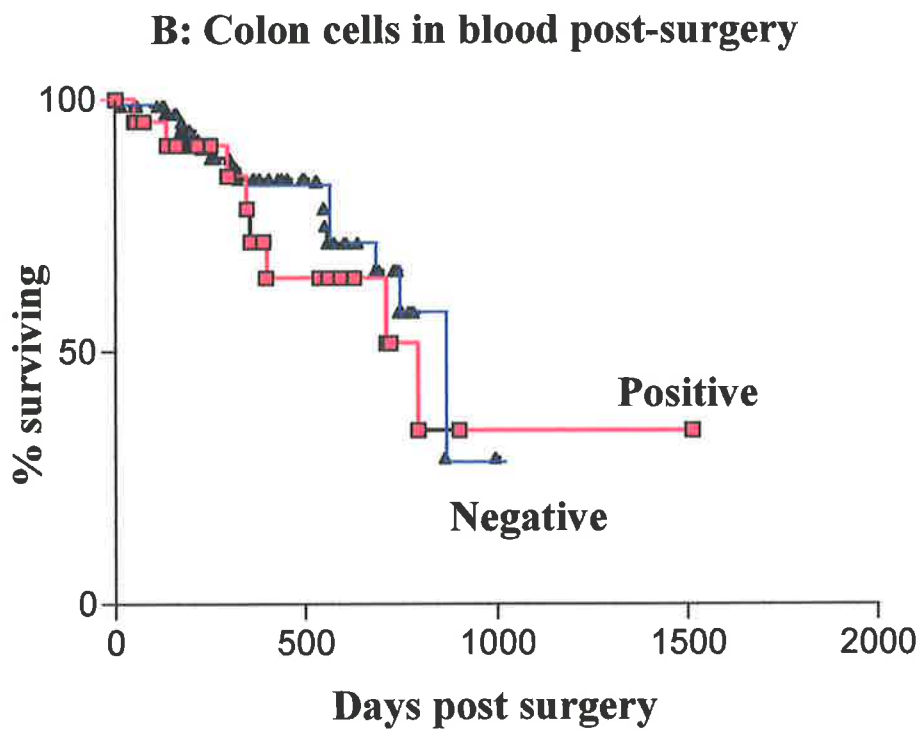
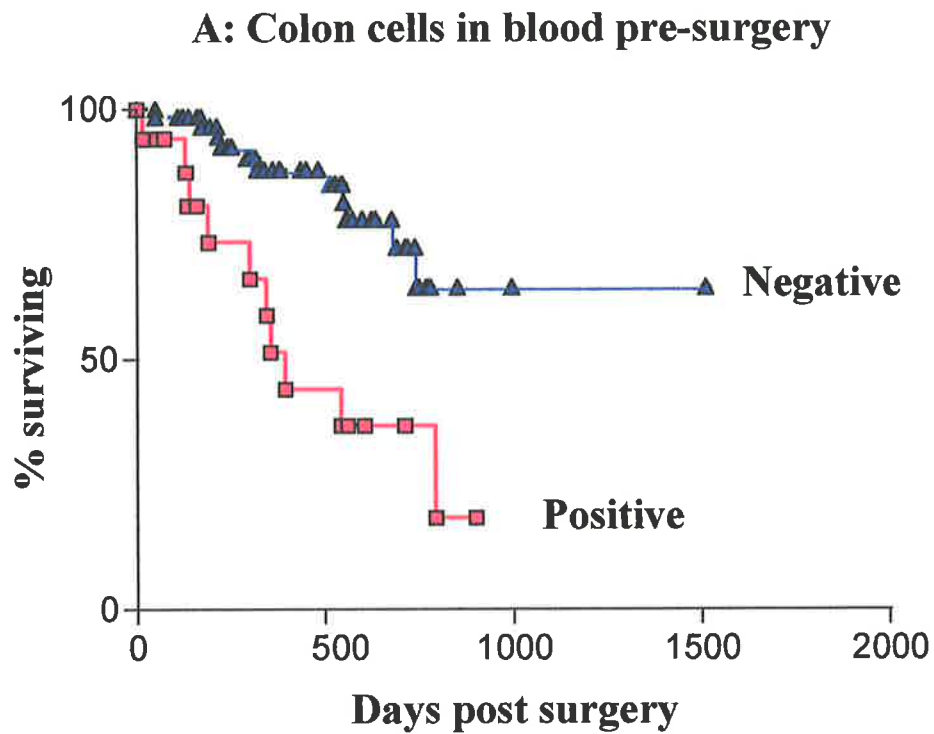
Autoradiograph of immunobead RT-PCR for MUC 2 from pre and post-operative PB samples: Lanes 1-2, RT-PCR positive control cell lines HT29, LIM 1863 (200 cells each); lanes 3, 4, negative controls; lanes 5-16 patients' pre and post-operative blood samples. Note the positive signals in lanes 7 and 8 (pre- and post-operative blood from a patient).

To investigate the question of whether tumour cells are shed into the bloodstream during colorectal resection, post-operative peripheral blood samples were analysed from patients undergoing resection for CRC (n=95), adenoma (n=28), and benign conditions (n=28). Twenty three of 95 (24%) CRC patients were positive in post-resection blood (12 with early stage disease). Four of 28 (14%) adenoma patients were positive post-surgery, one of whom was also positive in pre-resection blood, while 4/28 benign bowel resection controls were positive post-resection (Table 10.2). In cases of CRC in which both pre- and post-operative blood was obtained, 6/79 (1 Dukes' B, 5 Dukes' C) were positive before but negative post-resection, 10/79 (2 Dukes' A, 3 Dukes' B, 5 Dukes' C) were positive post-resection but negative pre-resection, while another 10 were positive in both samples (2 Dukes' A, 2 Dukes' B, 6 Dukes' C). None of 11 normal (non-surgical) volunteers were positive for epithelial markers in peripheral blood by immunobead RT-PCR. Kaplan-Meier survival curves comparing CRC patients positive or negative for epithelial markers in the blood post-operatively showed no difference in survival between the two groups (Figure 10.1B).

Table 10.4. Overall frequency of positive markers

RT-PCR marker	No. samples tested	No. positive (%)
CK 19	158	19 (12)
CK 20	126	9 (7)
MUC 1	50	7 (14)
MUC 2	112	14 (13)

Figure 10.1. Kaplan-Meier survival analysis



Comparison of survival data from patients positive or negative for colon cells in blood: A, pre-operative blood, $p=0.001$; B, post-operative blood, NS.

10.4 DISCUSSION

We have used the sensitive technique of immunobead RT-PCR, which is capable of detecting 1 tumour cell per 5-10 million white blood cells, to detect epithelial cells in peripheral blood before and after surgical resection for colorectal malignancy. In this study the presence of epithelial-marker positive cells in pre-operative peripheral blood correlated with reduced disease-free survival ($p=0.001$), suggesting that at least some of the cells detected were metastatic colon carcinoma cells in transit in the circulation. Only a very small percentage of carcinoma cells that intravasate into the bloodstream from the primary tumour are able to form metastases since most succumb to host immunological responses or entrapment in the microvasculature (Weiss, 1986). In post-operative blood analyses we found no difference in survival between patients positive or negative for the markers. This was probably due to the fact that the epithelial-specific markers used did not discriminate between tumour cells and normal colon cells that may have been shed into the circulation during surgery.

In patients from whom both a pre- and post-operative blood sample was obtained, 10/79 were positive in both samples (2 Dukes' A, 2 Dukes' B, 6 Dukes' C). One of the Dukes' A patients died of operative complications at day 54, while the other was lost to follow-up. Of the Dukes' B cases, one was disease-free at day 74 post-surgery, while the other suffered recurrent rectal carcinoma at day 159 post-surgery. All 6 of the Dukes' C patients developed recurrent or metastatic disease at median day 355 (range 135-793 days).

Interestingly, 6/79 patients (1 Dukes' B, 5 Dukes' C) were positive in pre-resection blood but negative in post-resection blood suggesting that removal of the primary tumour had removed the source of circulating tumour cells. Some of these

circulating cells had already seeded a metastatic deposit since all 5 of the Dukes' C patients developed metastatic disease at median day 191 post-surgery (range 16-542 days); the Dukes' B patient remained disease-free at 2 years post-resection. In comparison, 6/16 stage C patients who were negative in pre-operative blood developed a recurrence or metastatic disease at median day 532 (range 225-681 days). Ten of 79 patients (2 Dukes' A, 3 Dukes' B, 5 Dukes' C) were positive in post-resection blood but negative in pre-resection samples suggesting that epithelial cells (tumour or normal) had been released into the circulation during surgery. Some of the discrepant results between pre-and post-operative blood samples may simply reflect the sporadic nature of tumour cell shedding (Glaves, 1983) so that sampling effects come into play, or that the level of cells was at the lower limit of detection.

Among adenoma patients, 3 were positive in pre-resection blood: the first of these had multiple tubular adenomas and was also positive post-resection, the second had undergone a needle biopsy of a prostate carcinoma the day before colorectal surgery so the cells detected may have been prostatic in origin, and the third had a moderately dysplastic polyp removed trans-anally. An additional 3 adenoma patients were positive in post-resection blood: one patient also had chronic inflammatory changes consistent with Crohn's disease, one had recurrent adenoma in the rectum and one had an anterior resection for tubulovillous adenoma with no further recurrences to date. The positive pre-operative blood results indicate that adenomas may also spontaneously shed cells into the circulation. The benign control patient who was positive in pre-operative blood had diverticulitis with severe inflammation. Perhaps severe inflammatory changes and associated ulceration provides a means whereby normal colonic mucosal cells gain access to the bloodstream. Angiogenesis associated with tumours may also facilitate entry of tumour or normal cells into the circulation. A positive association has been

found between the extent of tumour angiogenesis and tumour cell shedding into effluent venous blood during breast cancer surgery. This study however did not include benign control patients (McCulloch et al., 1995). Tumour angiogenesis has also been found to be a predictor of recurrence and survival in patients with early stage breast (Weidner et al., 1992) and colon cancer (Frank et al., 1995). The suggestion is that the newly formed vessels facilitate entry of tumour cells into the circulation which may contribute to later recurrences and metastatic disease (reviewed in Woodhouse et al., 1997).

Surgical manipulation of the tumour may also play a role in tumour dissemination. One of the earliest studies, reported in 1955 by Fisher and Turnbull, found tumour cells in mesenteric blood during surgery for CRC in 8/25 cases, including 1/5 lesions classified as Dukes' stage A. This study was the basis from which they developed the "no-touch isolation" technique for colorectal resection, where preliminary venous ligation was used to avoid the possibility of dissemination of tumour cells. More recently it has been suggested that manipulation of the prostate during radical prostatectomy results in haematogenous dissemination of tumour cells which may explain the metastatic progression of some organ-confined prostate carcinoma after surgery (Eschwege et al., 1995).

Few other studies have correlated the finding of colon tumour cells in peripheral blood with prognosis. Recently, a study of 37 CRC patients, using CK 20 as a marker for tumour cells in bone marrow and blood, found significantly reduced survival in the bone marrow positive group compared to the negative group. No such difference was observed for patients positive or negative for tumour cells in blood (Soeth et al., 1997). Another study used RT-PCR to detect carcinoembryonic antigen (CEA) mRNA in peripheral blood samples from 27 patients with CRC. Follow-up and survival analysis showed that the frequency of cancer recurrence was significantly different between

CEA mRNA negative and positive groups (Mori et al., 1998). Our study has shown, in a larger group of CRC patients, that the presence of epithelial marker-positive cells in peripheral blood before surgical resection is predictive of shortened time to recurrence and overall survival ($p=0.001$). Recruitment of more patients and longer follow-up will be needed to determine if the presence of tumour cells in blood is independent of Dukes' stage as a prognostic factor.

Epithelial-specific markers were deemed appropriate for analysing pre-operative blood for the presence of disseminated tumour cells as there was no evidence in the literature to suggest that normal colon cells have access to the bloodstream in the absence of colorectal resection. I have now shown that non-malignant colonic mucosal cells may also be shed into the circulation both spontaneously, as was the case in 3 patients with adenoma and 1 patient with diverticulitis, and as a result of bowel surgery for these conditions.

CHAPTER 11

METASTASIS-ASSOCIATED MARKERS FOR CARCINOMA CELL DETECTION

11.1 INTRODUCTION

One of the questions I was addressing in this project was whether or not colon cancer cells were released into the bloodstream as a result of colorectal resection for malignancy. From the results in the preceding chapter, it was noted that normal colonic mucosal cells were gaining access to the bloodstream during bowel resection, in both malignant and non-malignant conditions. This was apparent in CRC patients in that the presence of epithelial cells in post-operative blood had no apparent effect on survival, whereas the detection of epithelial cells in pre-operative blood correlated strongly with shortened disease-free survival. The discrepancy in these findings can be explained on the basis that the epithelial cells detected pre-operatively were malignant cells in transit in the circulation i.e. micro metastatic disease was present, whereas the cells detected in post-operative blood in these patients included normal colon cells released on their own, or concomitantly with cancer cells as a result of surgical manipulation of the bowel. Therefore markers were needed that were able to distinguish between colon cancer cells and cells from the normal mucosa.

One possibility was to investigate the use of markers that were greatly over-expressed in tumour cells relative to their normal counterparts as a result of the acquisition of a metastatic phenotype. Metastasis, the final stage of CRC progression, involves multiple tumour-host interactions, and is the result of an accumulation of genetic changes, including up-regulated expression of genes whose products are involved in one or more aspects of metastasis. The importance of molecules involved in the multiple steps of metastasis have been discussed in Chapter 1. Three candidate markers potentially useful as expression markers of carcinoma cells are described in more detail below.

11.1.1 γ 2 chain of laminin-5

The γ 2 chain of laminin-5, a member of a family of trimeric adhesion molecules involved in epithelial cell-basement membrane adhesion, was found to be preferentially expressed in invading malignant cells in human colon and breast. The localisation of expression of markers is important as some of them e.g. MMP-9 (type IV collagenase) have been shown to be of macrophage origin in colorectal cancers (Pyke et al., 1993). In contrast only a weak signal could be detected in the luminal epithelial cells of normal colon mucosa, while six sarcoma specimens were negative (Pyke et al., 1994). The tumour cells had pseudopodial projections, referred to as 'tumour budding', which are the means whereby tumour cells migrate through tissue (Hase et al., 1993). The correlation between a "budding" cell, with concomitant expression of laminin-5 γ 2 and urokinase plasminogen activator suggested that laminin-5 γ 2 was involved in the invasive cell phenotype (Pyke et al., 1995).

Two different cDNA clones for the laminin γ 2 chain have been isolated, due to alternative splicing which results in chains differing in their carboxyl terminal ends. The longer transcript was strongly expressed in all epithelia in embryonic tissue, whereas the shorter had restricted distribution, in cerebral cortex, lung and kidney (Airenne et al., 1996). Pyke et al. (1995) determined that only the longer transcript was present in tumour samples. This sequence corresponds to the GenBank sequence Z15008 lodged by Kallunki et al. (1992).

In summary, the consistent finding of laminin γ 2 expression in invading carcinoma cells suggests an important function of this molecule in establishing a contact between the invading cells and a provisional matrix. As such it would appear to be a good marker of malignant cells.

11.1.2 Matrilysin

To accomplish invasion, a tumour cell must induce local proteolysis of the ECM either by expressing proteolytic enzymes itself, or by stimulating the tumour stromal cells to express them. Matrilysin (MAT), a member of the matrix metalloproteinases, is constitutively expressed in normal glandular epithelial tissue and it is thought that this enzyme acts to maintain the patency of the glandular lumen (an enzymatic 'pipe cleaner' -Saarialho-Kere et al., 1995). Matrilysin has been found to be over-expressed in adenoma and carcinoma tissue in breast, colorectum, stomach, prostate and lung, and in squamous cell carcinoma of lung and skin (reviewed in Wilson and Matrisian, 1996). Powell et al. (1993) showed that when the non-metastatic prostatic cell line DU-145 was transfected with human MAT cDNA, it became significantly more invasive in an *in vivo* model of tumour invasion. Interestingly, this finding did not hold for the colon tumour cell line SW480. In this case, transfection and over-expression of matrilysin resulted in an increase of tumorigenicity when injected into nude mice, rather than an increase in invasive potential (Witty et al., 1994).

11.1.2.1 Localisation of expression of matrilysin

Northern analysis of tumour tissue has detected MAT mRNA in the majority (>75%) of breast, gastric, colon and prostate carcinomas. In colorectal tumours, MAT mRNA was detected in 6/8 carcinomas. *In situ* hybridisation localised the mRNA specifically to the tumour cells in stomach, colon and prostatic neoplasms, rather than to the surrounding inflammatory cells or stromal cells (McDonnell et al., 1991) as has been the case for other MMPs (Newell et al., 1994; Heppner et al., 1996). Mori et al. (1995) used Northern blot analysis to show that expression was greater in carcinoma tissue than in paired normal mucosa in 39/47 cases. The expression level was also found to correlate

positively with increasing Dukes' stage, with the highest expression detected in metastatic liver lesions. Itoh et al. (1996) examined the level of matrilysin protein expression and showed that the level of secreted matrilysin was higher in cancer tissue than in adenoma, but was undetectable in hyperplastic polyps, normal colonic mucosa and mucosa from ulcerative colitis.

Using RT-PCR, expression of MAT was found in 9/10 colorectal adenocarcinomas, whereas none was detected in adjacent normal mucosa, nor in mucosa from 3 patients with ulcerative colitis. Interestingly, only 1/6 colon cancer cell lines tested was positive by RT-PCR. This cell line was BM314 (Yoshimoto et al., 1993). The results from the cell lines suggest that the tumour microenvironment plays a role in expression of matrix degrading enzymes as was first suggested by Nakajima et al. (1990). Matrilysin expression in particular has been shown to be induced by fragmented fibronectin or RGD containing peptides, RGD being the recognition amino acid sequence for matrix metalloproteinases (Yamamoto et al., 1994).

Thus, from the available evidence, matrilysin expression would appear to be a useful diagnostic marker for colon carcinoma cells. One possible down-side to its specificity for cells of epithelial origin is that it is also expressed by mononuclear phagocytes involved in severe inflammatory reactions such as cystic fibrosis. Basal expression of matrilysin in normal blood monocytes in culture was either non-existent or negligible, however, its expression was induced by exposure of the cells to LPS (lipopolysaccharide), a bacterial endotoxin (Busiek et al., 1995). Perhaps activated mononuclear phagocytes present in the tumour stroma may also express matrilysin, however the use of immunobead RT-PCR with the prior isolation of epithelial cells from blood would substantially reduce contamination by such cells.

11.1.3 Metastasis-associated gene *mta-1*

Toh et al. (1994) isolated a novel candidate metastasis-associated gene by differential screening of a cDNA library of metastatic and non-metastatic rat mammary carcinoma cell lines. In this system gene expression was compared between the non-metastatic cell line MTC.4 and the highly metastatic one MTln3. Ten clones were found to be differentially expressed and two of these were unrelated to known genes in GenBank. One, which they named *mta-1*, was sequenced and was predicted to encode a 703 amino acid protein. Several phosphorylation sites, a zinc-finger motif, and a proline-rich stretch at the carboxy-terminal end that completely matched the consensus sequences for the *src*-homology 3 domain-binding site, indicated that this protein may be involved in signal transduction. Further evidence for this function was obtained from *mta-1* antisense oligonucleotide inhibition of the growth of metastatic breast cancer cells in culture. Such an inhibitory effect was not seen in non-metastatic breast cancer cells (which had low expression of *mta-1*). The data suggest that the *mta-1* gene product may function in a signal transduction pathway important in the growth and progression of cancer cells (Toh et al., 1997).

11.1.3.1 Over-expression of the human *MTA-1* gene in carcinomas

Northern blot analyses of human breast cancer cell lines showed that expression of MTA-1, the human homologue of the rat *mta-1* gene, was greatest in the metastatic variant cell line MCF7/LCC2, intermediate in the invasive variant MCF7/LCC1, and low in the non-metastatic parent line MCF7, in a ratio of 4:2:1. Similarly, expression of the MTA-1 gene in the metastatic cell line MDA-MB-231 was 4 fold that of the non-metastatic MDA-MB-468 cells (Toh et al., 1995).

Using RT-PCR, expression of MTA-1 was examined in 36 colorectal carcinomas and 34 gastric carcinomas, relative to their matched normal mucosa. Over-expression (ratio >2:1) was found in 14/36 colorectal, and in 13/34 gastric carcinomas and correlated with more advanced stage i.e. deeper wall invasion and a higher rate of lymph node metastasis (Toh et al., 1997).

11.2 ASSESSMENT OF MARKERS

11.2.1 Expression of markers in tumour tissue and matched normal mucosa

Ideally, the selected marker would show marked over expression in tumour tissue while being negative for expression in the matched normal mucosa. Expression of laminin γ 2, MAT and MTA-1 was assessed using RT-PCR and Northern Blotting.

11.2.1.1 Selection of primers and assessment of candidate markers by RT-PCR

Primers were designed from the published mRNA sequences for laminin γ 2 (GenBank accession number Z15008), matrilysin (Z11887) and MTA-1 (U35113). Primer sequences were selected using the programs "Amplify" and "Primers for the World Wide Web" using the criteria of primer length between 20-24 bases, primer pairs to have similar melting temperatures (Tms), and approximately 50% GC content. Probe sequences were chosen internal to the primer binding sites. Primer and probe sequences are shown in Table 11.1.

RT-PCRs for each of the markers were performed on the colon cancer cell lines SW480, SW620, HT29, LIM-2412, LIM-1215, LIM-2099, LIM-2405, LIM-1899, LIM-2463, and LIM-1863 for optimisation of PCR conditions and to assess expression. Cells (approximately 200) were lysed in lysis buffer (Chapter 2.4.4) and freeze-thawed prior

to reverse transcription. PCR was initially carried out under standard conditions (Chapter 2.4.3). Optimisation experiments, varying the annealing temperature in each PCR, showed that the best results were obtained for each marker at the following annealing temperatures: laminin γ 2 68°C for 1 minute, matrilysin and MTA-1 both at 62°C for 1 minute. All 3 PCRs were carried out for 45 cycles. Genomic DNA controls were included in each PCR to ensure that the primers did not amplify a product from contaminating genomic DNA, which may have resulted in false positives.

Total RNA was extracted from colon tumours and paired normal mucosa from 8 CRC patients using TRIzol reagent as per the method described in Chapter 2.3. RT-PCR was performed for CK 19, MUC 2, matrilysin, laminin γ 2, and MTA-1, using an aliquot of the same cDNA mix for each PCR.

11.2.1.2 Assessment of candidate markers by Northern analysis

Northern blot analysis (Chapter 2.7) was performed using 10 μ g total RNA to compare expression of laminin γ 2, matrilysin and MTA-1 in matched tumour and normal colon RNA from CRC patients, including one with adenoma tissue, and in colon cancer cell lines.

11.2.2 Immunobead RT-PCR for metastasis-associated markers in blood

A total of 80 patients' blood samples were tested using immunobead RT-PCR for a panel of epithelial-specific markers (CK 19, CK 20, MUC 1, MUC 2) and metastasis-associated markers (LAM γ 2, MAT, MTA-1). The patients included 50 undergoing resection for primary adenocarcinoma of the colon or rectum, 17 patients undergoing bowel resection for inflammatory conditions such as Crohn's disease or diverticulitis, and 13 patients undergoing resection for adenoma. The colorectal tumours were staged

as 5 stage A, 29 stage B, and 16 stage C. Peripheral blood samples (20 mL) were collected into EDTA both before and 16-24 hours after surgery.

Table 11.1. Primer and probe sequences (metastasis-associated markers)

Oligonucleotide	Sequence 5'-3'	T _m °C
LAM2TS (laminin sense primer)	TGCAATGGGAAGTCCAGGCAGTG	71
LAM2TAS (antisense primer)	TGCCTCCTCTGTCCACACGGTAG	68
LAMININ PROBE	AAGACCAGAGACTGCTAGACTCC	63
MAT SENSE	TGGAATGTAAACTCCCGCGTCA	68
MAT ANTISENSE	CCTCATCGAAGTGAGCATCTCCT	64
MAT PROBE	AAGCCAATCATGATGTCAGC	59
MTA-1 SENSE	GTGCTTCGGTATCTTGAGACCCA	65
MTA-1 ANTISENSE	CACGCTTGGTTTCCGAGGAT	65
MTA-1 PROBE	CAACATGAAGAAGCGCCTCTTGA	66

11.3 RESULTS

11.3.1 RT-PCR

11.3.1.1 Cell lines

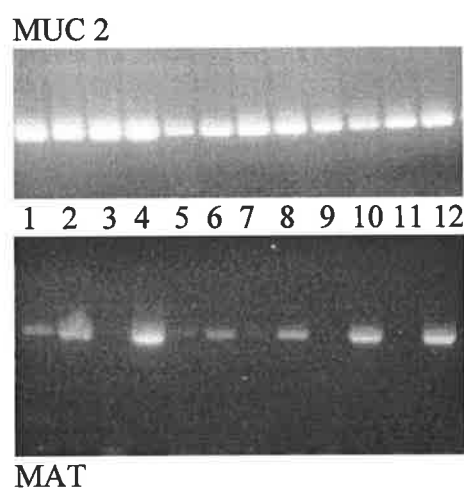
In replicate RT-PCR assays of the CRC cell lines, 10/10 were positive for laminin γ 2 expression, 9/10 were positive for MAT expression (LIM-2405 was negative) and 8/8 were positive for MTA-1 expression.

11.3.1.2 Matched tumour/normal tissue

Using an aliquot of the same cDNA sample for different PCR assays, all 6 sets of matched tumour/normal mucosa tissues showed equivalent expression of MUC 2, but over-expression of MAT in the tumour tissue compared to the normal mucosa (Figure 11.1, Table 11.2). This finding was very encouraging in the search for a discriminating marker, however subsequent analyses comparing expression of LAM γ 2, MAT, and MTA-1 (Figure 11.2, Table 11.3) showed variable results. CK 19 RT-PCR was performed first on an aliquot of the cDNA samples to ensure that the reverse transcription step had worked. We had expected that CK 19 would be expressed in all samples but this was not the case. The sample TRI normal mucosa (Figure 11.2, lane 12) did not amplify a CK 19 transcript at all, but was positive for MAT, indicating that the reverse transcription step had worked; MZ normal mucosa (lane 4) amplified poorly for CK 19 and not at all for the other markers, hence results for this patient were considered invalid and represented 'false negative' results.

LAM γ 2 was over-expressed in tumour tissue in 2 pairs (1 stage B, 1 stage C) over-expressed in normal compared to tumour in two and showed equivalent expression in the other pair (Figure 11.2 and Table 11.3). MAT expression was equivalent in all 5 evaluable pairs (Figure 11.2, Table 11.3). MTA-1 was over-expressed in tumours in 2/5 patients (1 stage B, 1 stage C) (Figure 11.2 and Table 11.3). The genomic DNA samples and 'no target' negative controls were negative for all the markers (data not shown).

Figure 11.1 RT-PCR: expression of MUC 2 compared to MAT



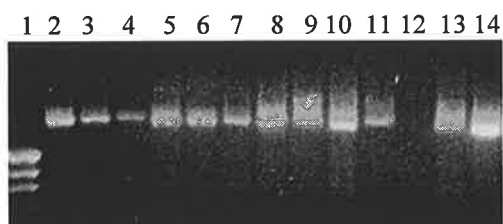
Ethidium bromide stained gels comparing expression of matched tumour-normal paired cDNA samples. Lanes and gel interpretation are shown in Table 11.2

Table 11.2 Interpretation of RT-PCR gels in Figure 11.1

Lane	Sample	Tumour stage	MUC 2	MAT
1	SC normal mucosa		4+	2+
2	SC tumour	B	4+	4+
3	NEV normal		4+	-
4	NEV tumour	B	4+	4+
5	TP normal		4+	1+
6	TP tumour	B	4+	2+
7	JC normal		4+	(+)
8	JC tumour	B	4+	2+
9	COL normal		4+	-
10	COL tumour	C	4+	4+
11	VOR normal		4+	-
12	VOR tumour	B	4+	4+

Figure 11.2 RT-PCR: comparison of expression of CK 19, LAM γ 2, MAT and MTA-1

CK 19 RT-PCR



LAM γ 2 RT-PCR



MAT RT-PCR



MTA-1 RT-PCR



Ethidium bromide stained gels comparing expression of CK 19, LAM γ 2, MAT and MTA-1 in normal/tumour paired cDNA samples. Lanes and gel interpretation are shown in Table 11.3

Table 11. 3 Interpretation of RT-PCR gels in Figure 11.2.

Lane	Sample	Tumour stage	CK 19	LAM γ 2	MAT	MTA-1
1	pUC19 MW markers					
2	JC normal mucosa		2+	4+	2+	1+
3	JC tumour	B	2+	1+	3+	-
4	MZ normal		1+	-	-	-
5	MZ tumour	C	4+	4+	3+	2+
6	TP normal		4+	4+	3+	1+
7	TP tumour	B	4+	4+	2+	1+
8	NEV normal		4+	-	3+	■
9	NEV tumour	B	4+	4+	4+	■
10	SC normal		4+	4+	3+	-
11	SC tumour	B	4+	2+	3+	1+
12	TRI normal		-	-	3+	■
13	TRI polyp		4+	-	4+	■
14	TRI tumour	C	4+	2+	4+	■

The invalid results (MZ) are shown in the shaded section. Markers showing distinct over-expression in the tumour compared to normal are highlighted.

11.3.2 Northern blotting

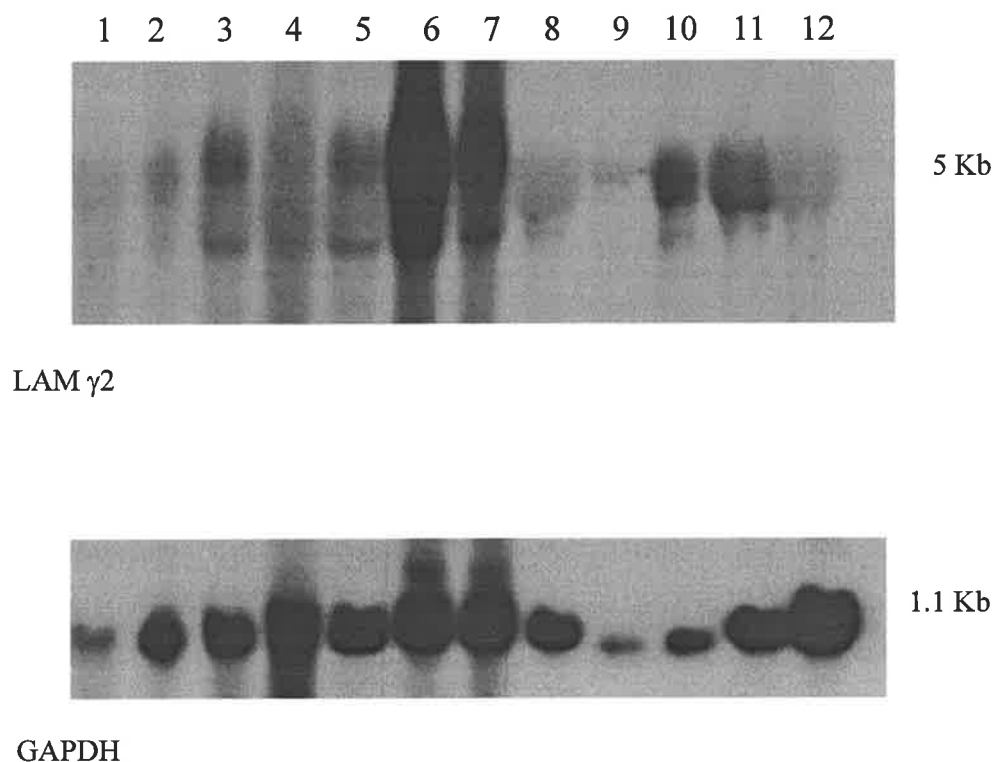
11.3.2.1 Cell lines

Laminin γ 2 has 2 transcripts, one of 5 kb, the other 4.5 kb (Kallunki et al., 1992), while MAT has one transcript of 1.2 kb (Yamamoto et al., 1994) and MTA-1 one of 3 kb (Toh et al., 1995). Northern analysis of the colon cancer cell lines LIM-1215, LIM-1899, LIM-2099, SW480 and SW620 (Figure 11.4) showed that they all express laminin γ 2, with LIM2099 and SW480 showing the greatest expression. It is of interest to note that the SW620 cells, which are the metastatic variant of SW480, show reduced expression

compared to SW480, suggesting that once the cells have metastasised they no longer need to express adhesion molecules such as laminin-5 which is involved in focal adhesion of tumour cells to the extracellular matrix during migration (Pyke et al., 1995). A separate Northern blot confirmed over-expression of laminin $\gamma 2$ in LIM-2099 and also over-expression in SW48 (Figure 11.5).

The cell lines LIM-2405, SW620 and HT29 showed high expression of MTA-1, whereas only HT29 showed any expression of MAT. These results were in contrast to the results using RT-PCR, however it would be expected that RT-PCR would detect low level expression which would not be detectable by Northern analysis.

Figure 11.4 Northern analysis of matched tumour/normal mucosa and cell lines



Lane 1, NEV normal; lane 2, NEV tumour; lane 3, SC normal; lane 4, SC tumour; lane 5, TRI normal; lane 6, TRI polyp; lane 7, TRI tumour; lane 8, LIM-1215; lane 9, LIM-1899; lane 10, LIM-2099; lane 11, SW480; lane 12, SW620.

11.3.2.2 Matched tumour and normal mucosa

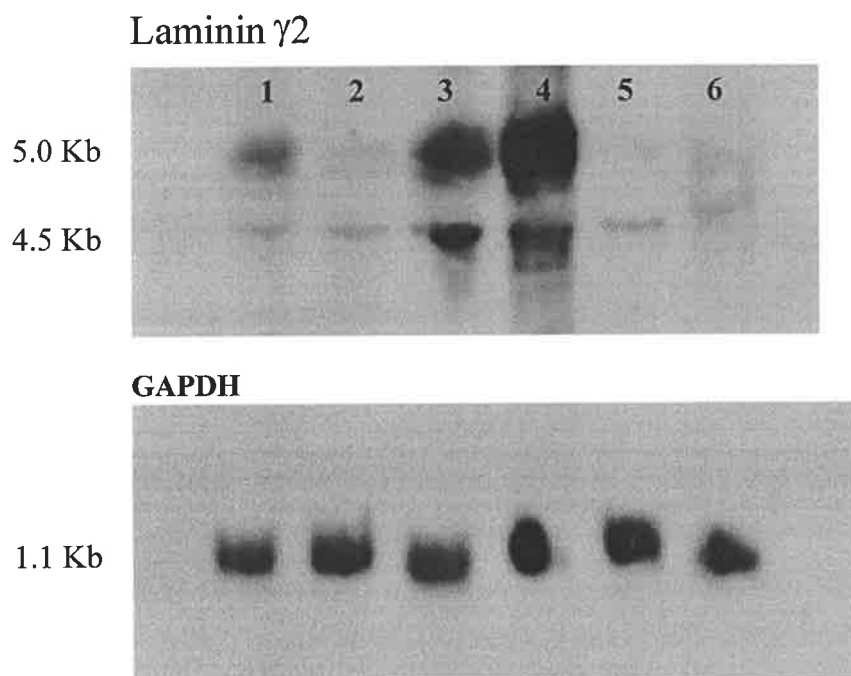
Northern analysis showed over-expression of laminin $\gamma 2$ in the tumour and adenoma in one of 3 patients, while in 2/3 expression was reduced or the same in the tumour compared to matched normal mucosa (Figure 11.4). The patient (TRI) showing over-expression of this metastasis-associated marker was Dukes' stage C, and so this result is consistent with the tumour staging (stage C patients have loco-regional lymph node involvement). Interestingly, I had also detected circulating epithelial-marker positive cells in this patient, further confirming that this patient had disseminated disease. The 2 patients *not* showing over-expression of LAM $\gamma 2$ were diagnosed as stage B, again suggesting that tumours confined to the bowel wall have not yet acquired invasive properties and hence do not express this molecule involved in migration.

The same stage C patient (TRI) also showed over-expression of MAT in adenoma tissue but not in tumour tissue, by Northern analysis (Figure 11.6), which is in contrast to the results obtained for this patient with RT-PCR in which all three tissues (tumour, adenoma, normal mucosa) showed increased expression. This patient did however show differential over-expression of MTA-1 and LAM $\gamma 2$ in tumour tissue by RT-PCR. On the same Northern (Figure 11.6) MAT expression was undetectable in the remaining normal-tumour pairs (all stage B) and in the cell lines LIM-2405 and SW620, while HT29 showed some expression (Figure 11.6).

In contrast, MTA-1 was over-expressed to the same extent in the adenoma and tumour tissue of the stage C patient, increased in tumour relative to normal in one patient (stage B), expressed at equivalent but low level in another patient (stage B) and not detectable in either tissue in a fourth patient (stage B) (Figure 11.6). Expression of GAPDH was used to standardise RNA loading so that a comparison of expression levels

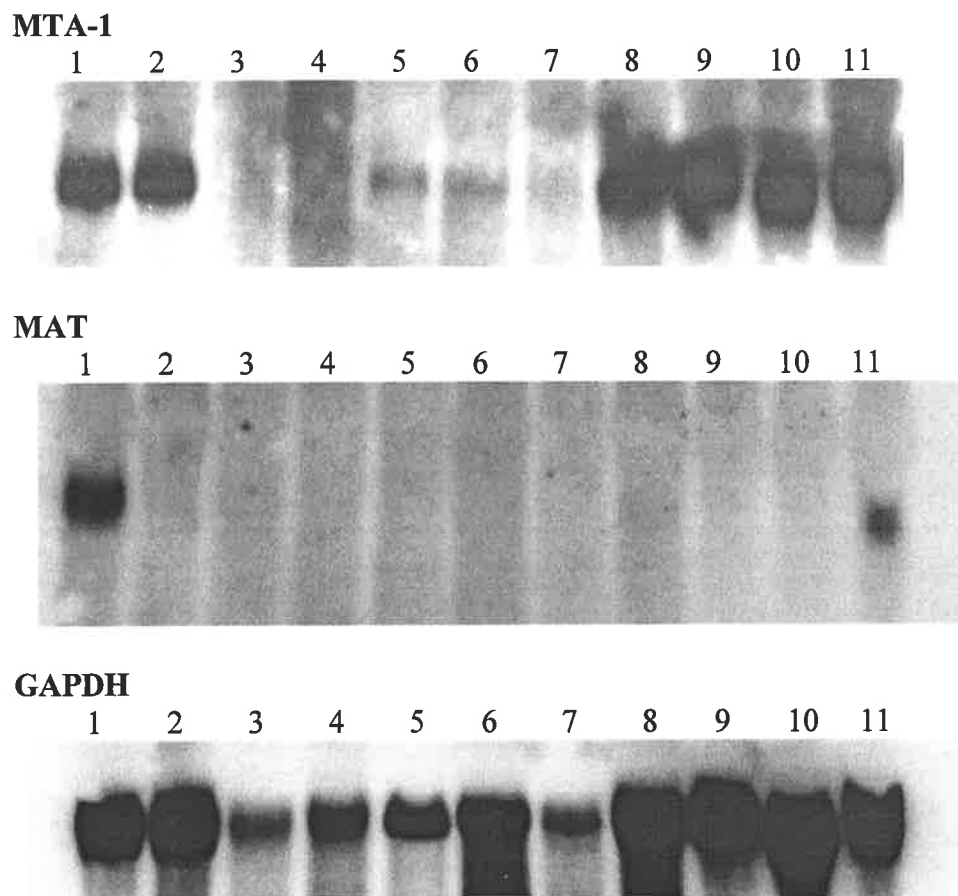
among patient samples could be made. The results of Northern analyses for patient samples is summarised in Table 11.5.

Figure 11.5 Northern analysis: comparison of laminin $\gamma 2$ expression in cell lines



Lane 1, LIM 2412; lane 2, LIM 1899; lane 3, SW48; lane 4, LIM 2099; lane 5, LIM 1215; lane 6, SW480.

Figure 11.6 Northern analysis: comparison of MTA-1 and MAT expression



Lane 1, TRI polyp; lane 2, TRI tumour; lane 3, JC normal; lane 4, JC tumour; lane 5, SC normal; lane 6, SC tumour; lane 7, NEV normal; lane 8, NEV tumour; lane 9, LIM-2405; lane 10, SW620; lane 11, HT29. GAPDH is the control for RNA loading.

Table 11. 5 Summary of Northern blot results for matched tissues

Sample	Tumour stage	LAM γ2	MAT	MTA-1
NEV normal mucosa		+	-	-
NEV tumour	B	+	-	4+
JC normal		nd	-	-
JC tumour	B	nd	-	-
SC normal		+	-	+
SC tumour	B	+	-	+
TRI normal		+	nd	nd
TRI adenoma		4+	4+	4+
TRI tumour	C	4+	-	4+

11.3.3 Immunobead RT-PCR analysis of peri-operative PB samples

Among CRC patients, 8/50 were positive for epithelial markers in pre-operative PB, with 2 patients (both stage B) also positive for MAT and MTA-1. In post-operative PB, 6/50 patients were positive for epithelial markers, with 4 of these (1 stage A, 2 B, 1 C) positive for metastasis-associated markers (Table 11.6).

In adenoma patients undergoing bowel resection, none were positive for epithelial markers in either pre- or post-operative PB, while 2 were positive in pre-operative PB for metastasis-associated markers only (one for MAT, one for MTA-1) (Table 11.6).

In the group of patients undergoing bowel resection for benign inflammatory conditions, one patient was positive for epithelial markers in both pre-and post-

operative PB. None of the patients were positive for metastasis markers in pre-operative PB, however 2 were positive post-operatively for MTA-1 only (Table 11.6).

Table 11.6 Immunobead-RT-PCR: detection of metastasis-associated and epithelial-specific markers in PB samples pre-and post-operatively

MARKERS	No. POSITIVE/No. TESTED	
<i>CRC n=50</i>	PRE-OP PB	POST-OP PB
LAM γ 2	0/36	1/35 (B*)
MAT	1/26 (B)	1/27 (B) same patient
MTA-1	1/14 (B)	2/14 (1 A, 1 C)
EPITHELIAL	8/50	6/50
<i>BENIGN n=17</i>		
LAM γ 2	0/9	0/11
MAT	0/11	0/13
MTA-1	0/10	2/10
EPITHELIAL	1/15	1/17 this patient also positive for MTA-1
<i>ADENOMA n=13</i>		
LAM γ 2	0/7	0/9
MAT	1/7	0/5
MTA-1	1/3	0/3
EPITHELIAL	0/10	0/10

* letter in brackets refers to ACPS stage

DISCUSSION

In these preliminary studies, expression of the putative metastasis-associated markers in matched tumour/normal mucosa and CRC cell lines was compared using Northern analysis and RT-PCR, and these results compared to previous reports in the literature.

Using Northern analysis to study expression of LAM $\gamma 2$, I found differential over-expression in tumour and adenoma tissue in only one patient (stage C), and over-expression in 3/7 CRC cell lines (LIM-2099, SW480 and SW48). The remaining cell lines showed a very low level of expression. Analysis of LAM $\gamma 2$ expression by RT-PCR confirmed over-expression in the stage C patient and additionally in a stage B tumour relative to the normal mucosa.

Pyke et al. (1994) showed that 16/16 colon carcinomas and 6/7 ductal breast cancers were positive by *in situ* hybridisation for laminin $\gamma 2$ chain expression and that expression was confined to the tumour cells, being highest at the invading front. In a second study Pyke et al. (1995) reported that 23/23 colon adenocarcinomas were positive for the $\gamma 2$ chain expression by immunohistochemistry in the tumour cells but not in the surrounding stromal cells. The staining was intracytoplasmic in all cases and extracellular in only one, indicating that in cancer tissue this laminin isoform is not laid down in the extra-cellular matrix but probably exerts its function at or near the tumour cell surface membrane. Pyke's group did not assess expression in matched normal mucosa from these cancer patients. There have been no reports in the literature using Northern analysis or RT-PCR to study expression of laminin $\gamma 2$.

In examining expression of matrilysin, Yamamoto et al. (1994) reported that this matrix metalloproteinase was expressed in all 10 colorectal adenomas tested by Northern blot hybridisation, whereas it was almost undetectable in adjacent paired

normal colon tissue, hyperplastic polyps and mildly inflamed colon tissue from ulcerative colitis patients. Expression between synchronous adenoma and cancer tissue was compared in 3 patients; the expression being lower in adenomas in all 3 cases (Yamamoto et al., 1994). In contrast with these findings I found, by Northern analysis, that matrilysin was over-expressed in the adenoma tissue but not the tumour of the patient with a stage C tumour (TRI) (Figure 11.5). Three other matched tumour-normal pairs had no detectable expression; these cases were all diagnosed as stage B tumours, which may explain the low level of MAT expression, as this has been found to increase with increasing Dukes' stage (Mori et al., 1995). By RT-PCR, 0/5 sets of matched tissues showed differential expression of MAT (Table 11.3), however this group consisted of 4 with stage B tumours and with 1 stage C.

Northern analysis of our samples for MTA-1 expression showed differential over-expression in 1/4 normal/tumour pairs; another set showed over-expression in adenoma and tumour tissue, however in this case there was insufficient normal RNA available to run on the gel. By RT-PCR, over-expression of MTA-1 in tumours was found in 2/5 patients (1 stage B, 1 stage C). Toh et al. (1997) also used RT-PCR, and found over-expression of MTA-1 (ratio >2:1) in about the same proportion (39%) of colorectal carcinomas compared to matched normal mucosa and this correlated with more advanced stage. Clearly, many more sets of matched tumour/normal tissue need to be examined before any firm conclusions can be drawn.

A word of caution is warranted in that RT-PCR in this study is by no means quantitative and any comparisons assume equivalent amplification efficiency between different PCR tubes. This point was illustrated in the separate RT-PCR assays for MAT in which the results could not be duplicated in 3/4 patients represented on both gels (Figures 11.1 and 11.2). One way to overcome this would be to use the recently

available “Real-time Quantitative PCR” technology (PE Applied Biosystems) for reliable quantitative RT-PCR. The principle of this technique is that the accumulation of PCR product is monitored throughout the PCR run by virtue of the fluorescent signals generated by specific cleavage of fluorogenic probes. When the probes are cleaved by the 5' nuclease activity of Taq polymerase, the reporter dye is separated from the quencher dye, generating a sequence-specific signal each cycle and this is displayed graphically. The software compares the product accumulation curve during early exponential amplification to that generated from standards and quantifies the yield of product.

Using the panel of markers to detect carcinoma cells in peri-operative PB in CRC patients, our study showed that of 8 patients in whom epithelial cells were detected, 5 were also positive for metastasis-associated markers. (Table 11.6). These 5 patients were diagnosed as stage A (1), stage B (3), and stage C (1) so perhaps 4 early stage patients have been identified who may be at risk of developing overt metastatic disease. The follow-up period for 4 of these patients is still less than 12 months, however one of the stage B patients died of disease 2 months after 'curative' resection.

In summary, whilst detection of over-expression of these putative metastasis-associated markers may be useful to phenotype colon tumours, the fact that expression is still detectable by RT-PCR in most of the matched normal colonic mucosal RNA, highlights the problem in attempting to use expression of these markers to identify small numbers of tumour cells isolated from post-operative blood where normal colon cells are also likely to be present. The problem is that even if the marker shows over-expression in tumour RNA compared to the same amount of normal RNA, a signal generated from 10 colon cancer cells may equate to one generated from 50-100 normal colonic mucosal cells. There is no data in the literature describing the release of normal

colonic cells into the peripheral blood circulation so we have no way of knowing whether normal cells are likely to be circulating in relatively large numbers (compared to tumour cells). This thesis describes for the first time the detection of normal colon cells in the peripheral blood in 3 patients with adenoma and one with diverticulitis (Chapter 10) and thus highlights the need for carcinoma- or metastasis-specific markers to answer the question of whether or not tumour cells have been dispersed into the bloodstream during resection for colorectal malignancy.

CHAPTER 12

CONCLUDING DISCUSSION

This thesis describes the development of a novel immunomagnetic molecular technique for the detection of colon cancer cells in peripheral blood and, in particular, its potential in predicting shortened disease-free survival in those patients in whom circulating cancer cells were detected. The technique of immunomagnetic bead enrichment of cells had been applied to the isolation of trophoblasts from the maternal circulation (Mueller et al., 1990), however, the combination of immunomagnetic bead isolation and molecular detection of specific markers to detect rare tumour cells in blood was, at the time of commencement of this work, a completely new idea. The earliest reports of the use of immunomagnetic beads was for *depletion* of cells in the field of bone marrow transplantation for haematological malignancies and some solid tumours (reviewed in Chapter 1).

Subsequent to our first publishing this technique as a means to sensitively detect free cancer cells in the peripheral blood in patients undergoing surgical resection for colorectal malignancy (Hardingham et al., 1993), the company manufacturing the magnetic beads, Dynal, adopted our immunomagnetic bead isolation procedure for isolating epithelial cells using the antibody Ber-EP4 that I had selected; they now market "Dynabeads[®] anti-Epithelial Cell" beads that have Ber-EP4 covalently bound to the surface.

12.1 Progress and pitfalls

The first marker I chose to use as a molecular determinant of colon cancer cells was a K-*ras* codon 12 mutation. This resulted in a very sensitive assay, capable of detecting 1 cancer cell in 10^6 leucocytes, as well as being specific for colon cancer cells bearing a K-*ras* codon 12 mutation. A pilot study of 27 CRC patients showed that detection of the

K-*ras* marker in blood taken peri-operatively was associated with a much poorer prognosis ($p=0.0001$). This marker was only applicable, however, in the 35% of our patients whose tumour carried this particular mutation.

The search began for markers that were applicable to a greater proportion of CRC patients. Mutations in the p53 gene were reported as being present in some 75% of colorectal cancer patients (Hollstein et al., 1991) which would have doubled our previous rate of detection of a tumour cell marker. PCR-SSCP was selected as the most appropriate method to use, however it was soon discovered that although the technique was suitable for screening tumour samples for point mutations in p53, it was not sufficiently sensitive to detect mutant alleles from one or a few tumour cells that may have been present in the cell lysate. The reason for this was inherent in the SSCP technique itself in that it relied on staining of the different conformers (DNA sequences that migrate through a gel at different rates dependent on their conformation, which in turn is dependent on their sequence). In certain tumour DNA the conformers representing the mutant sequence stained very weakly. Bands such as these from only 1-10 cells would not be detectable with this technique and thus it was abandoned for the purpose of detecting tumour cells in blood.

12.2 Development and use of epithelial-specific markers

In order to ensure that the markers were appropriate for all patients with colorectal cancer, RT-PCR assays for epithelial-specific markers were developed. CK 19 was already being developed for use as marker for breast cancer cells in blood in our laboratory (Eaton et al., 1997). CK 20, MUC 1 and MUC 2 were developed by myself specifically for colon cancer cells, although MUC 1 has also been useful as a marker to

detect breast cancer cells in peripheral blood stem cell harvests in this laboratory (Walsh et al., submitted for publication).

I have studied a cohort of 96 patients undergoing surgical resection for primary colorectal malignancy. Pre- and post-operative blood from these patients was tested by immunobead RT-PCR using the four markers CK 19, CK 20, MUC 1 and MUC 2. The patients were followed for a median of 392 days (range 16-1505) for disease-free and overall survival outcome. Kaplan-Meier survival analyses showed that the detection of cells positive for these markers in blood samples taken pre-operatively was strongly associated with reduced survival ($p=0.001$). This suggested that the cells being detected were malignant colon cancer cells which later gave rise to recurrent local disease and/or metastatic lesions, predominantly in the liver. These results are very exciting since this is the first large study applicable to all colon cancer patients to show that detection of cancer cells in the blood pre-operatively is a prognostic marker for disease outcome following 'curative' surgery. Our earlier study using *K-ras* as a marker of cancer cells also showed that the presence of cancer cells in blood correlated with much poorer prognosis but this marker was only applicable in a proportion of patients. Recently, Mori et al. (1998) reported a study on 27 CRC patients using CEA expression as a marker for colon cancer cells in PB and showed significantly reduced survival in patients positive for cancer cells. To address the question of whether the detection of cancer cells in blood at the time of surgery is a prognostic marker of metastatic disease independent of other prognostic (staging) factors, our study must await accrual of some 250 further patients. This number has been calculated to ensure that Kaplan-Meier survival analysis and Cox regression analysis can be performed with adequate power.

The detection of colon cells in post-operative blood did not show any correlation with disease-free survival probably due to the fact that normal mucosal cells were also

shed into the circulation during surgical resection of the bowel thus masking any effect shed cancer cells may have had on survival. The markers used were epithelial-specific and thus not able to differentiate normal colon cells from malignant ones.

This thesis describes for the first time the detection of 'normal' colonic mucosal cells in the peripheral blood in non-malignant conditions. Epithelial cells were detected in post-operative PB in 4 patients undergoing bowel resection for diverticular disease (3 patients) or Crohn's colitis, strongly suggesting that surgical resection of the bowel provides a means whereby colonic epithelial cells gain access to the bloodstream. Interestingly, epithelial cells were also detected in one of these patients in pre-operative blood; this patient was diagnosed as diverticular disease with severe inflammation. Importantly, none of 20 normal controls (normal subjects not undergoing surgery) were positive for epithelial markers in blood. Perhaps normal colon cells are able to gain entry into the bloodstream as a result of bowel pathology such as inflammation with increased vascularity, as was the case in the patient with severe diverticulitis. In the same way, transformed but non-metastatic cells from within adenoma tissue may gain entry to the circulation. Alternatively, cells from adenomas containing micro-deposits of *in situ* carcinoma may already have an invasive phenotype.

12.3 Future directions

To address the problem of identifying a marker specific for carcinoma cells or metastasising cells, recently developed commercially available technology based on comparison of hybridisation of tumour and normal cDNA probes to a filter array of human cDNA sequences could be used. The principle of the technique is that it will allow identification of mRNAs that are present in one RNA sample but not another.

Several commercially available kits are available that provide cDNA arrays on nylon membranes. The "Gene Discovery Array" (Genome Systems) consists of two identical 22x22 cm nylon filters that have been spotted with 18,376 non-redundant human cDNA clones (Lennon et al., 1996) with several control clones and an orientation marker. Each filter is hybridised with radio-labelled probes prepared from each RNA sample (normal, tumour, metastasis) by reverse transcription using a random oligo dT primer supplied with the kit. The resulting cDNA product is a mixture of labelled cDNAs representative of all mRNA species in the original RNA sample. The mixture is used to probe the filter array. Once hybridised the filters will be analysed by phosphor-imaging and specifically designed software for identification of the positive clones. These clones may then be obtained from Genome Systems for sequencing and identification. One or more clones that show over-expression in tumour samples with no expression detectable in the normal tissue would be selected as putative carcinoma or metastasis-associated markers. Primers for RT-PCR may be designed from the sequence information available for each cDNA clone. Differential expression would be confirmed between tumour tissue and normal mucosa from a series of patients using Northern blot analysis.

Using this technology it is possible that a gene or genes so far unknown may be identified as important markers for carcinoma cells since the array consists of 3,230 known full-length cDNA sequences, the remaining clones being from expressed sequence tags of unknown genes. Conversely, genes that are not expressed or show reduced expression in tumour tissue compared to normal mucosa may represent novel tumour suppressor genes.

Another company (Clontech) has a specific human cancer cDNA array available (Atlas Human Cancer cDNA Expression Array). Each array is a positively-charged nylon membrane on which 588 cDNA clones have been spotted. The selection of genes

was based on their functional importance in cancer and includes cDNAs from genes in the following categories: cell cycle and growth, angiogenesis, cell adhesion motility and invasion, apoptosis, DNA damage response, and cytokines.

Work is continuing on the accrual of tumour tissue and matched normal mucosa and, when available, adenoma tissue and metastases, to use as cDNA probes in the hybridisation of the arrays. Ideally we require a stage C (or D) tumour for the best chance of identifying a metastasis-specific marker. A positive signal with the tumour cDNA probe and a corresponding negative signal with the normal mucosa probe would indicate a marker of interest to us. From the position on the array, the cDNA can be identified (by reference to the company's web-site information). As the sequences of all the cDNAs on the arrays are known, primers could be designed for assessment of differential expression of the marker using patient tissue. There are difficulties however, in obtaining suitable tumour specimens. Patients diagnosed with stage D (metastatic) disease are usually not resected, while many patients with stage C rectal cancers are not operated on until after they have received adjuvant radiotherapy or chemo-radiotherapy. Tumour tissue so treated is unsuitable from which to obtain RNA for these studies.

Recently, Zhang et al. (1997) analysed expression of more than 300,000 transcripts from colon cancer cells, colon cancer cell lines and normal mucosal cells using the technique of SAGE (serial analysis of gene expression) (Velculescu et al., 1995). In colorectal cancers there were 548 transcripts identified as being differentially expressed between tumours and normal mucosa; 289 being over-expressed in tumour relative to normal tissue. This figure represented only 1.5% of the total number of detectable transcripts. Also these differences in expression were not always found in cultured colon cancer cell line cells emphasising the need for caution in the interpretation of cell line gene expression. Earlier this year, Zhang and colleagues

published their complete expression analysis profile on the World Wide Web (<http://welchlink.jhu.edu>). They have identified 24 transcripts showing over-expression in colon tumours and no expression in adjacent normal mucosa; 16 were from known genes lodged in GenBank, 5 were expressed sequence tags (ESTs) of unknown genes, and for 3 there were no matches in GenBank. The 2 transcripts identified as showing the highest level of expression by Northern analysis were from the IGF II gene (insulin like growth factor II). In contradiction to these results, Tricoli et al. (1986), also using Northern analysis, found over-expression of IGF II in colon tumours. However, there was still a low but significant level of expression in the matched normal mucosa which would cause a problem in using this as a specific RT-PCR marker for tumour cells.

12.4 Conclusions

This work has established a novel, highly sensitive technique-immunobead RT-PCR, employing a panel of epithelial-specific markers (CK 19, CK 20, MUC 1, MUC 2) that was applicable in the detection of circulating colon tumour cells in pre-operative blood. Survival analysis of this group showed that patients in whom these cells were detected had significantly shortened disease-free survival ($p=0.001$), and included 5 patients diagnosed with early stage disease. Thus there is now a diagnostic non-invasive blood test that can be done prior to surgery which will yield additional prognostic information and should result in a more appropriate selection of patients for adjuvant radiotherapy or chemotherapy. To answer the question of whether epithelial cells, dispersed either into the bloodstream or into the abdominal cavity as a result of surgery, are malignant cells that may be responsible for subsequent intra-abdominal recurrences or development of metastatic disease, carcinoma- or metastasis-specific markers are required. The ability to

sensitively and specifically detect disseminated carcinoma cells in peripheral blood and other body fluids after 'curative' surgery would be of major importance in the accurate staging of colorectal cancer, and indeed other cancers, and thus the ideal marker, or panel of markers for this purpose should be pursued.

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Appendix A: Detection of K-ras mutations in colon tumours

No.	Patient ID	K-ras	Patient ID	K-ras	Patient ID	K-ras		
1	254	NEG	75	707	NEG	149	675	NEG
2	490	NEG	76	287	NEG	150	263	NEG
3	475	NEG	77	18	NEG	151	776	NEG
4	374	NEG	78	803	NEG	152	686	NEG
5	795	NEG	79	649	NEG	153	302	NEG
6	216	NEG	80	429	NEG	154	270	NEG
7	932	NEG	81	143	NEG	155	954	NEG
8	568	NEG	82	74	NEG	156	581	NEG
9	643	NEG	83	674	NEG	157	965	NEG
10	692	NEG	84	213	NEG	158	71	NEG
11	233	NEG	85	482	NEG	159	241	NEG
12	107	NEG	86	17	NEG	160	241	NEG
13	372	NEG	87	669	NEG	161	971	NEG
14	950	NEG	88	165	NEG	162	723	NEG
15	323	NEG	89	216	NEG	163	816	NEG
16	933	NEG	90	886	NEG	164	975	NEG
17	250	NEG	91	384	NEG	165	864	NEG
18	118	NEG	92	484	NEG	166	709	NEG
19	811	NEG	93	200	NEG	167	613	NEG
20	3010	NEG	94	87	NEG	168	49	NEG
21	734	NEG	95	308	NEG	169	183	NEG
22	262	NEG	96	634	NEG	170	129	NEG
23	686	NEG	97	579	NEG	171	793	NEG
24	420	NEG	98	895	NEG	172	957	NEG
25	289	NEG	99	6	NEG	173	500	NEG
26	956	NEG	100	640	NEG	174	896	NEG
27	873	NEG	101	585	NEG	175	570	NEG
28	618	NEG	102	162	NEG	176	118	NEG
29	493	NEG	103	153	NEG	177	482	NEG
30	154	NEG	104	186	NEG	178	905	NEG
31	987	NEG	105	127	NEG	179	454	NEG
32	863	NEG	106	285	NEG	180	500	NEG
33	465	NEG	107	547	NEG	181	623	NEG
34	841	NEG	108	242	NEG	182	477	POS
35	926	NEG	109	111	NEG	183	703	POS
36	823	NEG	110	17	NEG	184	697	POS
37	388	NEG	111	574	NEG	185	352	POS
38	390	NEG	112	84	NEG	186	185	POS
39	710	NEG	113	104	NEG	187	870	POS
40	131	NEG	114	257	NEG	188	177	POS
41	392	NEG	115	67	NEG	189	451	POS
42	730	NEG	116	626	NEG	190	812	POS
43	327	NEG	117	556	NEG	191	26	POS
44	521	NEG	118	417	NEG	192	604	POS
45	358	NEG	119	765	NEG	193	512	POS
46	707	NEG	120	59	POS	194	581	POS
47	560	NEG	121	762	POS	195	604	POS
48	929	NEG	122	195	POS	196	661	POS
49	919	NEG	123	300	POS	197	356	POS
50	695	NEG	124	301	POS	198	886	POS

No.	Patient ID	K-ras	No.	Patient ID	K-ras	No.	Patient ID	K-ras
51	729	NEG	125	21	POS	199	47	POS
52	207	NEG	126	606	POS	200	712	POS
53	680	NEG	127	2171	POS	201	329	POS
54	900	NEG	128	966	POS	202	335	POS
55	827	NEG	129	743	POS	203	412	POS
56	373	NEG	130	271	POS	204	369	POS
57	285	NEG	131	878	POS	205	304	POS
58	36	NEG	132	711	POS	206	195	POS
59	78	NEG	133	988	POS	207	840	POS
60	689	NEG	134	664	POS	208	731	POS
61	524	NEG	135	769	POS	209	638	POS
62	209	NEG	136	400	POS	210	831	POS
63	940	NEG	137	777	POS	211	925	POS
64	648	NEG	138	954	POS	212	166	POS
65	613	NEG	139	711	POS	213	130	POS
66	946	NEG	140	535	POS	214	844	POS
67	895	POS	141	43	POS	215	625	POS
68	514	POS	142	956	POS	216	401	POS
69	21	POS	143	425	POS	217	896	POS
70	109	POS	144	292	POS	218	748	POS
71	233	POS	145	692	POS	219	414	POS
72	100	POS	146	169	POS	220	883	POS
73	173	POS	147	413	POS	221	177	POS
74	799	POS	148	983	POS			

Appendix B: Analysis of p53 and K-ras mutations in colon tumour samples

No.	PATIENT ID	DATE	STAGE ACPS	K-RAS	p53	EX5	EX6	EX7	EX8
1	254	Jun-92	B	NEG	NEG	NEG	NEG	NEG	NEG
2	932	Aug-93	A	NEG	POS			POS	
3	606	Aug-91	C	POS	POS	POS			
4	859	Oct-94	C	NEG	POS				POS
5	372	Mar-94	A	NEG	NEG	NEG	NEG	NEG	NEG
6	966	Nov-91	B	POS	NEG	NEG	NEG	NEG	NEG
7	3323	Mar-94	C	NEG	POS			POS	
8	933	Jun-93	B	NEG	NEG	NEG	NEG	NEG	NEG
9	271	May-92	C	POS	POS	NEG		POS	NEG
10	811	Jan-94	B	NEG	POS				POS
11	118	May-92	B	NEG	POS				POS
12	3010	Sep-92	C	NEG	POS			POS	
13	988	Mar-94	B	POS	NEG	NEG	NEG	NEG	NEG
14	420	Jun-93	C	NEG	NEG	NEG	NEG	NEG	NEG
15	493	May-94	C	NEG	POS	POS			
16	954	Jan-93	B	POS					
17	711	Dec-93	B	POS	NEG	NEG	NEG	NEG	NEG
18	154	Oct-92	D	NEG	POS	POS	NEG	NEG	NEG
19	535	Apr-93	A	POS	NEG	NEG	NEG	NEG	NEG
20	863	Apr-94	C	NEG	POS			POS	
21	43	Nov-91	C	POS	POS	POS			NEG
22	707	Jul-94	B	NEG	POS		NEG	POS	
23	477	Dec-93	C	POS	POS	NEG	NEG	POS	NEG
24	703	Jan-94	B	POS	POS				POS
25	697	Feb-94	B	POS	NEG	NEG	NEG	NEG	NEG
26	352	Mar-92	D	POS		NEG	NEG	NEG	smear
27	482	May-94	D	NEG	POS	POS			
28	165	Aug-92	B	NEG	POS			POS	
29	177	Aug-92	B	POS	NEG	NEG	NEG	NEG	NEG
30	216	Sep-93	D	NEG	POS				POS
31	26	Feb-94	A	POS	NEG	NEG	NEG	NEG	NEG
32	886	Oct-93	C	NEG	POS	POS	NEG	NEG	NEG
33	384	Jan-92	C	NEG	NEG	NEG	NEG	NEG	NEG
34	4087	Sep-92	D	NEG	POS		NEG		POS
35	308	Jul-93	B	NEG	NEG	NEG	NEG	NEG	NEG
36	512	Nov-93	D	POS	POS	POS	NEG		NEG
37	634	Nov-93	B	NEG	NEG	NEG	NEG	NEG	NEG
38	579	Jan-92	B	NEG	POS				POS
39	895	Oct-92	C	NEG			NEG	NEG	NEG
40	1006	Aug-93	B	NEG	NEG	NEG	NEG	NEG	NEG
41	585	Apr-92	C	NEG	POS				POS
42	356	Dec-93	B	POS	POS	POS			
43	547	Nov-93	B	NEG	POS	POS			
44	263	Aug-94	B	NEG	NEG	NEG	NEG	NEG	NEG
45	776	Feb-94	B	NEG	POS	NEG	NEG	NEG	POS
46	686	Apr-93	D	NEG	POS		POS		
47	581	Oct-92	D	NEG	POS			NEG	POS
48	329	Jun-94	D	POS	POS	POS			
49	412	Dec-92	D	POS	NEG	NEG	NEG	NEG	NEG
50	241	Mar-94	D	NEG	POS				POS
51	304	Feb-94	B	POS	POS				POS
52	816	Apr-94	C	NEG	NEG	NEG	NEG	NEG	NEG

No.	PATIENT ID	DATE	STAGE ACPS	K-RAS	p53	EX5	EX6	EX7	EX8
53	975	Dec-92	B	NEG	POS	POS	NEG		NEG
54	613	Oct-93	B	NEG	POS	NEG		POS	NEG
55	195	Oct-93	D	POS	na	smear	NEG	NEG	smear
56	840	Nov-93	D	POS	POS		POS		
57	6831	Dec-92	B	POS	na	NA	NEG	NEG	NEG
58	925	May-94	D	POS	NEG	NEG	NEG	NEG	NEG
59	118	Apr-93	B	NEG	POS	NEG	POS		NEG
60	895	Aug-92	C	POS	POS	POS	NEG	NEG	NEG
61	905	Apr-94	B	NEG	POS	POS			
62	21	Feb-94	C	POS	NEG	NEG	NEG	NEG	NEG
63	233	Apr-92	C	POS	POS			POS	
64	926	Feb-93	C	NEG	POS	NEG	NEG	POS	
65	823	Dec-93	B	NEG	NEG	NEG	NEG	NEG	NEG
66	433	Oct-92	B	POS	POS				POS
67	173	Mar-92	B	POS	POS		POS		
68	286	May-93	B		POS				POS
69	292	Aug-93	C	POS	NEG	NEG	NEG	NEG	NEG
70	358	May-94	B	NEG	POS		NEG	POS	NEG
71	692	Apr-90	C	POS	POS		POS		
72	900	Oct-93	B	NEG	NEG	NEG	NEG	NEG	NEG
73	285	May-94	C	NEG		NEG	NEG	NEG	SMEAR
74	689	Jul-94	B	NEG	POS	POS	NEG	NEG	
75	4078	Jun-94	A	NEG	POS	NEG	NEG	NEG	POS
76	209	Mar-93	D	NEG	NEG	NEG	NEG	NEG	NEG
77	940	Oct-92	C	NEG	POS	NEG	NEG	POS	
78	748	Dec-92	C	POS	NEG	NEG	NEG	NEG	NEG
79	17	Oct-93	C	NEG	NEG	NEG	NEG	NEG	NEG
80	104	Nov-92	C	NEG	POS	POS	NEG		NEG
81	765	Mar-94	B	NEG	POS	POS	NEG		NEG
82	625	Jul-92	D	POS	POS		NEG	POS	
83	679	Jun-93	B	NEG	NEG	NEG	NEG	NEG	NEG
84	149	Dec-93	D	NEG	NEG	NEG	NEG	NEG	NEG
85	281	Aug-92	A	NEG	NEG	NEG	NEG	NEG	NEG
86	970	Jun-93	C	POS	POS	POS		NEG	NEG
87	423	Jun-92	C	POS	NEG	NEG	NEG	NEG	NEG
88	830	Nov-93	D	POS	NEG	NEG	NEG	NEG	NEG
89	816	May-92	C	POS	NEG	NEG	NEG	NEG	NEG
90	747	Sep-93	A		POS		NEG	POS	NEG
91	283	Sep-92	B	POS	POS			POS	NEG
92	575	Sep-93	B	NEG	POS	NEG		NEG	POS
93	697	Mar-93	B	NEG	POS	POS	NEG	NEG	
94	933	May-93	B	NEG	NEG	NEG	NEG	NEG	NEG
95	271	May-92	C	POS	POS	NEG		POS	NEG
96	483	Feb-93	C	NEG	NEG	NEG	NEG	NEG	NEG
97	686	Apr-93	C	NEG	NEG	NEG	NEG	NEG	NEG
98	752	Feb-93	C	NEG	NEG	NEG	NEG	NEG	NEG
99	1675	Aug-90	C	NEG	NEG	NEG	NEG	NEG	NEG
100	878	Mar-94	A	POS	NEG	NEG	NEG	NEG	NEG