Candidate Tumour Markers and Potential Therapeutic Targets in Colorectal Cancer

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Abstract

Aim: To identify candidate tumour-specific molecular markers for the detection of disseminated tumour cells in peripheral blood and intra-peritoneal lavage samples from patients undergoing surgical resection for CRC and as potential therapeutic targets.

Results: cDNA microarray screening identified Dipeptidase-1 (*DPEP-1*) to be over-expressed by ≥ 2 fold in colon tumour compared to normal colonic mucosal tissue in 56/68 (82%) patients. The laminin gamma-2 chain of laminin-5 (*LAM-γ2*) and Matrilysin (*MAT*) were also identified as potential candidate molecular markers and found to be over-expressed in 22/30 (73.3%) and 47/53 (88.7%) patient matched samples respectively. Immunobead RT-PCR found *DPEP-1*, *LAM-γ2* and *MAT* positive cells in 82 of 168 (48.8%) CRC patients (14 Stage A, 32 Stage B, 17 Stage C and 19 Stage D). Of patients who were positive for one or more marker in any sample, 41 suffered disease relapse (recurrence) or death resulting from cancer progression within the follow-up period. Kaplan-Meier survival analysis, conducted on 110 early (A and B) stage patients, found those who were positive for any marker had significantly shorter disease-free survival than patients who were negative (P=0.026) and patients who were positive for any marker in their post-operative lavage samples also had a poorer survival outcome (P=0.038). Multivariate analysis showed that detection of disseminated tumour cells with any molecular marker remained significant (P=0.015, hazard ratio 3.459, 95% CI 1.272-9.410) and was independent of other risk factors of disease relapse, indicating patients that were positive for any marker were 3.5 times more likely to suffer relapse than patients who were negative. Further characterisation of *DPEP-1* and *LAM-γ2* identified that HT29 cells transfected with the *DPEP-1* construct migrated through a Matrigel™

invasion assay in greater numbers than untreated cells (P=0.007). RNA interference of *DPEP-1* found a significant difference in migration capacity between the mock transfected (MT) cells when compared to *DPEP-1* siRNA treated cells (P=0.034). Fluorescent immunohistochemistry located *DPEP-1* expression in the crypts of colon tumour tissue. Anti-LAM-γ2 treated LIM 2099 cells migrated through the Matrigel™ invasion assay in significantly reduced in numbers when compare to non-treated and normal IgG_1 antibody treated cells (P=0.0006) and siRNA-mediated gene silencing of *LAM-γ2* significantly reduced the number of cells migrating through the Matrigel™ invasion assay (P=0.007).

Conclusions: *DPEP-1* and *LAM-γ2* are potential targets for tumour-specific therapeutic intervention. Immunobead RT-PCR using a panel of molecular markers has the ability to identify early stage CRC patients at risk of disease relapse.

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, to be available for loan and photocopying.

Cassandra M. McIver

Publications associated with this PhD research project:

Appendix II

Cassandra M. McIver, Julia M. Lloyd, Peter J. Hewett and Jennifer E. Hardingham. *Dipeptidase 1: A Candidate Tumor-specific Molecular Marker in Colorectal Carcinoma.* Cancer Letters, 209: 67-74, 2004.

Appendix III

Julia M. Lloyd, **Cassandra M. McIver**, Sally-Anne Stephenson, Peter J. Hewett, Nicholas Rieger, and Jennifer E. Hardingham. *Identification of early-stage colorectal cancer patients at risk of relapse post-resection by immunobead reverse transcription-PCR analysis of peritoneal lavage fluid for malignant cells*. Clinical Cancer Research, 12: 417-423, 2006.

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Chapter 1

Introduction & Literature Review

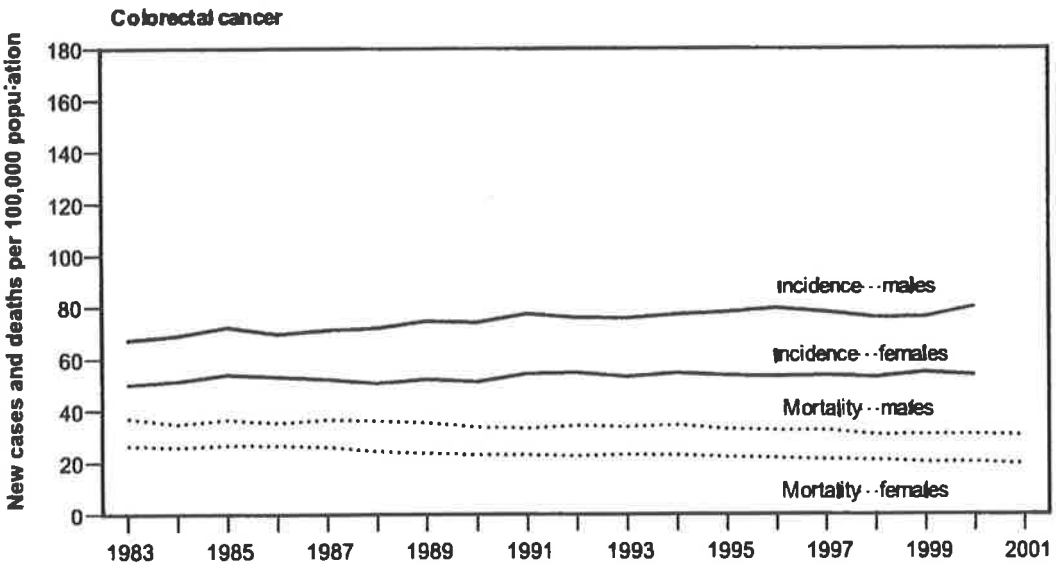
1. Introduction

Colorectal carcinoma (CRC) is the second leading cause of cancer related death in the western world. There have been great advances in the past decade in the understanding of large bowel cancer, including improvements in primary and secondary prevention, screening, and more effective adjuvant therapies. However, the development of recurrent and metastatic disease in early stage patients remains a major problem. Up to 30% of patients diagnosed with early stage disease (ACPS Stage A and B) do not survive beyond 5 years after potentially curative surgery. Clearly additional prognostic markers are needed in conjunction with current staging techniques to more accurately predict which patients are at risk of disease relapse following surgery. Here I will discuss the use of immunobead RT-PCR to detect disseminated tumour cells in the circulation and peritoneal cavity, the identification of tumour-specific molecular markers to improve the specificity of the immunobead RT-PCR technique and the characterisation of genes over-expressed in colorectal cancer as potential therapeutic targets.

1.1 *Colorectal Cancer Epidemiology*

In Australia, there were 11,000 new cases in 2000, and over 3,000 CRC related deaths reported annually (1). Interestingly, the incidence of CRC is slowly increasing but mortality is on a gradual decline (Figure 1.1). Over the past decade there have been many improvements in primary and secondary prevention, screening, equipment for diagnosis and more effective adjuvant therapies for CRC (2). Prior to this it was proposed that the trend in incidence may reflect changing dietary habits including the increased popularity of "fast food", and the effects of environmental toxins (3). However, now there is also the

increasing awareness of cancer, and better disease detection at earlier stages due to improved screening practices (2, 4) which may account for the declining mortality despite the increased incidence. In Australia and other developed countries, similar rates of incidence and mortality relative to population size can be seen (5). Reports from the United States of America indicate that CRC is the most frequent form of cancer among persons aged over 75 years and due to increasing life expectancies and the aging population there will be an obvious impact on the incidence and social burden of CRC (4).



Source: Cancer in Australia 2000, AIHW & AACR, 2003.

Figure 1.1. Trends in age-standardised incidence and mortality rates for colorectal cancer, Australia, 1983-2001 (Australian Institute for Health and Welfare).

1.2 *Tumour Cell Dissemination in Colorectal Cancer Patients*

The development of disseminated (metastatic) disease accounts for the majority of deaths in CRC and is therefore a major concern for patients diagnosed with this disease. Tumour cell dissemination primarily occurs to the surrounding lymph nodes and/or the liver, via portal venous and lymph drainage (6). However, this is the endpoint of a complex multi-step process of metastasis.

Experimental studies in humans and animals have shown that tumour development proceeds via a succession of genetic changes, sequential and interrelated steps, enabling a subpopulation of cells to gain a growth advantage, leading to the progressive transformation of normal cells into cancer cells. These cells also acquire capabilities to enable them to dislodge from the primary tumour, migrate and form secondary tumour foci at distant sites (7, 8). Mammalian cells have multiple safeguards to protect them against potentially lethal effects of mutations in the three types of genes that are responsible for tumourgenesis: oncogenes, tumour-suppressor genes and stability genes. Only when several genes are defective does an invasive cancer develop thus mutated cancer genes contribute rather than cause cancer (9).

Our understanding of the molecular changes that underlie CRC have been significantly advanced by the genetic model for colorectal tumourgenesis proposed by Fearon & Vogelstein in the early nineties. The model suggests that colorectal tumours arise as a result of mutational activation of oncogenes coupled with the inactivation of tumour suppressor genes. That mutations in at least four to five genes are required for the

formation of a malignant tumour and that the total accumulation of changes, rather than their order with respect to one another, is responsible for determining the tumours biological properties (10).

Therefore for CRC to develop, a single mucosal cell must undergo an initial genetic transformation event (11) and there must be a selective but progressive growth advantage of the neoplastic cells over adjacent normal cells (12) involving tumour promoting genes (oncogenes) and the loss of expression of genes which suppress tumour formation (tumour suppressor genes), for example, genes that regulate apoptosis. Extensive vascularisation takes place to enable the tumour to exceed 2mm in diameter and several angiogenic factors play important roles in enabling this to occur. Tumour cells must also acquire the ability to detach from the primary tumour, migrate through the basement membrane and extracellular matrix, intravasate and travel in the circulation to the new site, before reattachment, extravasation, the development of a new focus and neovascularisation (12, 13) (Figure 1.2). The immune system is also stimulated to initiate an antigenic response against the carcinoma cells (14) that metastatic cells must overcome.

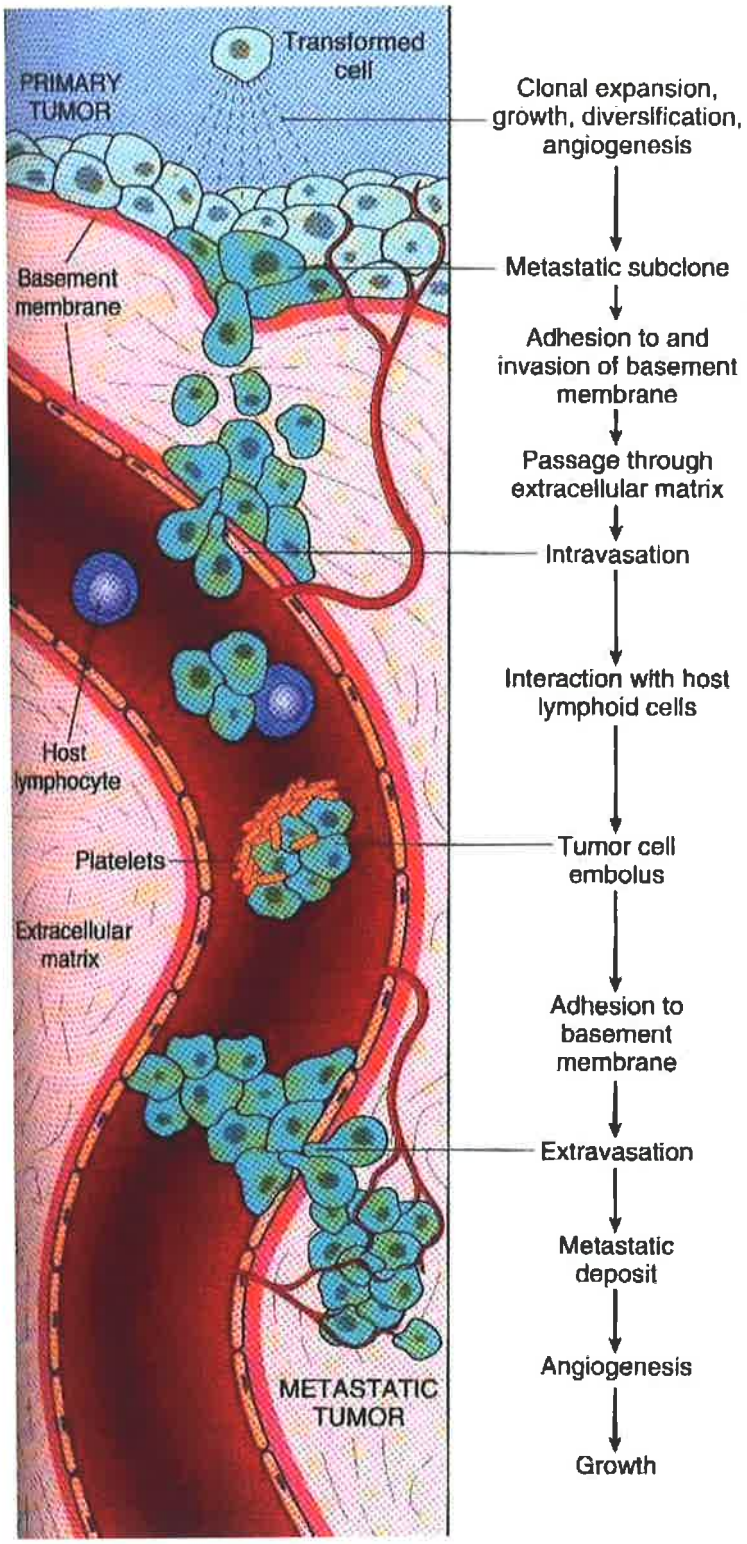


Figure 1.2 Schematic diagram of the metastatic cascade (15) p303.

For some CRC patients, tumour cell dissemination may occur at an early stage of cancer development, as currently up to 30% of patients who present with early stage disease (ACPS Stage A and B) die within 5 years, despite potentially curative surgery (16-18). This suggests that viable metastatic tumour cells escaped from the primary tumour before resection, or surgical manipulation of the tumour enabled cancer cells to disseminate into the circulation or the peritoneal cavity. These cells may have the potential to develop into recurrent or metastatic tumours. At least two-thirds and perhaps as much as 90% of colorectal cancers arise from benign adenomatous polyps lining the wall of the bowel, with those that grow to a large size and having a villous appearance or containing dysplastic cells being most likely to progress to cancer (4).

1.3 *Colorectal Cancer, Recurrence and Disseminated Disease*

To date, the staging of CRC is determined by the histological examination of the tumour tissue and surrounding lymph nodes, by ascertaining the depth of invasion through the bowel wall and the presence or absence of tumour cells in the lymph nodes (ACPS staging, as shown in Figure 1.3) (19, 20). Staging systems based on these variables are not always reliable for the prediction of patient outcome, particularly as patients diagnosed within the same stage may have varying disease outcomes (21).

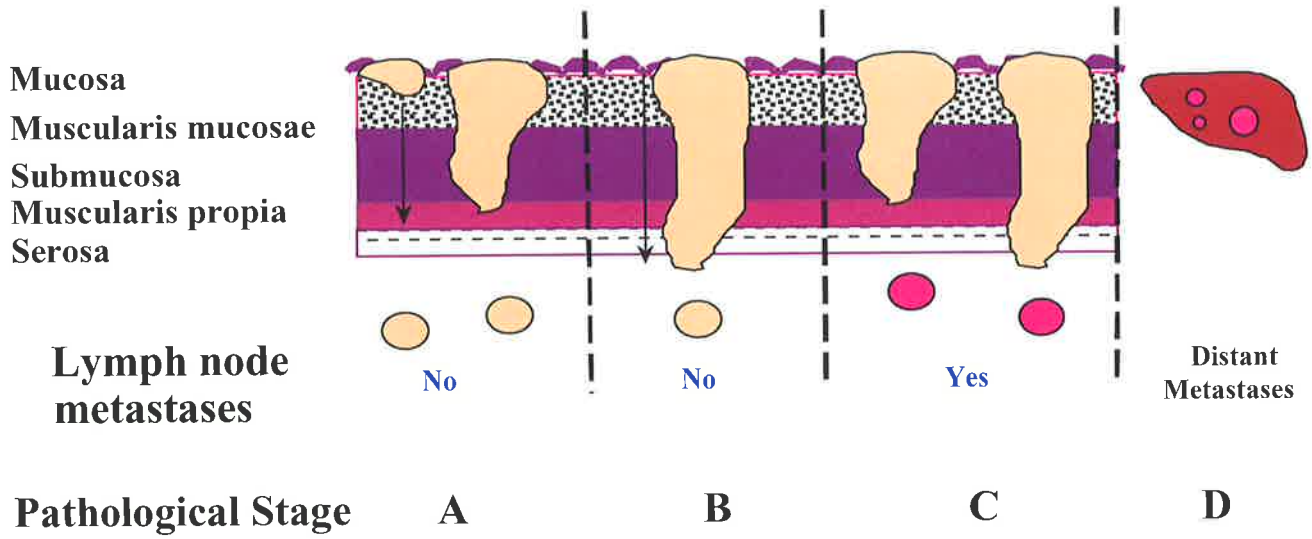


Figure 1.3 Schematic Diagram of the Australian Clinical Pathological Staging (ACPS) classification system for the staging of colorectal carcinoma. Adapted from Gastroenterology p236 (22).

At present, standard treatment of CRC includes surgical resection and adjuvant chemotherapy for stage C patients (defined by the presence of lymph node metastases) but surgery alone for patients with stage A or B (23). However, patients whose tumours have lymph node metastases have a 50-60% tumour recurrence rate, and adjuvant chemotherapy given to all C stage patients reduces the recurrence rate by 15%. In comparison, patients with tumours extending into (Stage A) or through the colonic wall but with no lymph node metastases (Stage B) do not receive adjuvant therapy, although 30% of these patients (10% Stage A and 20% Stage B) will develop tumour recurrence or metastasis. Liver metastases are also present in some 25% of patients at the time of initial

colorectal resection (Stage D) and over 50% of patients will eventually develop them. Of patients who die from colorectal cancer, 90% will have liver metastases (6).

For early stage patients, improved techniques that are reliable and sensitive, and that can detect disseminated tumour cells are required to establish accurate staging to aid in the management of a patient's disease. Early detection of tumour cell dissemination may have important diagnostic and prognostic implications, altering the initial staging of the disease, and resulting in the early identification of patients at high risk of disease relapse. By identifying genes that are involved in colorectal carcinogenesis and understanding the mechanisms that may be altered in the development of this disease, it will be possible to use gene markers that are sensitive and specific, to detect circulating tumour cells and/or to use as therapeutic targets for this particular type of cancer.

1.4 *Tumour Cell Dissemination in Cancer Patients*

The detection of circulating tumour cells is not a new concept with the first report of tumour cells observed in the blood as early as 1869 (24). Since then there have been several reports using cytological techniques to detect tumour cells circulating in the bloodstream (25-27) and in the peritoneal cavity of CRC patients (28-30).

Utilisation of molecular techniques, in particular polymerase chain reaction (PCR) and more recently reverse transcription (RT)-PCR, has improved the sensitivity and specificity of tumour cell detection in solid tissues and blood samples (31). Gene mutations have previously been studied using PCR to determine the significance of

disseminated tumour cells in the circulation (21, 32). *p53* and *K-ras* mutations have been identified as significant molecular events that contribute to the malignant phenotype. A mutation in the p53 gene causes inactivation of the p53 tumour suppressor protein, enabling tumour cells to acquire resistance to apoptosis (7). It has been found that a mutation in codon 12 of the *K-ras* gene is an early event in the development of up to 50% of colorectal tumours (33, 34). The cellular responses to activated Ras vary depending on the cell type. Activated Ras expression can often induce normal cells into pathways that lead to cell growth arrest, senescence, and/or apoptosis (35). These important protective responses restrict the propagation of cells bearing activated oncogenes. Therefore a mutation in the *K-ras* gene will enable tumour cells to gain the physiological traits of unrestricted growth, evasion of senescence and resistance to apoptosis.

A problem that has been identified with the use of these mutations as markers for circulating disseminated tumour cells is that they are only applicable for patients that have the mutation. Hardingham *et al.,* (1995) found only 35% of CRC patients to have the *K-ras* mutation (32) and *p53* mutations have also been reported to occur in up to 80% of these patients (36, 37). The use of gene mutations as molecular markers, like *K-ras* and *p53*, to detect circulating tumour cells is therefore limited by the number of patients that have the mutation, as many patients will be excluded.

To further improve the sensitivity of detection of epithelial-derived tumour cells in blood and intra-peritoneal wash samples collected from CRC patients, our laboratory developed immunobead RT-PCR (38, 39). This technique enriches for epithelial cells by using

magnetic beads coated with an epithelial-specific monoclonal antibody Ber-Ep4. The sensitivity of this technique is such that it has the ability to detect gene expression from 10 tumour cells in 10 mL of blood (40). By enriching for epithelial cells, this technique reduces the potential to detect very low levels of illegitimate expression (41) from other cell types including white blood cells that are present in peripheral blood and intra-peritoneal wash samples, which may produce false positive results. The need to use nested PCR to increase sensitivity is also alleviated, a technique which has previously caused problems in assessing Cytokeratin 19 (*CK 19*) expression as a marker for epithelial cells in lymph nodes of breast cancer patients (42, 43).

More recently it was found that CRC patients with positive expression for the cytoskeleton components Cytokeratin (*CK*) *19* and *CK 20*, which are expressed in epithelial cells of the gut, also the epithelial glycoproteins Mucin 1 (*MUC 1*) and Mucin 2 (*MUC 2*) in peripheral blood samples taken before surgery, had a significantly shorter overall survival ($P<0.0001$) than patients who were negative for these markers (40). It was also found that this technique has the ability to differentiate patients with a poorer prognosis, within the same stage classification as demonstrated in Figure 1.4. Patients with stage C disease who were positive for these markers were identified as having a significantly shorter overall survival ($P=0.02$) than patients who were negative. This indicates that the technique has the ability to stratify patients with C stage disease that are at increased risk of disease relapse. However, due to small patient numbers, patients with stage A and B disease could not be analysed separately. It is therefore important to

investigate whether this technique is useful in these patients to help identify those that may benefit from further adjuvant therapy.

Yamaguchi *et al.* (2000), used carcinoembryonic Antigen (*CEA*) and *CK 20* on mesenteric venous blood samples, and found that the prognosis of the PCR-positive group was significantly poorer than that of the PCR-negative group (P=0.003) (44). *CEA* is a membrane glycoprotein and an antigenic component in cancers derived from gastrointestinal tract epithelium. It has been widely used as a tumour marker in the management of bowel cancer (45). *CEA* mRNA expression has also been used by Castells *et al.,* (1998) to detect circulating tumour cells in CRC patients. Thirty-nine peripheral blood samples were positive in 95 colorectal cancer patients tested. They found a significant correlation between *CEA* mRNA positivity and the presence of distant metastases at the time of surgery (P=0.05) and serum CEA levels were significantly higher (P<0.02) than those patients with negative *CEA* expression (46).

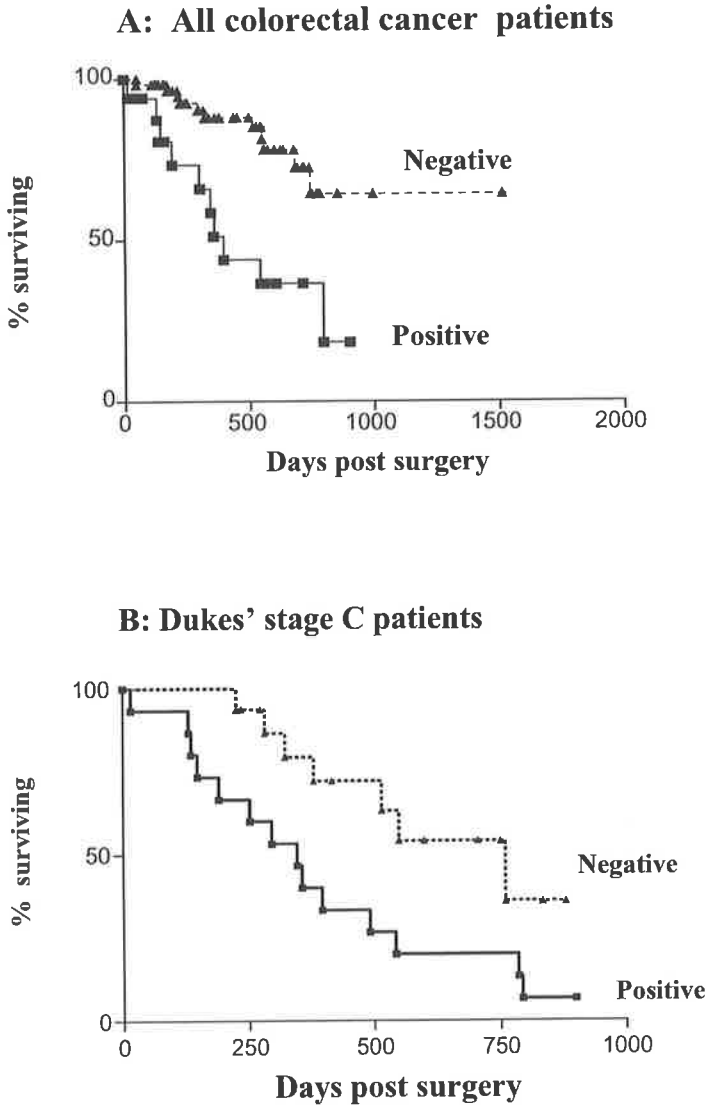


Figure 1.4 Kaplan-Meier survival analysis of colon cancer cells detected using immunobead RT-PCR. A: All patients. B: Stage C patients only, Hardingham *et al.*, (40).

A variety of epithelial-specific markers have been used previously, such as *CEA* (44, 46-50), *CK 20* (18, 21, 40, 44, 49, 51-56), *CK 19* (21, 40, 48, 57), *MUC 1* (40, 48, 58, 59), and *MUC 2* (40) in RT-PCR based assays to detect tumour cells in colon, breast and gastric carcinomas. However, a number of problems relating to specificity have been identified with the use of these markers.

Five of 9 patients with benign inflammatory bowel disease were positive for *CEA* expression (46), while 4/34 were positive for *CK 20* and *MUC 2* expression (40). These results suggest that the inflammatory process and ulceration may enable normal colonic epithelial cells to gain access to the circulation (40). Thus, the molecular markers used in these assays are epithelial specific but not tumour-cell specific. It is therefore appropriate to identify epithelial markers that are tumour-cell specific, particularly for use with the immunobead RT-PCR technique.

1.5 *Tumour Cell Dissemination into the Peritoneal Cavity*

A number of techniques have been developed to detect tumour cells in blood, bone marrow and lymph nodes, though little investigation has been directed at the identification of tumour cells in the peritoneal cavity before and after surgical resection in colon cancer patients. Umpleby *et al.*, (1984) (60) suggested that the presence of large numbers of tumour cells at the site of intestinal anastomoses could be responsible for suture-line recurrence. This was supported by Leather *et al.* (1994) (28) who also proposed that free malignant cells within the peritoneal cavity could cause implantation of tumour cells at the anastomosis or within the peritoneal cavity itself with the potential

to develop into recurrent tumours. Using conventional cytology, malignant cells were identified in the peritoneal washings from 15/35 (43%) patients (3 pre-resection only, 4 post-resection only and 8 both pre and post-resection). They concluded that tumour cells may have leaked out from lymphatics cut during the resection.

Solomon *et al.,* (1997) (29) used cytology to detect cancer cells in 15/103 (14.6%) patients on the peritoneal or peri-rectal surface of the bowel. They concluded that the presence of free surface cancer cells gives indirect support to the theory of trans-coelomic route to port-site metastasis, which may occur from laparoscopic removal of the colorectal tumour. More recently, Vogel *et al.,* (1999) (61) used immunohistochemistry to detect *CEA* antibody stained cells (tumour cells) in peritoneal lavage samples from 31 of 135 (23%) CRC patients'. They also found that the detection rate of tumour cells increased with the tumour stage from 15% (3/23) in early stage patients to 43.6% (17/39) in late stage patients.

Detection of tumour cells, using the sensitive immunobead RT-PCR technique, in intra-peritoneal wash samples collected before and after surgery may indicate whether manipulation of the bowel during resection enables viable cells to escape into the peritoneal cavity or whether cells had been shed prior to surgery. These cells may have the potential to cause local recurrence or escape into the circulation and lymphatics to cause distant metastases.

1.6 *Identification of Genes Over-expressed in Colorectal Cancer*

1.6.1 cDNA arrays

Recent advances in complementary DNA (cDNA) array technology have enabled the investigation of differential gene expression in CRC (62-66) and many other carcinomas including breast, lung, bladder, and melanoma (67-69). Previously, differences in gene expression between tumour and normal cells were detected using techniques such as differential screening and subtractive hybridisation. Genome sequencing and expressed sequence tag (EST) projects have also provided a wealth of data to study the molecular genetics of tumour progression.

cDNA microarray technology presents parallel expression analysis of thousands of these genes in a single experiment without prior knowledge of gene function (64, 67, 70). Tumour and normal colonic mucosal tissue can be hybridised simultaneously on a nylon membrane or glass slide array containing human cDNA sequences. By using fluorescent dyes, for example Cy3 (green) and Cy5 (red) to label the different RNA, differentially expressed genes can be determined between the two tissues. Due to the large numbers of cDNA sequences spotted on these arrays (up to 20,000 elements), many studies have used clustering and proprietary algorithms (62, 65, 66, 71) and other statistical approaches (64), to analyse the vast amount of data that can be gained from this technology, in order to identify those genes that may have the most significant role in tumour development, progression or metastasis.

The first report of the use of this technology to investigate differential gene expression in colon carcinoma was by Backert *et al.* (1999). A cDNA array was used to analyse human cell line samples derived from normal mucosa, non-mucinous and mucinous colonic carcinomas. They identified 10 genes with altered expression and 6 were confirmed by northern blot analysis. Kitahara *et al.,* (2001) also used cDNA array analysis to identify a set of genes involved in the development of colorectal carcinogenesis. However, instead of analysing whole tumour tissue samples they used laser-capture microdissection (LCM) to collect homogenous cell populations. LCM, under direct microscopic visualisation, permits rapid one-step procurement of selected human cell populations from a section of complex, heterogenous tissue (72). RNA can be extracted from these cell populations and reverse transcribed for cDNA array analysis. The LCM technique enables genes that are up- or down-regulated in tumourgenesis to not be masked by the expression of genes in non-tumour cells. Forty-four genes were found to be commonly up-regulated (from 8 patient tissue samples) and 191 commonly down-regulated using this technique (65). However, the amount of RNA generated using this technique is small and therefore needs to be amplified to generate enough RNA product to use for array analysis. By amplifying RNA to generate enough for experimental use, sequence errors may be introduced. This is a potential problem with using this technique as sequence errors will produce inaccurate results.

Notterman *et al.,* (2001) suggested that relatively few changes in transcript expression are associated with colon tumourgenesis, however, these change may be significant. Many of the transcripts they identified were already known to be abnormally expressed,

demonstrating the consistency of this method with others used previously. The results of these studies provided important genetic information, particularly the differential expression of genes that distinguish premalignant from carcinoma and normal tissue. They found 19 transcripts with at least a 4 fold increase in expression in carcinoma compared to normal tissue samples and 47 transcripts with a 4 fold or greater decrease in expression.

Investigation of genes that show differential expression in colon tumour compared to normal mucosa has the potential to identify important, novel genes that may be involved in the development of the metastatic phenotype. Genes found to be over-expressed may be useful as tumour-specific markers for tumour cell dissemination. These genes may also be used as specific targets for gene-mediated therapy, for example, siRNA or antibody-mediated therapy.

1.6.2 SAGE Map/CGAP website

Serial analysis of gene expression databases of the Cancer Genome Anatomy Project (CGAP) website (http://cgap.nci.nih.gov) can also be used to identify novel genes that are over-expressed in colon tumour compared to normal colonic epithelium, using the digital gene expression displayer (DGED) tool (73). The DGED tool compares gene expression between two pools of SAGE (serial analysis of gene expression) libraries, for example, one from colon tumor tissue and one from normal colon tissue and reports statistically significant differences. A virtual northern blot tool can then be used to show the expression pattern across a range of human tissues.

1.7 *Potential Tumour-specific Markers Identified*

To date, I have identified a number of potential tumour-specific markers. The gamma-2 chain of laminin-5 (*LAM-γ2*) was identified from the literature as a candidate tumour-specific molecular marker. Laminin-5 is a basement membrane adhesion protein important for epithelial attachment, cell motility and migration. *LAM-γ2* has been found to be highly expressed at the leading edge of migrating colon tumour cells (74) and will be described in detail in Chapter 5.

Matrilysin is the smallest member of the matrix metalloproteinase family (*MMP-7*) and was also identified from the literature. This gene was selected as a candidate tumour-specific marker because it is expressed in epithelial-derived tumour cells rather than mesenchymal-derived cells which is distinct from other MMPs (75) and will also be further described in Chapter 6.

The third candidate tumour-specific marker is Dipeptidase-1 (*DPEP-1*). This gene was identified by cDNA array analysis conducted in our laboratory and confirmed by the DGED tool from the CGAP website (described in Chapter 4).

1.8 *Candidate Genes as Potential Therapeutic Targets*

Current adjuvant therapy is less than ideal for the treatment of later stage (Stage C and D) disease and is not offered to patients with early stage A and B tumours, who are considered "cured" by surgery alone, due to the problem of toxicity outweighing the potential benefits of its use. It is given in an attempt to eradicate micrometastatic tumour

cells by targeting cells undergoing proliferation, i.e. by the inhibition of DNA replication (76, 77). The problem with this treatment is that many micrometastatic cells circulating in the blood are in the dormant (G0) phase of the cell cycle and therefore not proliferating (76). It has also been well documented that the non-specific nature of chemotherapy causes many undesirable, toxic side effects including vomiting, mucositis, diarrhoea, and leukopenia (77, 78).

Carcinomas of the large bowel are distinctly classified into two groups, colon cancer and rectal cancer. The major difference in treatment is the routine incorporation of combined modality therapy (chemotherapy plus radiation) in the adjuvant management of rectal cancer, due to the dual imperatives of optimising both overall survival and local recurrence-free survival. Colon cancer adjuvant treatment is focused more on the risk of distant metastatic recurrence and is based on chemotherapy alone (79). The efficacy of chemotherapy for colorectal cancer has often been judged by the so-called objective regression, usually defined as 50% reduction in the tumour mass. This however, does not necessarily correlate with improvement in survival or quality of life for patients undergoing chemotherapy (78). Chemotherapy treatment for colon cancer is received after the initial tumour mass is resected, therefore candidates for post-operative adjuvant therapy are patients at high risk of disease relapse, as judged by clinical evaluation, surgical exploration and removal of the tissue, and the pathological evaluation of resected specimens (extent of invasion) (80).

Chemotherapy for colorectal cancer can be dated to the introduction of the fluorinated pyrimidines, fluorouracil and its deoxyriboside, floxuridine. These drugs were experimentally found to result in tumour regression (reviewed in Moertel, 1994). The single agent fluorinated pyrimidine 5-fluorouracil (5-FU) remains the primary agent used to treat this type of cancer (80).

A new approach to fluorouracil therapy involves its combination with substances that increase the chemotherapeutic cytotoxic effects. The most persuasive evidence for the benefit of a biochemical modulation of fluorouracil comes from studies with leucovorin, a chemical that can stabilise the ternary complex of fluorodeoxyuridine monophosphate, 5,10-methylenetetrahydrofolate, and thymidylate synthase, augmenting the activity and toxicity of fluorouracil (78). In patients with C stage colon cancer, several trials have shown that a 6 month post-operative treatment course with combined 5-FU and leucovorin improves survival compared to either no adjuvant therapy or a 12 month course of either agent alone, a regime now adopted as the treatment of choice (81).

Controversy, however, surrounds whether adjuvant therapy should be given to early stage patients, particularly Stage B. The results of 3 published trials showed only a small reduction in mortality for these patients, also the trials lacked sufficient power to show a significant effect (82). In the United States, a subsequent review of pooled data from four trials of adjuvant chemotherapy conducted by the National Surgical Adjuvant Breast and Bowel Project has suggested a similar survival advantage to patients with C stage tumours (83). Thus although improvements in survival can be made with adjuvant

chemotherapy following surgery, current mortality rates indicate that these treatment options are not ideal and that more specific (and less toxic) therapies are warranted.

It has been suggested by Jansen and Zangemeister-Wittke (2002) that there is limited effectiveness of conventional treatment strategies for many cancers. Therefore identification of nucleotide sequences of potential cancer-related genes has paved the way for tailored anticancer agents that lack many of the toxic side-effects of conventional therapy (84).

1.9 *Alternative Therapeutic Strategies*

1.9.1 Antibody-Mediated Therapy

Monoclonal antibodies were the first successful targeted therapy for cancer. As early as the 1970's they were identified by clinical studies to be viable therapeutic tools. Antibodies bind with high specificity to cell-surface antigens, resulting in the targeted killing of malignant cells, with relative sparing of normal tissues, and low toxicity (85).

This type of cancer therapy has become particularly successful in the treatment of hematologic malignancies, especially B-cell non-Hodgkins lymphoma by targeting the CD20 antigen. However, the advancement in the treatment of solid tumours has been slow to progress (85). The recent targeting of the epidermal growth factor receptor (EGFR) by monoclonal antibody therapy (Cetuximab) is producing some promising results in the treatment of head and neck and colorectal cancer. The EGFR monoclonal antibody specifically binds to the receptor with high affinity, blocking growth-factor

binding, receptor activation and subsequent signal-transduction events leading to cell proliferation. This antibody therapy has been evaluated both alone and in combination with radiotherapy and various cytotoxic chemotherapeutic agents in a series of phase I/II studies in patients with either head and neck or colorectal cancer (86). Cetuximab is now being trialled in combination with chemotherapy in phase II and phase III trials to determine its efficacy as a treatment option for solid tumours.

1.9.2 Antisense Oligonucleotide Gene Silencing

Another experimental type of therapy involves antisense oligonucleotides (ASOs). ASOs are short single-stranded synthetic sequences of DNA, complementary to specific strands of RNA. Once delivered into the target cell they have the ability to bind to the mRNA by the process of Watson-Crick base pairing, allowing hybridisation with its RNA complement. The best-characterised antisense mechanism results in cleavage of the targeted RNA by endogenous cellular nucleases, such as RNase H. This process results in the inhibition of mRNA which, in turn, inhibits the expression of the corresponding disease-associated protein through various mechanisms including prevention of mRNA transport, splicing, and translational arrest (reviewed in (84, 87).

The most extensively studied *Bcl-2* antisense oligonucleotide has been G3139, or oblimersen sodium (Genasense). This treatment strategy appears to be having success in the treatment of breast cancer by down-regulating *Bcl-2*. By reducing *Bcl-2* levels, apoptosis can be increased in tumours exhibiting chemoresistance. It has been found that systemic treatment with G3139 markedly increases the cytotoxic actions of

chemotherapy. A phase I/II study of docetaxel plus G3139 for patients with metastatic breast cancer has shown that the combination was safe and affective (reviewed in (88).

There are a number of limitations with the use of ASOs for inhibition of gene expression. These include possible degradation by nucleolytic enzymes, affecting the molecules' stability and resulting in the poor cellular uptake of oligonucleotides due to the difficulty of ASOs in crossing the cell membrane (87-89). These molecules are polyanionic, therefore the uptake of ASOs into the cells occurs through active transport, which, in turn depends on temperature, structure and concentration (reviewed in Biroccio *et al.,* 2003). The effective intracellular delivery of these molecules remains an important issue for the clinical application of this technique, as they have to reach the cytoplasm to effectively access their mRNA target (84).

Both antibody-mediated therapy and antisense oligonucleotide gene silencing have the potential to improve adjuvant therapy for CRC patients. Each technique, however, has demonstrated to be problematic and their use in clinical trials has been disappointing. Therefore the discovery of new techniques that target specific gene sequences continues to occur.

1.9.3 RNA interference (RNAi)-mediated gene silencing

RNAi-mediated gene silencing is triggered by exogenous double stranded (ds) RNA and has been termed RNA interference (RNAi). A schematic representation of how dsRNA can be used as a molecular trigger to mediate changes in gene expression is demonstrated

in Figure 1.5. As indicated (90), these dsRNA functions include sequence specific gene-silencing by RNAi via small interfering RNAs (siRNAs) that target cognate mRNA degradation. RNA duplexes of 21 – 23 nucleotides with 2 nucleotide 3′ overhangs, the siRNAs, have recently been shown to mediate sequence-specific inhibition of gene expression in mammalian cells via this post-transcriptional gene silencing mechanism (91, 92). The naturally occurring RNAi mechanism is believed to enable cells to degrade foreign RNA, for example, virus infection, as most mammalian cells exhibit a very potent response to exogenous dsRNA. It has also been suggested that RNAi and related processes have a more generalised function in gene regulation (reviewed in Caplen, 2003). RNAi can be introduced to cultured mammalian cells by transfection of siRNA molecules of 21 nucleotides (91), or by short hairpin RNA (shRNA) molecules.

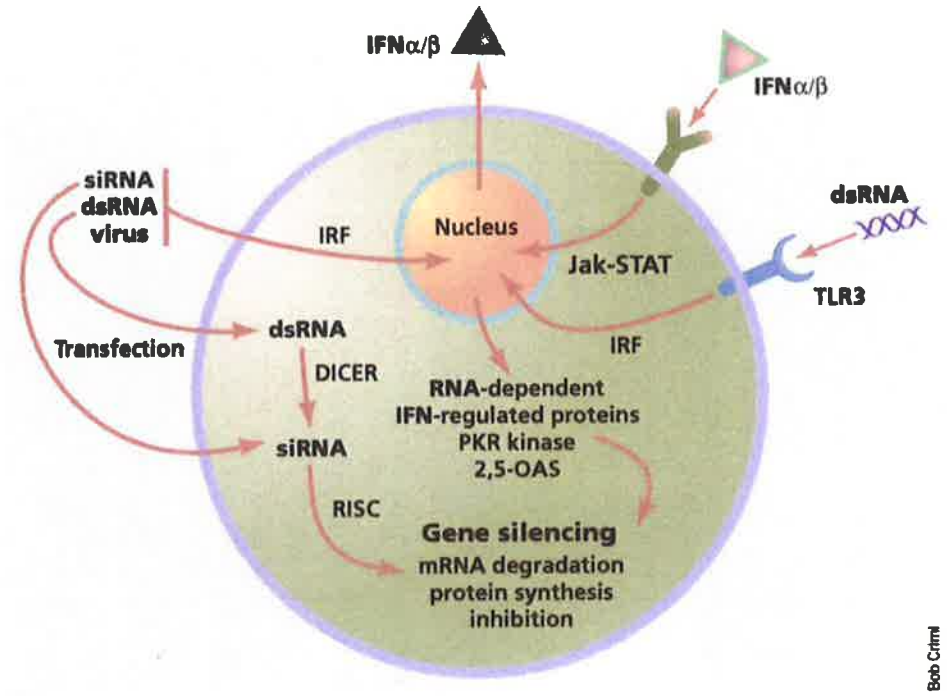


Figure 1.5 Schematic diagram taken from Samuel (2004), indicating the mechanisms by which dsRNA function as a molecular trigger to mediate changes in gene expression patterns inside the cell.

It has now been demonstrated that siRNAs act as a guide to ensure specific interaction with the target transcript and are incorporated into a multi-subunit complex called the RNA-induced silencing complex (RISC; reviewed in Caplen, 2003). An important aspect of siRNA is their ability to avoid activation of the interferon response to long (>30bp) dsRNA, whereby protein kinase R (PKR), which phosphorylates and inactivates the eukaryotic translation initiation factor (eIF2α), leading to a generalised suppression of protein synthesis and a significant alteration to the cellular physiology. The interferon

system is the first line of defence against viral infection and is normally induced when the by-product of viral replication (double-stranded RNA) activates a multi-component signalling complex. When activated, the system essentially shuts down the cellular operations and the cell eventually undergoes apoptosis and dies (93).

As described by Elbashir (2001) and Caplan (2003), siRNA is believed to direct the specific down-regulation of endogenously expressed proteins. However, as suggested by Samuel (2004), Moss & Taylor (2003) and Bridge *et al.*, (2003) (94), siRNAs can also, in some instances, activate the interferon system, potentially complicating the interpretation of RNA interference in mammalian cells.

Current therapeutics use small molecule-based drugs to block protein function or inhibit cellular processes. The effect however, is often non-specific, reducing the efficacy and increasing the potential for unwanted side effects. RNAi targets the encoding RNA transcript, resulting in specific gene inhibition. This would overcome the limitations of current therapies, for example, chemotherapy and hopefully eliminate the unwanted side effects of these regimes (92). As shown in the schematic diagram in Figure 1.6, the siRNA sequence directs the RISC complex to the complementary mRNA strand resulting in specific gene knockdown.

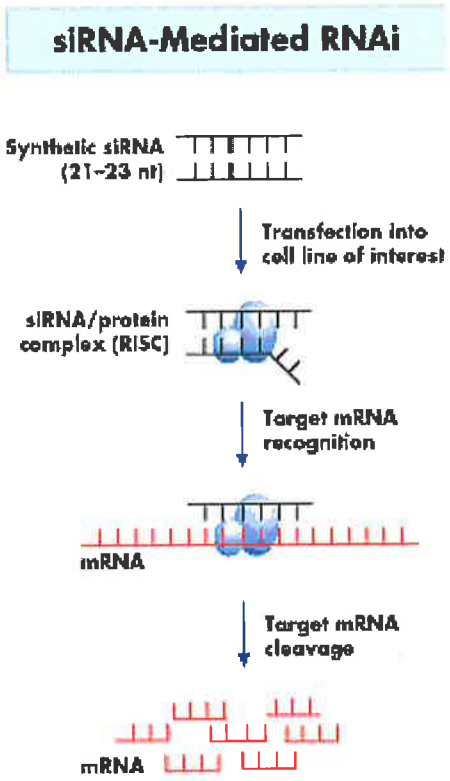


Figure 1.6 Schematic diagram of siRNA-mediated gene silencing process (picture taken from Qiagen siRNA design information at www.qiagen.com).

It has been suggested that RNAi may be more potent than antisense RNA mechanisms in reducing target gene expression in human cancer cells lines. Aoki *et al.,* (2003) compared the two effects in human cancer cell lines, hepatoma and pancreatic cancer, by targeting the exogenous Luciferase gene. The effect of 22 nucleotide RNA duplexes (RNAi) was stronger by one order than antisense DNA (95).

Knockdown of *STAT3* by siRNA mediated gene silencing was investigated by Konnikova *et al.,* (2003). *STAT3* has been found to be constitutively activated in astrocytomas. By

using RNAi they were able to assess the role of *STAT3* in apoptosis, cell proliferation and gene expression following knockdown. *STAT3* expression knockdown resulted in the reduction in expression of *Bcl-xL* and Survivin and lead to the induction of apoptosis in astrocytoma cells, a common type of primary central nervous system tumours (96).

CXCR4 is a chemokine receptor which plays an active role in the metastasis of breast cancer. Recently, Lapteva *et al.,* (2004) used siRNA against *CXCR4* to effectively down-regulate gene expression in human MDA-MB-231 breast cancer cells. The study found that down-regulation lead to a significant decrease in breast cancer cell adhesion and invasion, and also resulted in proliferation of the cells at a much slower rate than control cells. Tumour cells lacking *CXCR4* expression also failed to grow into significant tumours in SCID mice. RNA interference was therefore able to demonstrate that the *CXCR4* molecule plays a significant role in breast tumour growth in addition to its role in breast cancer metastasis (97).

Liu *et al.,* (2004) also used siRNA to investigate the possible role of a gene, *RhoA*, in regulating the malignant phenotype of gastric cancer cells. They designed and constructed a *RhoA*-specific siRNA vector and found that it could specifically and stably reduce *RhoA* expression up to 90% in AGS cells. This resulted in the inhibition of proliferation of AGS cells and enhanced the sensitivity of the cancer cells to Adriamycin and 5-fluorouracil (97).

The siRNA-mediated gene silencing technique not only has the potential to be used as a tumour-specific therapeutic option it also has the potential to identify the functional properties of recently discovered human genes identified via the human genome project and the development of cDNA arrays. Davis *et al.,* (2003) used siRNA knock-down to investigate p120-catenins role in epithelial cells. P120-catenin stabilises epithelial cadherin (*E-cadherin*), the main cell-cell adhesion molecule in epithelial tissues. Using this technique they were able to identify the core function of *p120* in cadherin complexes, which is to regulate cadherin turnover as *E-cadherin* levels depend absolutely on *p120* expression. The timing and location of *p120* action indicates that *p120* regulates adhesion via controlling cadherin turnover at the cell surface. These results have implications for malignant tumour cells, which have the ability to lose cell-cell adhesion in order to migrate from the primary tumour site (98).

More recently RNA interference was used by Sloan *et al.,* (2004) to identify tumorigenic properties of specific genes in human cell lines. A chemically synthesized siRNA was used to investigate whether knockdown of *CD155* affected cellular morphology in HT1080 (human fibrosarcoma) cells. They found that knockdown cells appeared more elongated, irregular in shape and exhibited significantly larger perimeters than control cells, suggesting that *CD155* plays a role in cell size shape. The researchers hypothesise that this may occur by regulating adhesion of cells to their substrate (99).

Although the therapeutic potential of RNAi may revolutionise cancer treatment in the future, a major rate-limiting step currently being examined is the method of delivery of

siRNA to the cancer cells. This problem has been reviewed by Pardridge (2004) (100) who believes that RNA-based therapeutics are currently not practical, due to the instability of RNA *in vivo*. However, as mentioned previously DNA (and as Pardridge describes) plasmid DNA can be engineered to express short hairpin RNA (shRNA). Intravenous, non-viral RNAi-based gene therapy is enabled due to new gene targeting technology, which encapsulates the plasmid DNA inside receptor-specific pegylated immunoliposomes (PILs). This approach was deemed to be feasible after demonstrating that it was possible to achieve 90% knockdown of brain tumour-specific gene expression with a single intravenous injection in adult rats or mice with intracranial brain cancer. Pardridge suggests that this research demonstrates that RNAi-based gene therapy can be coupled with gene therapy that replaces mutated tumour suppressor genes to build a polygenic approach to gene therapy of cancer. However, as mentioned earlier, Bridge *et al.,* (2003) and Moss & Taylor (2003) have indicated that under certain conditions, siRNA may also activate components of the interferon system, thus potentially affecting gene expression more broadly. Therefore reports which demonstrate gene knockdown using this technique should be viewed with caution if induction of this system is not assessed.

These combined results show that there are many potential applications for RNAi-mediated therapy. With the appropriate delivery technique, for example that demonstrated by Pardridge (100), siRNA-mediated gene silencing has the exciting potential to improve the therapeutic options for clinicians and will result in improved and more specific treatment for patients with cancer.

1.10 *Hypotheses*

1. Haematogenous and lymphatic spread of colorectal carcinoma occurs at an early stage of tumour development.
2. Surgical manipulation during resection enables viable tumour cells to disseminate into the peritoneal cavity. These cells may be responsible for later recurrence.
3. Silencing candidate tumour-specific genes using RNAi will reduce the ability of a colon carcinoma cell line to invade an artificial basement membrane (Matrigel) in an *in vitro* 2-chamber invasion assay.

1.11 *Aims*

Aim 1. To identify tumour-specific molecular markers using cDNA arrays, the CGAP website and the current literature.

Aim 2. To validate the markers chosen using relative quantitative reverse transcription (RT) - polymerase chain reaction (PCR) in a large cohort of patient matched tumour and normal colonic mucosal tissue.

Aim 3. To detect disseminated tumour cells using immunobead RT-PCR in pre and post-operative blood and in intra-peritoneal wash samples.

Aim 4. To determine if the presence of disseminated tumour cells is a prognostic factor independent of tumour stage.

Aim 5. To determine the effect of over-expression of selected markers using transfected cell lines and an *in vitro* trans-migration model of invasion.

Aim 6. To determine the effect of knock-down of gene expression using transfected cell lines and an *in vitro* trans-migration model of invasion.

1.12 *Significance and expected outcomes*

This study will investigate genes that are over-expressed in colorectal cancer to establish a panel of tumour-specific markers. Particular emphasis will be placed on identifying novel genes to be used in the immunobead RT-PCR assay to detect tumour cell dissemination in patients undergoing colorectal surgery. Detection of tumour cell dissemination at the time of surgery should more accurately identify patients at high risk of recurrent or metastatic disease, particularly for early stage (stage A and B) CRC patients who would otherwise be considered cured by the surgery alone. This research will assist clinicians in determining the most appropriate treatment options for patients following surgery by predicting whether the patient would benefit from adjuvant therapy, particularly for patients diagnosed with early stage disease. This research would also improve our current understanding of the development and progression of CRC, the genes involved in this process and their biological function. RNAi and antibodies (where possible) will be used as a method to knock-down the expression of candidate over-expressed genes in metastatic colon tumour cell lines to assess the effect on tumour cell growth and invasion. This may also lead to the development of novel therapeutic agents to be used in the treatment of CRC.

Chapter 2

Materials & Methods

2. Materials and Methods

2.1 Specimen Collection

2.1.1 Tissue Samples

Human colon tissue samples were collected from patients aged between 55 – 92 yrs undergoing colorectal surgery at The Queen Elizabeth Hospital between 2000 and 2003. Samples were obtained from tumour and matched normal mucosa adjacent to the resected margin. Normal colonic mucosa was dissociated from the underlying muscle and connective tissue. Samples were cut immediately after colon resection and snap frozen in liquid nitrogen. The remaining resected colon was sent for histopathological analysis. Tumours were staged according to the Australian Clinical Pathological Staging (ACPS) system (19). The study was approved by The Queen Elizabeth Hospital Ethics of Human Research Committee and informed consent was obtained from all patients.

2.1.2 Blood and Intraperitoneal Lavage Samples

Peripheral blood (20 mL) samples were collected before and after colorectal surgery from CRC patients at The Queen Elizabeth Hospital. Intraperitoneal saline lavage samples (50 mL) were collected from the tumour bed and pelvic floor regions of CRC patients before and after manipulation of the bowel. Lavage samples were also collected from the area of obstruction in benign inflammatory bowel disease patients. A 500 mL saline solution, warmed to 37°C, was poured into the peritoneal cavity by the surgeon and a 50 mL aliquot was drawn from the two regions. Samples were then placed into 50 mL tubes containing 75 mg dipotassium EDTA (Sigma, St. Louis, MO) to prevent blood coagulation. Samples were then centrifuged at 1000 rpm for 5 minutes and the supernatant poured off. The cell pellet was resuspended in 10 mL phosphate buffered

saline (PBS) and four million immunomagnetic beads (Dynal, Oslo, Norway) were added.

2.2 *Immunobead RT-PCR*

2.2.1 Patient Samples

The epithelial cell isolation technique developed by Hardingham *et al.*, (1993) involves immuno-magnetic isolation of epithelial cells using Dynabeads (Epithelial Enrich, Dynal) coated with the anti-epithelial antibody Ber-EP4 (38). Four million beads were added to each 10 mL patient peripheral blood or intra-peritoneal lavage sample. Each 10 mL sample was incubated for 2 hours at 4°C with gentle mixing. The samples were then placed against a magnetic field to localise the cell-bead pellet. Epithelial cells bound to the Dynabeads were retained and washed 3 times using PBS (schematic diagram shown in Figure 2.1). The pellet was stored in a 15 µL cell lysis buffer to release RNA, containing UPW (Fisher-Biotech, Perth, Australia), 10mM dithiothreitol (DTT), 0.1% non-ionic detergent (IGEPAL CA-630, Sigma), and 20 U RNasin (Promega) at -80°C until required for reverse transcription (RT).

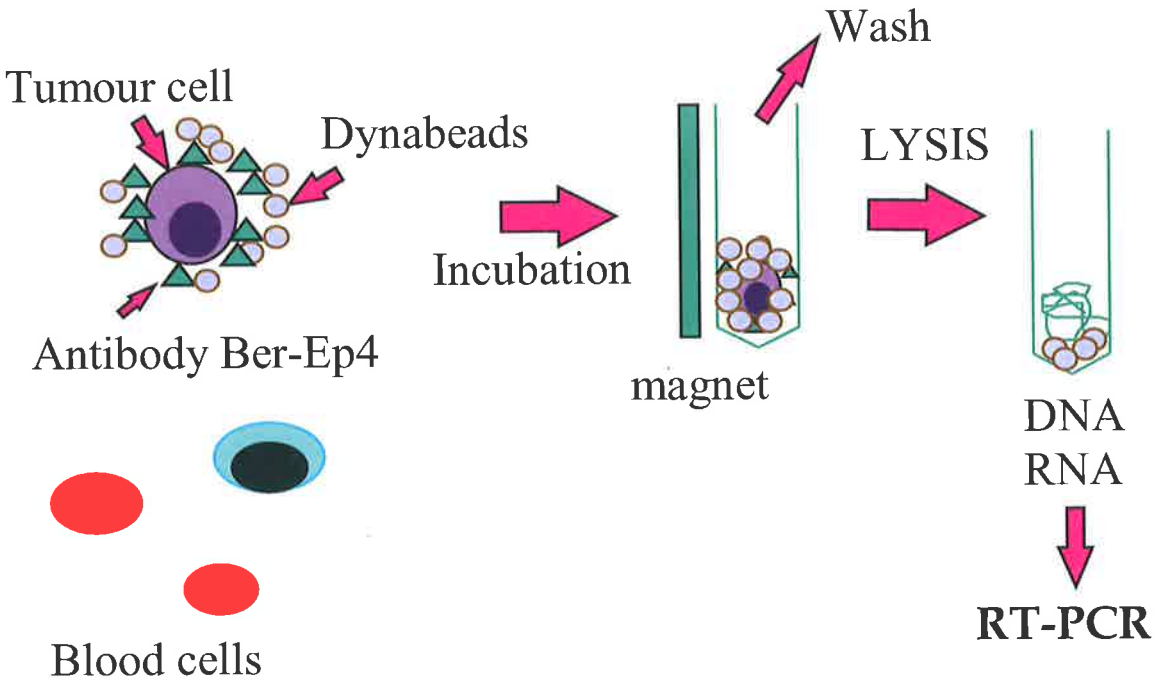


Figure 2.1. Schematic diagram of the Immunobead RT-PCR Technique.

2.2.2 Control Samples

Peripheral blood samples (20 mL) were also collected from healthy age-matched patients not undergoing surgery for bowel disease, as control samples for the immunobead RT-PCR technique for each of the chosen markers. White blood cells (WBC) were also isolated from normal peripheral blood using lymphoprep (Nycomed Pharma AS, Oslo, Norway). RNA was then extracted using TRI reagent (Sigma).

2.3 *Cell Culture*

Ten colon cancer cell lines, derived from varying stages of the disease, were tested in RT-PCR assays to investigate gene expression. The LIM cell lines (LIM-2412, LIM-1215, LIM-2099, LIM-2405, LIM-1899 and LIM-1863) were kindly provided by Dr R. Whitehead, Ludwig Institute for Cancer Research, Melbourne, Australia. SW48, SW480, SW620, and HT29 cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Cell lines were maintained in RPMI-1640 medium (Gibco BRL, Invitrogen Corporation, Bethesda, MD) or Dulbecco's Eagle's minimum essential medium (DMEM) containing amino acids (Sigma) and supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS), 100 U/mL penicillin, 100 mg/mL streptomycin and 1mM glutamine. Cells were cultured in 75 cm^2 vented tissue flasks at 37°C in 5% CO_2. OPTI-MEM® I Reduced Serum Medium (Gibco BRL) was used for siRNA transfection experiments.

2.4 *RNA extraction*

2.4.1 Tissue Specimens

Tumour and normal mucosa tissues were disrupted and homogenised using a mortar and pestle under liquid nitrogen and a 0.9 mm needle and syringe respectively. RNA was extracted from samples using TRIzol Reagent (Invitrogen, Carlsbad, USA), 1 mL per 50-100 mg of tissue, according to the manufacturer's instructions. RNA was redissolved in 100 mL of UPW and the concentration was determined using a SmartSpec™ 3000 spectrophotometer (Bio-Rad, Hercules, USA).

2.4.2 RNA Extraction for Microarray Hybridisation

Following homogenisation, RNA from four patients' tumour and matching normal mucosa samples were isolated using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. An RNA check gel was used to determine sample intergrity. Samples (20 μg) were pelleted and stored in ethanol. Hybridisation of 8K human slides was carried by the Adelaide Microarray Facility (Adelaide University, Adelaide, Australia) using slides printed at the facility. Patients' tumour and normal mucosal samples were labelled with either Cy3 (green fluorescent label) or Cy5 (red fluorescence label) on each slide. Only one patients' matched samples were hybridised to each slide.

2.4.2 i Agarose Gel Electrophoresis of RNA

RNA (2 μL) from each patients' matched tumour and normal mucosa tissue samples was mixed with 2 μL of 2X loading buffer (500 μL reagent grade formamide, 100 μL 10X MOPS buffer pH 7.0, 167 μL 37% formaldehyde, 100 μL glycerol, bromophenol blue, 3 μL 10 mg/mL ethidium bromide and diethyl pyrocarbonate (DEPC, Sigma)-treated H_2O, made up to 1 mL). Samples were heated at 68°C for 3 minutes then placed on ice immediately. Sample tubes were pulse spun before 4 μL was loaded and run on a 1% agarose gel (1 g agarose and 73 mL DEPC-treated H_2O dissolved by boiling in the microwave, then once cooled 10 mL 10X MOPS and 17 mL formaldehyde was added) in a clean electrophoresis tank. Samples were run to about 1 cm from the bottom edge of the gel. The agarose gel was photographed under UV. Samples were chosen for use if distinct 28s and 18s bands were observed on the gel indicating good quality RNA.

2.4.3 RNA Extraction from Cell Culture Cells

Cultured cell lines growing as monolayers were washed twice with PBS and disrupted with 2 mM EDTA. Cells were resuspended in 10 mL culture medium and spun at 1000 rpm for 5 minutes. The culture medium was removed and the cells were resuspended in 2 mL of fresh media. Tri Reagent (Sigma) was added directly to the resuspended cells and RNA was extracted according to the manufacturer's instructions. RNA was eluted in 100 µL of UPW and the concentration was determined using a SmartSpec™ 3000 spectrophotometer (Bio-Rad).

2.4.4 RNA Extraction from siRNA Treated Cell Culture Cells

1-2 x 10^5 LIM-2099 cells were disrupted using 2 mM EDTA as described earlier. The cells were then resuspended in 100-500 µL of fresh media and 175 µL SV lysis buffer (containing β-mecaptoethanol) was added according to the manufacturers instructions for SV Total RNA Isolation kit (Promega, Madison, USA). RNA samples were eluted in 50 µL UPW. Samples were re-precipitated and resuspended in 20 µL of UPW. The RNA sample (20 µL) was reverse transcribed and 2 µL used in subsequent PCR assays.

2.5 *Gene Identification from cDNA microarrays*

2.5.1 Hybond™ Atlas™ Human 1.2 III Array (Human Cancer Array)

The relative expression levels of a given cDNA from two different RNA sources can be assessed by comparing the signal obtained with a probe from one RNA source to that obtained with a probe from another source. Clontech Atlas cDNA expression arrays (BD Bioscience) include hundreds of cDNAs spotted on positively charged nylon membranes.

Plasmid and bacteriophage DNAs are included as negative controls to confirm hybridization specificity, along with several housekeeping cDNAs as positive controls for normalising mRNA abundance. The Hybond™ Atlas™ Human 1.2 III Array includes 1176 human cDNAs, 9 housekeeping control cDNAs and negative controls.

RNA was extracted from colon tumour and matched normal mucosa using TRIzol (Invitrogen). Purification of poly A+ RNA from total RNA was achieved using a poly A+ extraction kit (Qiagen). 32-P labelled cDNA probes were prepared from 1 μg of poly A+ RNA, the probes were hybridised to separate Atlas cDNA expression array membranes according to the manufacturer's instructions. Array images were viewed using a phosphoimager after 24 hour exposure.

2.5.2 cDNA Microarray (glass slides)

8K Human cDNA slides were purchased, hybridised and analysed by the Adelaide Microarray Facility (Adelaide University) and the top 100 differentially expressed genes were identified for each of the 4 patients. To validate the microarray results gene expression databases, available at the CGAP website (http://cgap.nci.nih.gov) (73), were used to confirm the differential expression of genes in colon tumour compared to normal mucosa. We used the digital gene expression displayer (DGED) tool from serial analysis of gene expression (SAGE) databases. Libraries from colon tumour were compared with libraries from normal colon tissue to identify genes that were over-expressed in the tumour but not expressed in normal epithelium. The Digital Gene Expression Displayer (DGED) tool analyses the differences in gene expression between two pools of libraries

and finds only statistically significant differences, based on the sequence odds ratio and the Fisher Exact test. A virtual northern blot demonstrated the expression pattern across a range of human tissues. This enabled the selection of the gene with the greatest differential expression. Gene expression was then further validated using relative RT-PCR.

2.6 *Primer Design and Specificity*

Primers for polymerase chain reaction (PCR) were designed using the Primer3 web-site primer design program (http://frodo.wi.mit.edu/) to amplify specific transcripts for the candidate markers *DPEP-1*, *LAM-γ2*, *MAT* and the internal control markers *MUC2* and *Cyclophilin-33A*. The software program Amplify was also used to optimise primer design. PCR was performed on genomic DNA specimens to confirm that a cDNA product was not amplified of the same size from amplification of genomic DNA. Table 2.1 demonstrates the primer sequences developed to amplify PCR products.

Table 2.1. Relative RT-PCR Primer Sequences

Oligonucleotide		**Sequence 5′-3′**
DPEP-1 (1)	sense	CCTGAGGCTG-GTGAAACAGACA
DPEP-1 (1)	anti-sense	GGGACTCTGGTCTCCCAGGTTT
LAM-γ2 (1)	sense	TGCAATGGGAAGTCCAGGCAGTG
LAM-γ2 (1)	anti-sense	TGCCTCCTCTGTCCACACGGTAG
MAT	sense	TGGAATGTTAAACTCCCGCGTCA
MAT	anti-sense	CCTCATCGAAGTGAGCATCTCCT
MUC2	sense	TGGCTGCGTGGTGGAGAAGGAA
MUC2	anti-sense	TTGGAG-CAGGTGACGCCCGTAGT
Cyclophilin-33A	sense	TAAAAAGGCCCGCTCAAATCCT
Cyclophilin-33A	anti-sense	TCTCCAAACACCACATGCTTGC

2.7 *Reverse Transcription (RT)-PCR*

2.7.1 Immunobead sample RT

Total lysed immunobead samples were denatured at 70°C for 3 minutes then reverse transcribed at 37°C for 1hour in 1x First strand buffer (50mM Tris-HCL pH 8.3, 75 mM KCL, 3 mM $MgCl_2$) and 200U of M-MLV (both from Invitrogen), 750 ng $pD(N)_6$ random hexamers (Pharmacia, Uppsala, Sweden), 0.6 mM of each deoxynucleotide triphosphate (Promega) and UPW to a volume of 30 µL.

Table 2.2 Immunobead RT-PCR Primer Sequences

Oligonucleotide		**Sequence 5′-3′**
DPEP-1 (2)	sense	AGGGCAGCAGTGCACACAGGT
DPEP-1 (2)	anti-sense	CTGCCAGGGGAGGTCATTGT
LAM-γ2 (2)	sense	CTCCAGGAGGGAAGTCTGTG
LAM-γ2 (2)	anti-sense	GCCATCAGTGTTGTCATTGC
MAT	Same as for relative RT-PCR	

2.7.2 Relative RT-PCR

Total RNA (2 μg each of tumour and normal mucosa samples) was denatured at 70°C for 3 minutes then reverse transcribed at 37°C for 1 hour in 1X First Strand Buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 200 U of M-MLV (both from Invitrogen), 750 ng $pD(N)_6$ random hexamers (Pharmacia, Uppsala, Sweden), 0.6 mM of each deoxynucleotide triphosphate (Promega) and UPW (Biotech International) to a volume of 30 μL.

MUC2 and *Cyclophilin-33A* were used as internal control markers after it was determined that the expression in normal colon mucosa and tumour tissue samples were similar across multiple samples. The primers used for *DPEP-1* (product size 502 bp), *LAM-γ2* (product size 751 bp), *MAT* (product size 373 bp), *MUC 2* (product size 320 bp), and *Cyclophilin-33A* (product size 402 bp) are demonstrated in Table 2.1. Primers were used in a mulitiplex PCR performed on a 2 μL aliquot of cDNA from patient tissue samples or cell lines. Reaction conditions included 100 ng of each primer (except *MUC 2* where only 10 ng of each primer was required), 0.75 U of Tth plus Taq polymerase (Biotech

International), 5 µl of 10X PCR buffer (670 mM Tris-HCl pH 8.8, 166 mM $[NH_4]_2SO_4$, 4.5% Triton X-100, 2 mg/mL Gelatin), 1.5 mM $MgCl_2$ (Biotech International), 200 µM of each deoxynucleotide triphosphate (Promega) and UPW (Biotech International) to a final volume of 50 µL. The exponential phase of the amplification reaction was established for each of *DPEP-1*, *LAM-γ2* and *MAT* at (32, 30 and 32 cycles respectively). Cycling conditions involved an initial denaturation at 94°C for 5 minutes, then 30 seconds at 94°C, gene annealing temperature and 72°C for each of the cycles with a final extension of 7 minutes at 72°C. Following the identification of positive expression in cell lines, the cDNA from these were subsequently used as positive controls. Negative controls consisted of reagent only (no target).

The amount of expression (ng) of the PCR product was normalised relative to the amount of expression (ng) of the MUC2 or Cyclophilin-33A product and the expression of each patient tumour-normal pair was expressed as a ratio T:N (Figure 2.2). Cyclophilin-33A was used as an internal control when the size of the MUC2 product was similar to the size of the gene of interest, for example, for *MAT* samples.

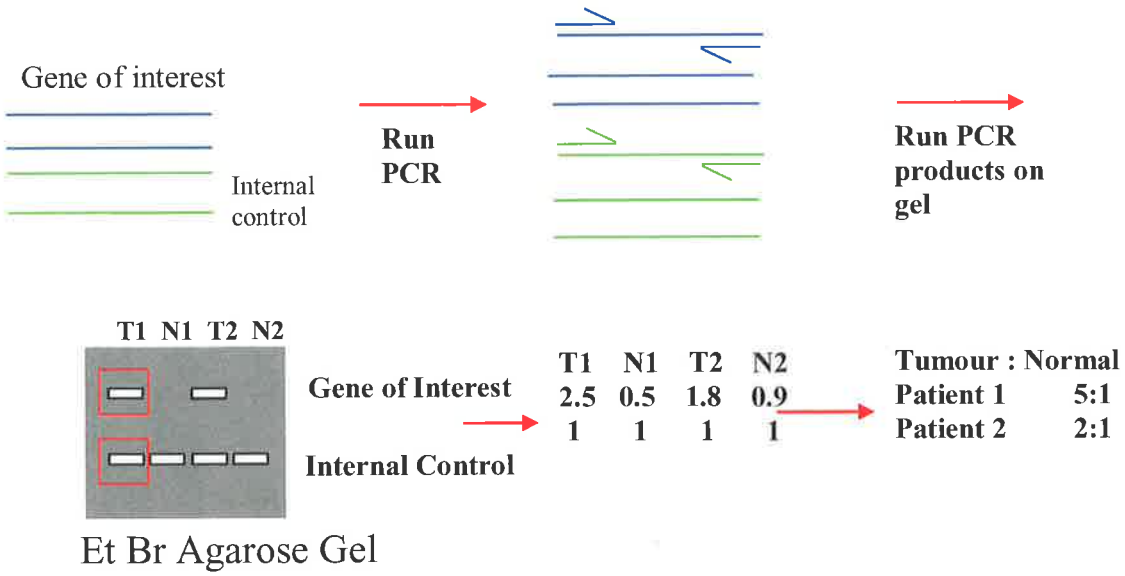


Figure 2.2 Schematic diagram of Relative RT-PCR technique.

2.7.3 Agarose Gel Electrophoresis

PCR products were run on a 1.5% agarose gel and stained with ethidium bromide. Gel analysis was performed using Kodak 1D image analysis software (Eastman Kodak Company, New Haven, CT).

2.7.4 Statistical Analysis of Over-Expression

Comparison of the expression of *DPEP-1* between colon tumor and normal mucosa was performed using a paired students *t*-test ($P<0.05$). Analysis of colon tumor expression between the three stages of the disease was performed using ANOVA. A difference between groups of $P<0.05$ was considered significant.

2.7.5 Southern Blotting

Following gel electrophoresis DNA products from immunobead RT-PCR assays were transferred to a nylon membrane, Hybond N+ (Amersham), and hybridised to a 32P end-labelled internal oligonucleotide probe. Autoradiographs were exposed for 6-72 hours.

2.7.6 Quantitative real-time RT-PCR

Real-time PCR was performed on a 2 µL aliquot of cDNA in a total reaction volume of 25 µL with the following conditions: 0.5 U HotStarTaq®, 2.5 µL 10X Reaction Buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl^{2+}$) and additional $MgCl^{2+}$ to a reaction concentration of 2 mM (each from Qiagen), 50 ng of each sense and anti-sense primer, 100 µM of each deoxynucleotide triphosphate (Promega), 0.2 µL SYBR® Green (Adelab Scientific, Adelaide, Australia) and UPW.

2.8 *Cloning*

2.8.1 Primer Design (Xi-Clone™ Conversion Kit)

Primers were designed following the manufacturers instructions for the Xi-Clone™ Conversion Kit. Primers were designed in front of the start and behind the stop codon of the gene of interest (*DPEP-1*), to amplify the open reading frame of the gene. The 5' PCR primer contained 28 nucleotides overlapping with the 5' end of the linear vector plus 20 nucleotides that were gene specific sequence. The 3' PCR primer contained 28 nucleotides that were complementary to 3' end sequence of the linear vector plus 20 nucleotides that were gene specific sequence (Table 2.3). The PCR product was amplified

using Expand Long Template PCR System (Roche, Mannheim, Germany) containing a proofreading polymerase according to the manufacturers' instructions.

Briefly, primers were used in a PCR assay performed on a 2 μl aliquot of cDNA from colon cancer cell lines. Optimal reaction conditions were carried out for PCR products between 0.5-12 kb in length (*DPEP-1* product size 1.3 kb). The manufacturer recommended the preparation of two separate master mixes, to avoid the enzyme mix interacting with the primers or template without dNTP's which could lead to partial degradation of the primer and template through the 3'-5' exonuclease activity of the proofreading polymerase. Master Mix 1 comprised of 350 μM of each deoxynucleotide triphosphate (Promega), 300 nM of each primer, 500 ng of template DNA and up to 25 μL of UPW. Master Mix 2 comprised of 5 μL of 10x PCR buffer (system 1; 2 mM Tris-HCl, 100mM KCl, 1mM DTT, 0.1mM EDTA, 0.5% Tween20, 0.5% Nonidet P40 and 50% glycerol) with $MgCl^{2+}$, 0.75 μL of enzyme mix and up to 25 μL of UPW. Thin-walled (0.2 mL) PCR tubes were also recommended. Cycling conditions included an initial denaturation for 2 minutes at 94°C, then for 10 cycles, denaturation at 94°C for 10 seconds, annealing at 65°C for 30 seconds and elongation at 68°C for 1 minute. For the remaining 20 cycles the elongation step included an additional 20 seconds for each cycle. PCR fragments were purified using the provided PCR Cleanup Spin Column. The recovered PCR product was resuspended in 50 μL of TE buffer.

Table 2.3 Xi-Clone PCR Primers 5′-3′

DPEP-1 (Xi-clone) sense

CGGACTCAGATCTCGAGCTCAAGCTTCGAGGTCCCCGGGGACCCCACC

DPEP-1 (Xi-clone) anti-sense

GCCCCGCGGTACCGTCGACTGCAGAATTGGGGACTCTGGTCTCCAGGTT

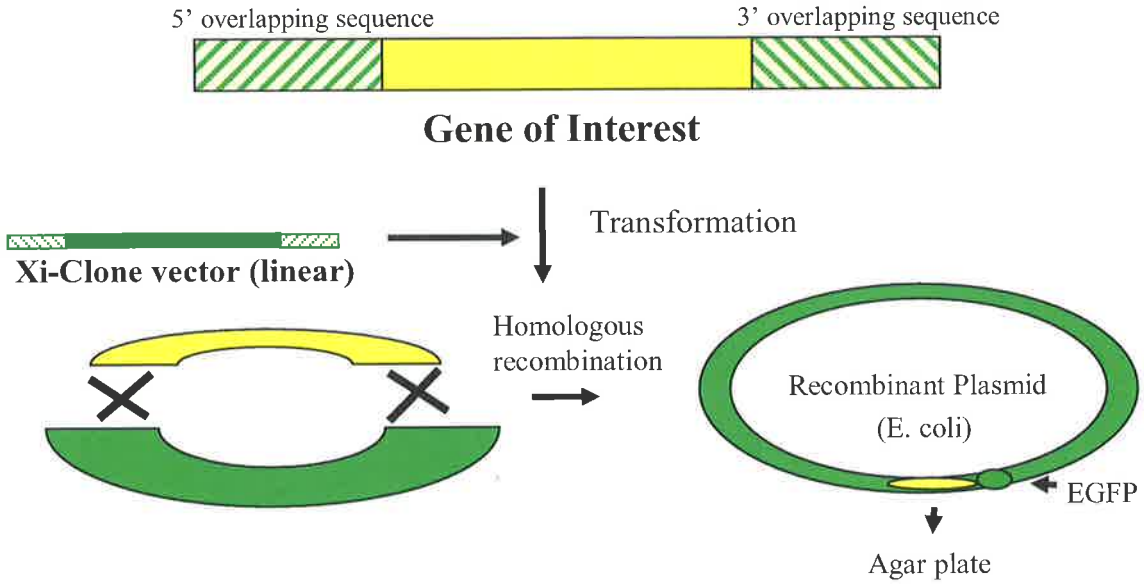


Figure 2.3 Schematic diagram of the Xi-clone conversion kit method.

The Xi-Clone™ PCR Cloning Technology (Figure 2.3) allows rapid, efficient, and directional cloning of PCR products. Using the conversion kit, a Xi-Clone-ready vector does not require the use of a ligase or multiple restriction digests for cloning experiments. The technology simplifies directional cloning by using only a single restriction digest in the multiple cloning site (MCS) of the vector of choice, in this instance the pIRES-EGFP vector (Figure 2.4).

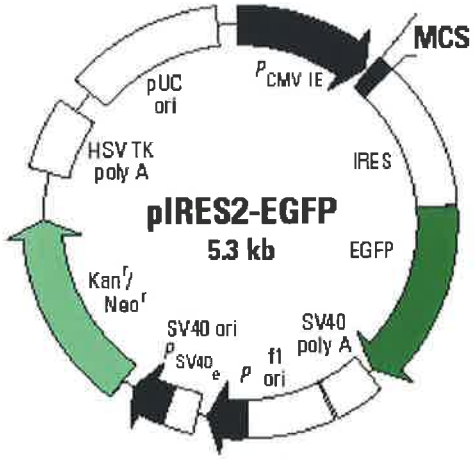


Figure 2.4 pIRES-EGFP mammalian expression vector (Clontech, BD Biosciences)

2.8.2 Plasmid Eco-RI restriction digest

The pIRES-EGFP vector was linearized using Eco-RI restriction enzyme (MBI Fermentas) according to the methods and procedures to convert the plasmid into a Xi-Clone™-ready vector. Briefly, a restriction digest using 1 μg of plasmid, digest buffer (50 mM Tris-HCL, 10 mM $MgCl_2$, 100 mM NaCl, 0.02% Triton X-100, 0.1 mg/mL BSA), UPW and 20U enzyme was carried out in a final volume of 40 μL. The digest was incubated at 37°C for 3 hours. An additional 1 μL of the restriction enzyme was added after the initial incubation and the digest was continued for another 3 hours. The addition of another 1μL of restriction enzyme was followed by an overnight incubation at 37°C. The linearized vector was then purified using a supplied DNA cleanup Spin Column. The DNA was resuspended in 50 μL of filter sterilized TE buffer and stored at -20°C.

2.8.3 Transformation

SmartCells™ (competent *E. coli*) were thawed on ice for 10 minutes. 10 µL of purified PCR fragment were mixed with 2 µL of purified linear Xi-Clone vector in a pre-chilled tube. 10 µL of SmartCells™ were added to the PCR product and vector mix and incubated on ice for 30 minutes. The mix was then heated at 42°C for 1 minute. 100 µL of SOC medium was added to the mix and incubated at 37°C for 1 hour in an air incubator. The transformation mixture was then spread on LB Agar plates containing Kanamycin antibiotic (50 µg). Colonies were picked and purified using a Qiagen Plasmid Mini-Prep kit and resuspended in 100 µL UPW. Unused SmartCells™ were stored at -70°C.

2.8.4 Sequencing

Sequencing was carried out at the Sequencing Facility, Flinders Medical Centre, Adelaide, Australia to confirm the amplification of bases for the gene was correct (Appendix I).

2.9 *Transfection*

2.9.1 Gene Therapy Systems Lipid Transfection

$2x10^6$ HT29 cells were incubated overnight at 37°C in 6-well culture plates. GenePORTER™ 2 Transfection Reagent (GeneTherapy Systems) was diluted with serum-free medium. In a separate tube, plasmid DNA was diluted with diluent B (GeneTherapy Systems), mixed well by pipetting and incubated for 5 minutes at room temperature. The plasmid DNA solution was added to the diluted GenePORTER2 reagent

and incubated at room temperature for 5 minutes to form GenePORTER2/DNA complexes. The complexes were added directly to the cells in serum-free medium in a final transfection volume of 1 mL. Twenty-four hours post-transfection fresh serum-containing medium was added. Forty-eight hours after transfection 2.5 mg/mL G418 (Gibco) was added for stable transfectant selection.

2.9.2 Electroporation

1 x 10^7 cells were resuspended in 1mL of culture media. 5 µg of either pIRES-EGFP vector only (VO) or pIRES/EGFP-DPEP was mixed with 400 µL of HT29. Electroporation conditions were 975 µF and 300 V. A Chinese hamster ovary (CHO) cell-line was also transfected with VO or pIRES/EGFP-DPEP as a control, as CHO cells do not express *DPEP-1*.

2.10 RNA interference

2.10.1 Design

siRNA oligonucleotide template sequences were designed with the aid of Ambion's siRNA template design tool (101), and synthesised by an *in vitro* T7 transcription method according to the manufacturers instructions of the Silencer Construction Kit (Ambion, Austin, USA) for *DPEP-1* and *LAM-γ2* (Table 2.4).

Table 2.4 siRNA Oligonucleotide Template Sequences

DPEP-1 sense template	5′- AAGTTGGGCAGGAAATGTAACCTGTCTC - 3′
DPEP-1 anti-sense template	5′- AATTACATTTCCTGCACCAACCCTGTCTC - 3′
LAM-γ2 sense template	5′- AAATCGACACCTATCACAGCGCCTGTCTC - 3′
LAM-γ2 anti-sense template	5′- AACGCTGTGATAGGTGTCGATCCTGTCTC - 3′

2.10.2 siRNA Transfection

LIM-2099 cells were optimised for transfection efficiency according to instructions outlined for the transfection reagent siPORT Lipid (Ambion). For *LAM-γ2* transfections, cells (0.75×10^5) were seeded onto a 24-well culture plate and incubated for 24 hours. For *DPEP-1* transfection 1×10^5 cells were seeded onto the culture plates and also incubated for 24 hours, siRNA was then transfected into the cells with siPORT lipid transfection reagent according to the manufacturer's instructions. Briefly, 10 nM of siRNA was incubated with siPORT Lipid for 20 minutes. The complex was then added to the cells in a final volume of 200 µL with the addition of OPTI-MEM (Gibco Life Technologies) reduced serum media and incubated at 37°C for 24 hours, normal DMEM media with 10% FCS was then added. The cells were harvested after 48 hours for analysis by real-time PCR or used in the Matrigel Invasion Assay. Adherent cells were collected from their culture wells by a 5 minute exposure to 2 mM EDTA. For real-time PCR analysis, RNA from adherent cells was extracted using an SV total RNA isolation kit (Promega) and for the reverse transcription 0.5 µg of Oligo d(T) (Promega) were used in the place of random hexamers.

2.10.3 Statistical Analysis

A paired students T-test was used to compare the invasion capacity of *DPEP-1* siRNA treated LIM 2099 cells with mock transfected and scrambled (control siRNA) treated LIM 2099 cells. A value of $P \leq 0.05$ was considered significant in all statistical analyses performed.

2.11 Invasion Assay

2.11.1. Matrigel-Coated Two-Chamber Invasion Assay

The effect of treatment conditions (over-expression of *DPEP-1*, RNAi, and incubation with *LAM-γ2* antibodies) on cell migration and invasion through a basement membrane-like substance (Matrigel™, BD Biosciences) was determined using a 2-Chamber Transwell system, a schematic diagram of this system is shown in Figure 2.5. Polycarbonate filters (pore size 8 μm, diameter 6mm; Costar, Cambridge, MA, USA) were coated with 70 ug/cm2 Matrigel (1:3, diluted in culture medium), as optimised previously in our laboratory by PhD student Evelyn Douglas. A total of $1x10^5$ cells (LIM-2099 or HT29, treated and untreated) in 100 μL of culture media were added per well in the upper compartment, and the lower compartment was filled with 600 μL of culture medium containing 10% FCS. Antibodies (5 μg anti-*LAM-γ2* or 5 μg anti-IgG_1) were added to the upper compartment, together with the cells. Following a 48hr incubation at 37°C (5% CO_2) the cells remaining in the upper chamber were removed with cotton swabs, the cells that had migrated to the underside of the membrane filter were fixed and stained with Diff-Quick. Filters were removed from the transwell and mounted on slides to count (9 fields of view, 100X magnification).

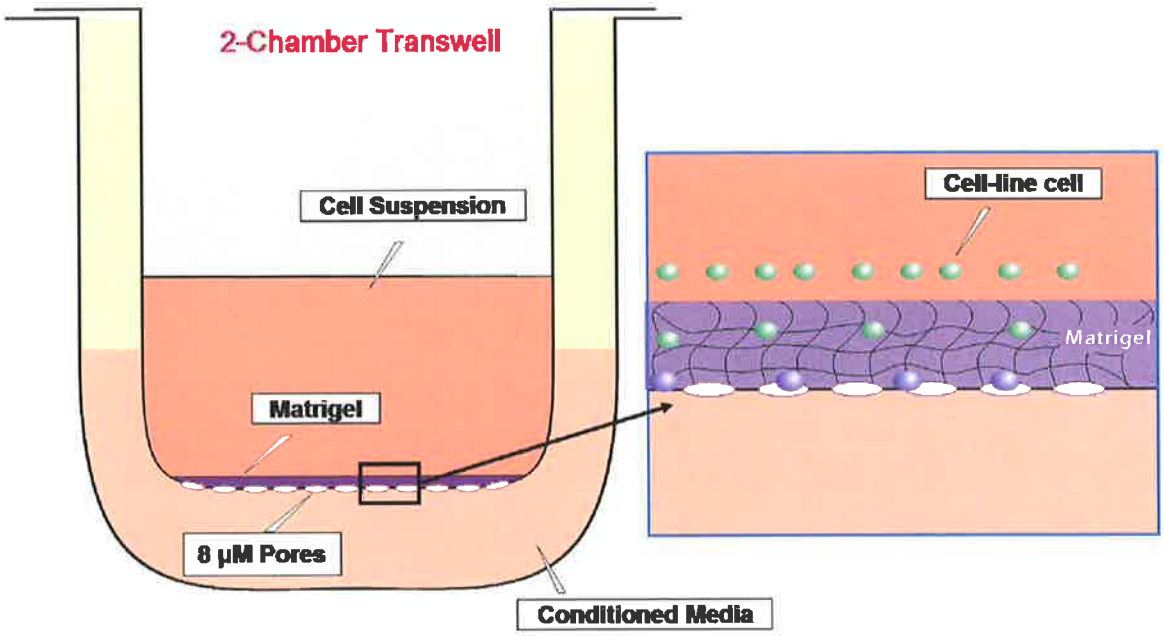


Figure 2.5 Schematic diagram of the 2-Chamber Transwell Invasion Assay with Matrigel™. Picture courtesy of Christopher Ross.

2.11.2 Transfectants

HT29 cells either transfected with VO, or pIRES/EGFP-DPEP or cells alone were seeded into the upper-chamber of the 2-well chamber system coated with Matrigel. Cells were fixed and stained after 48 hours.

2.11.3 Inhibitors and Antibodies

The *DPEP-1* antibody (MDP-Membrane Dipeptidase antibody) was a kind gift from Dr N. M. Hooper (The University of Leeds, UK). The *LAM-γ2* antibody was purchased from Chemicon (Temecula, CA). IgG_1 normal antibody was purchased from R&D

Systems, Inc. LIM 2099 cells ($1x10^5$) were seeded into the top chamber of the transwell in 100 µL of serum-containing media. Antibodies, 5 µg of *LAM-γ2* or 5 µg of IgG_1 were also added to the upper chamber and incubated for 48 hours at 37°C in 5% CO_2.

2.11.4 Statistical Analysis

An Analysis of Variances (ANOVA) was used to determine the significance in invasion capacity between *DPEP-1* transfected HT29 cells and HT29 untreated or vector only control transfected cells.

2.12 Fluorescent Immunohistochemistry

2.12.1 Frozen Tissue Sections

Snap frozen colon tumour and normal mucosal tissue were cut (10 µM and 20 µM thick sections) using a cryostat. Three tissue sections were placed onto each Poly-prep™ (Sigma), labelled appropriately and stored at -70°C. Slides were fixed by placing upright in a staining jar containing 100% ethanol for 5 minutes. Slides were then transferred to a PBS bath and washed 3 times for 5 minutes each. They were dried using KimWipes to remove the PBS without drying out the section. Each tissue section was circled using a PAP pen to trap antibody solutions. 50 µL of 10% normal serum was added per slide to the sections. Serum was from the host animal used to raise the secondary antibody (anti-mouse for *LAM-γ2* and anti-rabbit for *DPEP-1* antibodies). Each slide was dried and then the antibody solution added. Slides were then placed on a tray in a humid environment (sealed lunchbox containing 2 cm high pure water) and incubated at room temperature for 30 minutes. Excess solution was blotted from the section using KimWipes and 50 µL of

primary antibody (1:200 for *LAM-γ2* and 1:2000 for *DPEP-1*) was added to each slide. The slides were then placed back into the humid environment for 1 hour at room temperature then overnight at 4°C. The secondary antibody was added to the sections in the same way as the primary antibody following three washes with PBS. Slides were left in the humid environment for 2 hours at room temperature. The slides were dried and washed 3X with PBS, then dried again before 50 μL of fluorescent streptavidin was added and incubated in the humid environment for 2 hours. Slides were counter-stained after being dried and washed again 3X with PBS. After drying, 50 μL of DAPI (3 μM) was added and the slide was incubated in the humid environment at room temperature for 10 minutes. Slides were then dried and washed once with PBS. Once slides were dried, 2 drops of fluorescent mounting medium was added and a cover slip placed on top. Slides were stored in the dark at 4°C.

2.12.2 Paraffin Embedded Tissue Sections

Sections of human colon cancer biopsy (cut by TQEH Department of Pathology) were rehydrated through a histolene™ and ethanol series before washing in 0.1 M PBS three times for 5 min each. Antigen retrieval was performed in acetate buffer (0.1 M, pH 4.75) for 10 min at 121°C. Sections were washed as before and incubated with 10% normal donkey serum (Sigma, St Louis, MO) for 30 min at room temperature before incubation overnight at 4°C in a cocktail of *DPEP-1* antibody and *LAM-γ2* antibody (Chemicon) diluted in 10% normal donkey serum to final concentrations of 1:2000 and 1:100 respectively. The sections were washed and incubated for 2 hours at room temperature with a combination of Cy2 conjugated donkey anti-mouse IgG and Cy3 conjugated

donkey anti-rabbit IgG (Jackson ImmunoResarch, West Grove, PA) at concentrations of 1:100 and 1:200 respectively in PBS (0.1 M, 0.01% azide). After washing, the sections were incubated with 3μM DAPI for 10 minutes at room temperature and washed a final time. All slides were mounted with glass cover slips using an antifade aqueous mounting medium (DAKO, Carpinteria, CA). To confirm the specificity of the staining for the antibodies, primary omission controls were performed as well as a pre-absorption control for the DPEP antibody. Substitution of the monoclonal *LAM-γ2* antibody with another monoclonal antibody against a protein not expressed by the colon was performed to confirm the specificity of the *LAM-γ2* antibody. Staining was visualised using an Olympus BX50 fluorescent microscope and the images were captured with a Spot RT CCD camera. Fluorescent immunohistochemistry was conducted with the assistance of PhD student Kirsten Farrand, Department of Physiology, Adelaide University. Matched paraffin-embedded normal colonic mucosa tissue was unavailable for comparison.

2.13 Apoptosis Assay

An Annexin-V-FLUOS Staining Kit (Roche, Mannheim, Germany) was used to determine whether treatment with the anti-*LAM-γ2* antibody effects the rate of cell death in LIM 2099. Briefly, 20 μL of Annexin-V-FLUOS labelling reagent and 20 μL propidium iodide solution was pre-diluted in 1mL of incubation buffer (HEPES). Cells were washed once with PBS and 100 μL of solution was added. Cells were incubated for 10 minutes at room temperature, then analysed by fluorescent microscopy.

Chapter 3

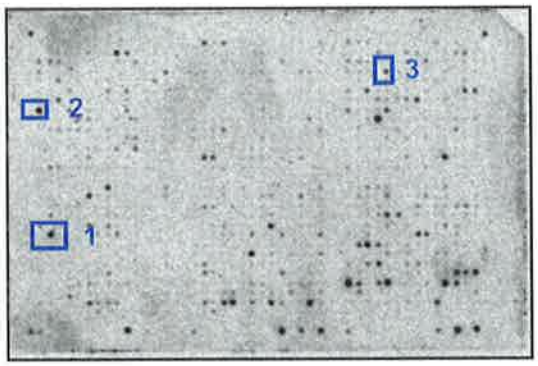
Identification of Genes Over-Expressed in Colorectal Cancer

3.1 *cDNA Nylon Membrane Array*

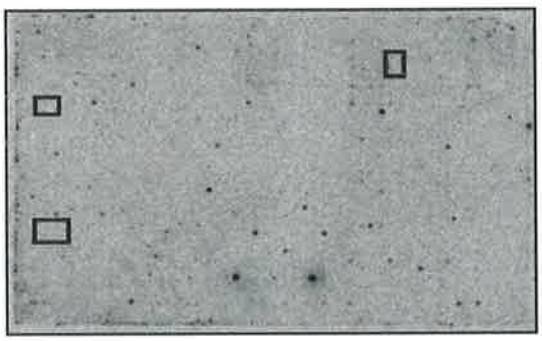
3.1.1 Hybond™ Atlas™ Human 1.2 III Array (Human Cancer Array)

To detect disseminated tumour cells in CRC patients' peripheral blood or intra-peritoneal wash samples, candidate colorectal tumour-specific gene markers were identified. Nylon membrane cDNA arrays (Clontech, BD Biosciences) were originally used to identify candidate gene markers. As described in Chapter 2, cDNA arrays were probed with radioactively labelled samples derived from colon tumour mRNA and normal colonic mucosal mRNA. The Human Cancer Arrays are spotted with cDNAs in a pattern according to functional classes that represent many current areas of cancer research i.e. oncogenes, tumour suppressor genes, cell cycle regulators, transcription activators and apoptosis related genes. Labelled arrays with both tumour and normal colonic RNA are demonstrated in Figure 3.1.

TotalLab Array analysis software (AtlasImage™), available on the internet, was used to identify genes over-expressed in matched colon tumour samples compared to normal colonic mucosal samples. The amount of expression for each of the spotted cDNAs was determined by the software program after normalisation to the housekeeping genes. By comparing genes expressed in colon tumour tissue to normal colonic mucosa a number of potential markers with the greatest difference in expression values were identified.



Tumour mRNA
1, Laminin 37kD receptor precursor
2, Human Interferon-Inducible Protein *(HIIP 9-27)*
3, Oligophrenin-1



Normal mRNA

Figure 3.1 Hybond™ Atlas™ Human 1.2 III Array (Human Cancer Array) and potential candidate markers identified. Arrays probed with 50 μg total RNA from matched patient tumour and normal mucosal tissue. Images were viewed after 24 hour exposure and gene expression was analysed using AtlasImage™.

Potential candidate tumour-specific molecular markers identified included the Laminin 37kD receptor precursor gene, human interferon-inducible protein 9-27 (*HIIP 9-27*), and Oligophrenin-1. Table 3.1 demonstrates the National Centre for Biotechnology Information (NCBI) GenBank sequence identification numbers for each of the potential candidate genes, the primer sequences used and whether they have previously been reported to be over-expressed in colorectal carcinoma.

Table 3.1 Potential candidate colon tumour expression markers as identified by the Hybond™ Atlas™ Human 1.2 III Array.

Nylon Membrane cDNA Array

Gene Name	NCBI sequence ID	Previously Reported in CRC	Primer Sequences Used 5' - 3'	Product size
Human Laminin Receptor 1 (67kDa Ribosomal Protein)	BC005391	Sanjuan *et al.*, (1996)	F1-TCA CAA TGT CCG GAG CCC TT R1- CAT TCA GTG GCC TGA GCA GTG F2- GCT GAC CAC CAG CCT CTC AC R2- TCC CGA GCC AGC A TC CAC CA	863bp 158bp
Human Interferon-Inducible Protein 9-27	j04164	Kitahara *et al.*, (2001)	F- TTG GTC CCT GGC TAA TTC AC R- TTG GGG AAG GAA GTG TTG AG	498bp
Oligophrenin-1	AJ001189	Pinheiro *et al.*, (2001)	F1- TGC TTA CCT TGG TTG GAA GGGA R1- AAA GGG ACC TGG CAA GGG TTT A F2- TGG AAG CCA TGG ATG GGA AAG A R2- CTC GCA GCA AGC GCT TTG AAA T	991bp 716bp

Ideal candidate genes were selected on the basis of the most suitable markers to use in the immunobead technique. Genes were selected that were not previously reported to be expressed in human white blood cells. This criterion was established as a precaution, to prevent false positive results due to the potential for the cells to be captured non-specifically by the immunobeads, although the cell-bead pellet is washed three times in PBS to remove any potentially contaminating cells.

3.2 *Potential Candidate Genes Identified*

3.2.1 Laminin 37kD Receptor Precursor

The 67kD laminin receptor is a cell surface protein whose expression has been identified as up-regulated in the progression of human colorectal carcinomas (102). This protein, which binds laminin with high affinity, was previously found to be expressed in a wide variety of mammalian cells and to be particularly abundant on the cancer cell surface. The interaction between tumour cell surface laminin receptors and their ligand laminin as a component of the extracellular matrix appears to play a prominent role in the highly complex and multistage mechanism of tumour cell invasion and metastasis (reviewed in (103)). The 37-kDa polypeptide is a precursor of the mature 67-kDa laminin receptor and exhibits both a ligand binding domain and a membrane-associated domain, consistent with the properties of the mature laminin receptor (104). It is now believed that the 37-kD laminin receptor precursor is actually a ribosome-associated molecule involved in protein synthesis that acquired the additional novel functions of the laminin receptor during evolution (105). As shown in Figure 3.1 the human 37-kD laminin receptor precursor

gene was identified from a nylon membrane cDNA microarray as over-expressed in colon tumour.

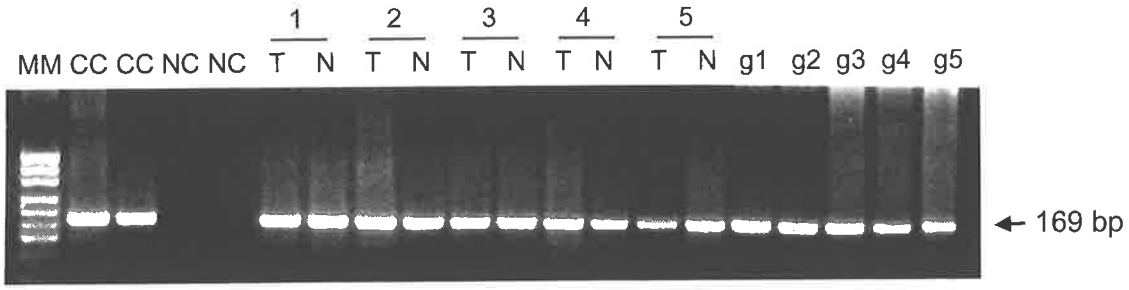


Figure 3.2 Laminin receptor precursor gene expression in five matched patient tumour and normal mucosal mRNA samples. DNA samples from five patients were included in the PCR and were positive at the same position as mRNA samples. MM; molecular marker pUC19. CC; cell line control cDNA, NC; negative control (no template), T; tumour cDNA, N; normal mucosa cDNA, g; genomic DNA.

Pseudogenes for the human laminin receptor precursor have previously been identified (106). For the current study, numerous primers were designed using Primer3 and Amplify primer design software, to distinguish the longer DNA product compared to the cDNA product. Unfortunately a PCR product of the same size was amplified for both cDNA and genomic DNA samples, as demonstrated in Figure 3.2. Due to the presence of these reported pseudogenes, and the inability to amplify a PCR product band of differing size for mRNA and DNA samples, the laminin receptor precursor gene was determined to be inappropriate for use as a candidate marker for tumour cell dissemination. DNAse treatment of the samples was not deemed appropriate to avoid the problem due to the low number of cells that the immunobead technique captures and therefore small amount of RNA, which may have been reduced further by DNAse treatment.

3.2.2 Human Interferon-inducible protein (HIIP 9-27).

Interferons are multifunctional cytokines that are normally associated with immune defence against viral or parasitic infections (107). Up-regulation of expression of the Human interferon-inducible protein *(HIIP 9-27)* in colon tumour tissue was first identified by Kitahara *et al.*, (2001). They used laser capture microdissection and cDNA glass slide arrays to identify a number of up-regulated genes in CRC (65). The interferon-inducible membrane proteins of approximately 17-kDa have been suggested to play a role in the anti-proliferative activity of interferons. This is based on their pattern of induction in interferon-sensitive and interferon-resistant cell lines and the ability of a membrane fraction enriched in 17-kDa proteins to inhibit cell growth. Results by Deblandre (1995) indicate that there is a novel level of cellular regulation by interferons involving a membrane protein, encoded by the *HIIP 9-27* gene which associates with other proteins at the cell surface, forming a complex relaying growth inhibitory and aggregation signals. It is unclear what the role of this gene plays in colorectal carcinoma. However, interferon alpha-inducible protein 27 (*IFI27*) has been identified as up-regulated in psoriatic skin and certain epithelial cancers (108). *IFI27* was found in the proliferating subpopulation of keratinocytes during normal wound repair and was observed to be stimulated by IFN-gamma, TNF-alpha or TGF-beta1. These results suggest that *IFI27* is a novel marker of epithelial proliferation and cancer. *HIIP 9-27* has also been identified to be stimulated by Angiotensin-II, a molecule which has been identified to induce proliferation in zona glomerulosa cells (109).

In the current study, *HIIP 9-27* was found to be over-expressed in colon tumour tissue compared to normal colonic mucosa as identified in Figure 3.1. Expression for this gene was tested in colon cancer cell lines with 9/10 showing positive expression. However, LIM 2412 and LIM 2099 were only slightly positive, and SW620 demonstrated negative expression as shown in Figure 3.3.

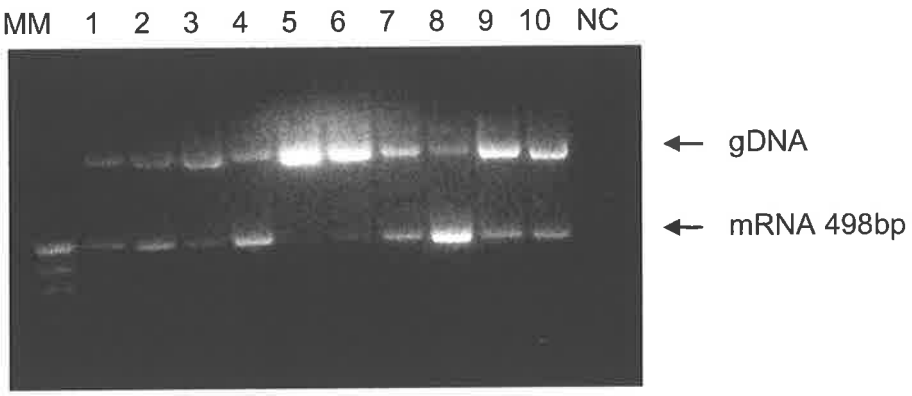


Figure 3.3 The expression of *HIIP 9-27* was tested in colon cancer cell lines. MM; molecular marker, pUC19, Lane 1; LIM 2405, Lane 2; HT-29, Lane 3; LIM 2412, Lane 4; SW 480, Lane 5; SW 620, Lane 6; LIM 2099, Lane 7; LIM 1863, Lane 8; LIM 1215, Lane 9; LIM 1899, Lane 10; SW 48 and NC; No target control. 9/10 cell lines were positive.

HIIP 9-27 expression was examined in patient DNA samples. A distinctive band, greater in size than that amplified from mRNA samples, was identified as shown in Figure 3.4. Patient tumour and matched normal mucosal samples were then tested for *HIIP 9-27* expression.

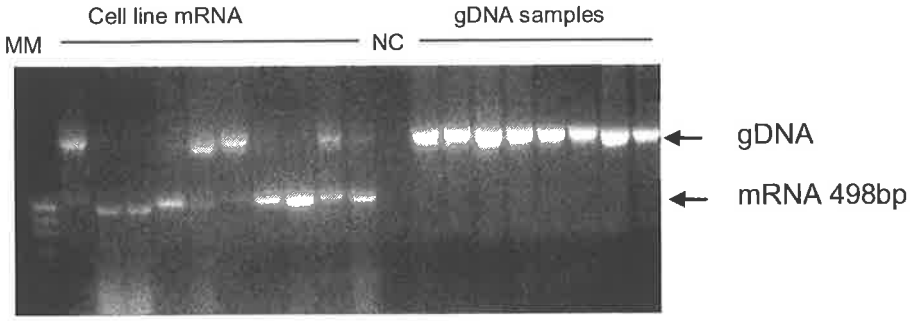


Figure 3.4 Expression of *HIIP 9-27* in colon carcinoma cell lines and DNA samples.

An initial semi-quantitative PCR was run to identify whether *HIIP 9-27* expression did appear to be over-expressed in colon tumour samples. Some samples appeared to be up-regulated while others appeared to be down-regulated. Further testing of matched patient tumour and normal samples was conducted to determine if *HIIP 9-27* was a suitable marker for this study.

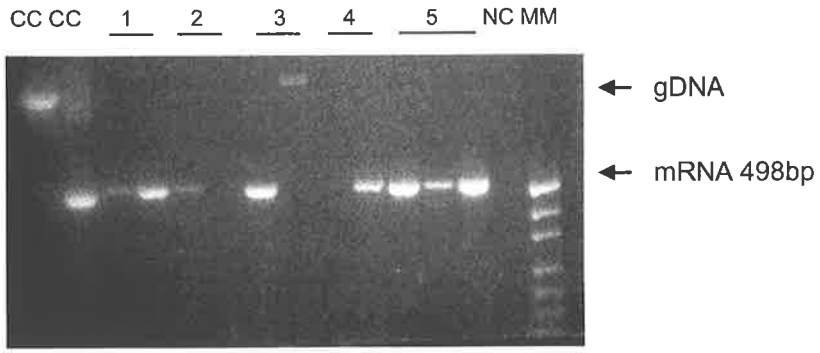


Figure 3.5 *HIIP 9-27* Semi-quantitative PCR of patient matched tumour and normal colonic samples. 3/5 patients appeared to be positive for *HIIP 9-27* over-expression in tumour compared to normal (Patient's 2, 3 and 5). CC; cell line control, Patient 1-4; Tumour and Normal colonic RNA samples, Patient 5; Tumour, Normal and Liver Metastasis, NC; Negative Control, MM; Molecular Marker, pUC19.

To quantitate and compare gene expression between matched colon tumour and normal mucosa, a multiplex PCR assay adapted from the Relative Quantitative RT-PCR technique (Ambion) was used. The technique involves using primers for an internal control in conjunction with primers for the gene of interest. The assay is quantified within the linear range of amplification of the PCR product for the gene of interest. The linear range of the reaction is defined as the period of the PCR in which the amplification efficiency is at its maximum and remains constant over a number of cycles. At some point during the reaction, the amplification efficiency falls and the rate of product accumulation slows or plateaus. To obtain meaningful results, relative RT-PCRs must be terminated for product quantification when all samples are in the linear range of amplification (Ambion Relative RT-PCR manual). An example of a PCR assay to determine the linear range for *HIIP 9-27* is shown in Figure 3.6.

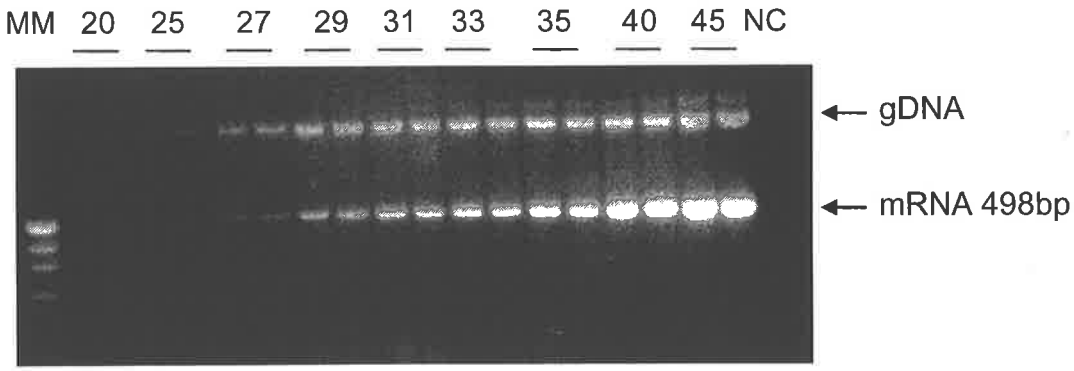


Figure 3.6 *HIIP 9-27* Linear range. MM; molecular marker, pUC19, NC; No target control, 20-45; PCR cycle numbers.

The *HIIP 9-27* PCR was determined to be in the linear range at 30 cycles. Relative RT-PCR was used to determine if *HIIP 9-27* was over-expressed in a majority of patient matched tumour compared to normal samples.

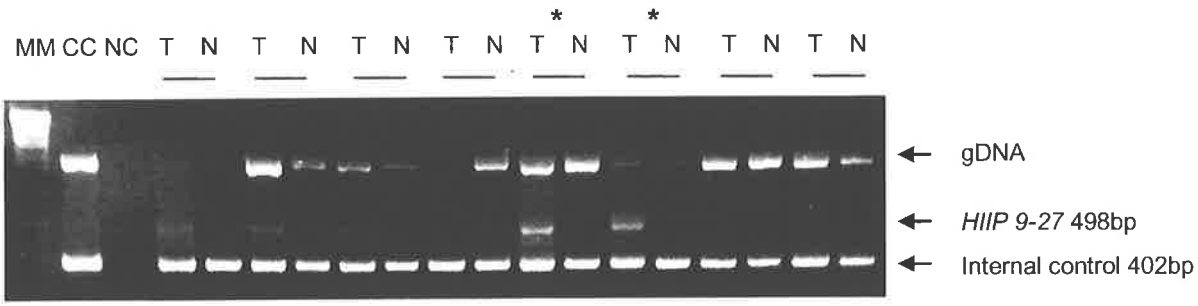


Figure 3.7 Expression of *HIIP 9-27* in 8 matched patients' tumour and normal colonic tissue samples. MM; molecular marker (SPP1), CC; Cell line control, NC; negative control. Kodak software was used to normalise HIIP 9-27 expression against the internal control for each sample.

Expression of *HIIP 9-27* was investigated in 24 patients' matched tumour and normal mucosa tissue samples. Ten of 24 (42%) patients' samples demonstrated over-expression of *HIIP 9-27* by 1.5X or greater. Only 5 patients showed over-expression greater than 2X or more, as demonstrated in Table 3.2.

Table 3.2 Normalised Expression (net intensity of band) of *HIIP 9-27* to the internal control

Patient ID	Stage	Normalised tumour	Normalised Normal Mucosa	Ratio T:N
001	D	0.46	0.54	0.85:1
002	D	2.28	0.98	2.33:1
003	C	0.19	0.07	2.7:1
004	B	0.12	0.14	0.85:1
005	B	0.16	no expression	>100X:1
006	B	1.51	0.95	1.6:1
007	B	no expression	no expression	0:0
008	B	0.8	0.49	1.6:1
009	A	0.92	0.64	1.44:1
010	B	0.71	2.1	0.34:1
011	B	0.22	0.82	0.35:1
012	B	0.43	0.29	1.5:1
013	B	1.07	1.26	0.8:1
014	B	no expression	no expression	0:0
015	B	0.77	1.02	0.7:1
016	D	0.24	0.15	1.6:1
017	C	0.03	0.72	0.04:1
018	A	0.15	no expression	>100X:1
019	C	0.47	0.28	1.7:1
020	C	0.67	0.75	0.89:1
021	A	no expression	no expression	0:0
022	C	no expression	no expression	0:0
023	B	0.07	no expression	>100X:1
024	B	no expression	no expression	0:0

From these results it was determined that *HIIP 9-27* was unsuitable as a candidate tumour marker for colon cancer and would not be used in the immunobead RT-PCR assay. The over-expression of this gene was not consistent across a large number of matched tumour and normal mucosa samples.

3.2.3 Human Oligophrenin-1

Human Oligophrenin-1 is expressed at high levels in the developing neuroepithelium of the neural tube and loss of function of this gene has been identified to be responsible for X-linked non-specific mental retardation (110). The oligophrenin-1 transcript was identified to be most abundant in RNA from fetal brain. Results also indicated that inactivation of oligophrenin-1 might affect the activity of interacting proteins or cause constitutive activation of Rho family members which in turn could effect cell migration, axon outgrowth and morphogenesis *in vivo*. Using cDNA microarrays, Pinheiro *et al.*, (2001) also found this gene to be strongly up-regulated in colorectal tumours. This was the first time this gene had been implicated in the development and progression of colorectal tumours (111). Semi-quantitative RT-PCR was used in their study on six patient paired tissue samples from normal colonic mucosa and colon tumour to confirm the over-expression of this gene.

In the current study, Oligophrenin-1 was also identified as over-expressed in colon tumour tissue using cDNA microarrays. Figure 3.1 demonstrates its location on the cDNA microarray. The oligonucleotides F2 + R2 were determined to be the most appropriate primers for amplification of Oligophrenin-1. Figure 3.8 demonstrates the location of the 716 base pair PCR product within the Oligophrenin-1 gene.

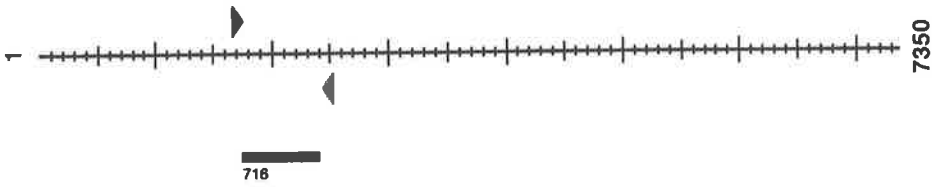


Figure 3.8 Schematic diagram of the 716 bp PCR product that will be used to determine if Oligophrenin-1 is over-expressed in colon cancer. The full length of the Oligophrenin-1 mRNA sequence is 7,350 bp.

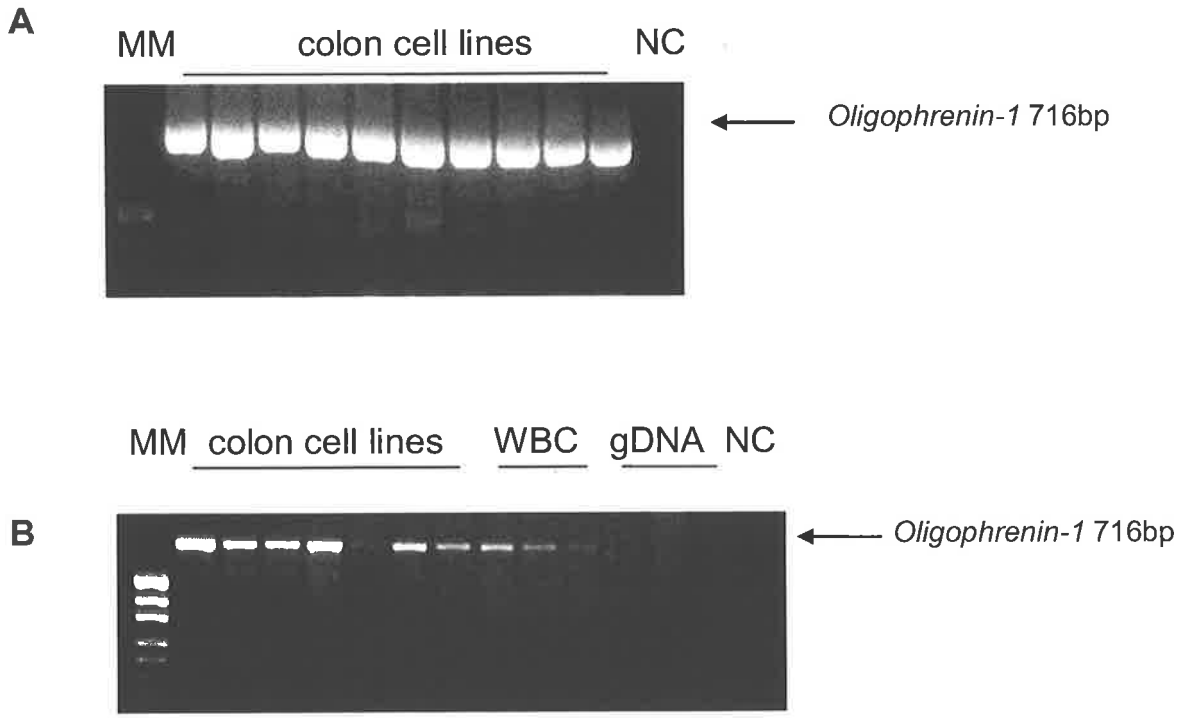


Figure 3.9 (A) Expression of Oligophrenin-1 in all 10 colon cancer cell lines, (B) Expression of *Oligo-1* in colon cancer cell lines, patient white blood cell (WBC) RNA and patient DNA samples. Three of 3 WBC samples were positive for Oligophrenin-1 expression. There was no amplification of a PCR product from patient DNA samples.

As demonstrated in Figure 3.9, Oligophrenin-1 was expressed in 3 of 3 patients' white blood cell samples and was therefore excluded from further analysis.

3.3 *Problems using Nylon cDNA arrays*

A number of technical issues were identified with using the nylon cDNA arrays as a means to identify genes over-expressed in CRC. The probing and stripping of each of the arrays created problems on subsequent reuse particularly with the hybridisation, as indicated by the housekeeping genes not being exposed and considerable time was taken to get acceptable exposure. This is despite the manufacturer advertising that the arrays could be reused. Software analysis of the arrays used the intensity of the housekeeping genes to quantify gene expression. With the establishment of the Adelaide Microarray Centre, Adelaide University, it was decided to pursue further over-expressed genes using glass slide cDNA arrays using this facility.

3.4 *cDNA Glass Slide Array (Adelaide Microarray Centre)*

3.4.1 Array Hybridisation

To overcome hybridisation problems encountered with using nylon membrane arrays, patient total RNA samples were hybridised at the Adelaide Microarray Centre (AMC). Twenty micrograms of total RNA was extracted and four 8K human glass slide cDNA arrays were fluorescently probed with matched patient tumour and normal colonic mucosa. Prior to hybridisation, RNA integrity was tested by gel electrophoresis as demonstrated in Figure 3.12. Bioinformatics was carried out by the AMC to identify the

top genes over-expressed in each individual patient and the top 100 genes over-expressed across all samples.

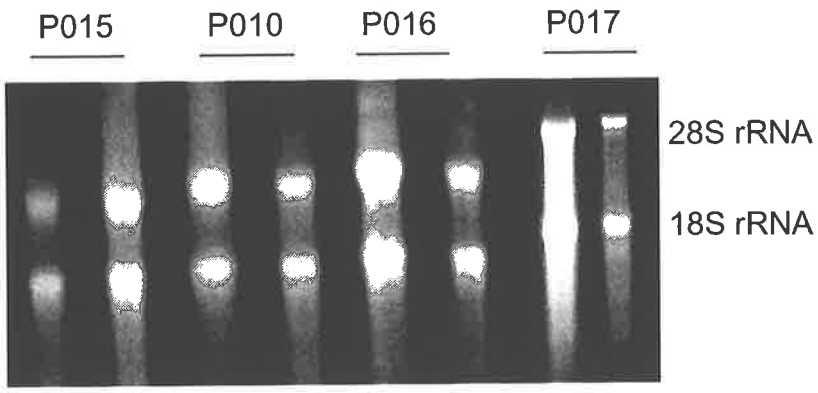
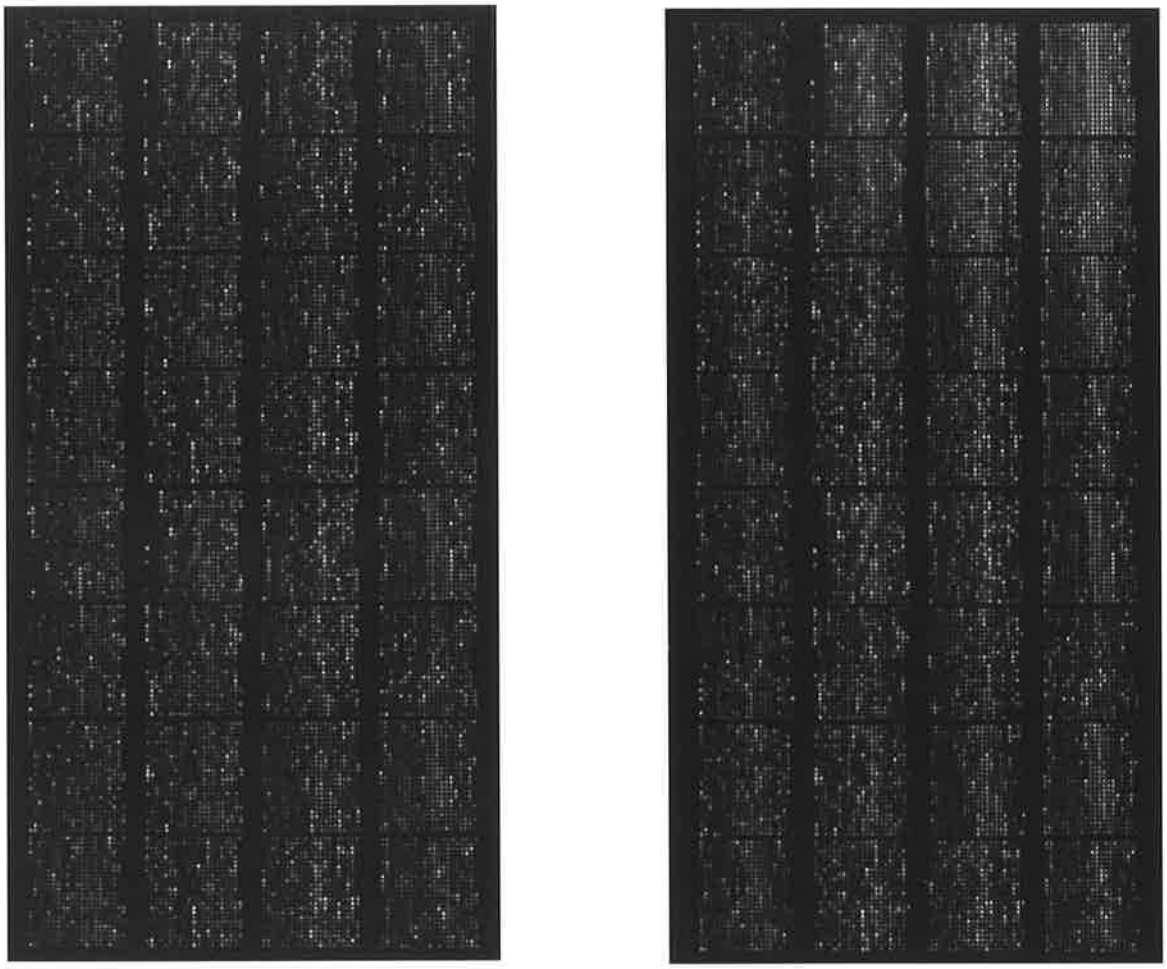


Figure 3.12 Gel electrophoresis of patient total RNA samples (2 μg) demonstrating the integrity of the ribosomal 18S and 28S RNA bands. Patient 015, Patient 010, Patient 016 & Patient 017, matched colon tumour and normal mucosa samples.

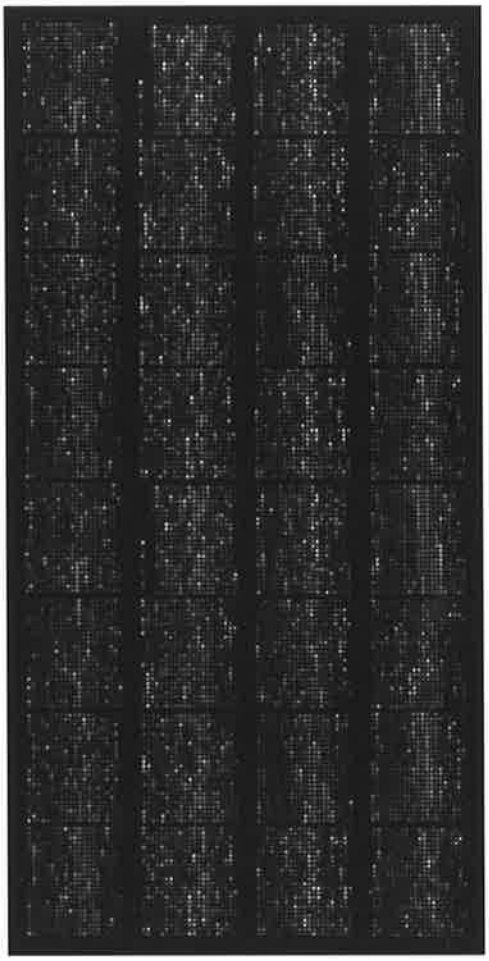
As demonstrated in Figures 3.13 and 3.14, patient tumour and normal mucosa samples were reverse transcribed (RT) and labelled with either Cy3 (green fluorescence) or Cy5 (red fluorescence). Both samples were then hybridised to an array.



Slide AMC 010 Slide AMC 015

Figure 3.13 cDNA 8K human slides for patient 010, colon tumour (Cy5 - red) and colon normal (Cy3 - green), and patient 015, colon tumour (Cy3 - green) and colon normal (Cy5 - red).

Slide AMC 016



Slide AMC 017

Figure 3.14 cDNA 8K human slides for patient 016, colon tumour (Cy3 - green) and normal colon (Cy5 - red), and patient 017, colon tumour (Cy5 - red) and normal colon (Cy3 - green).

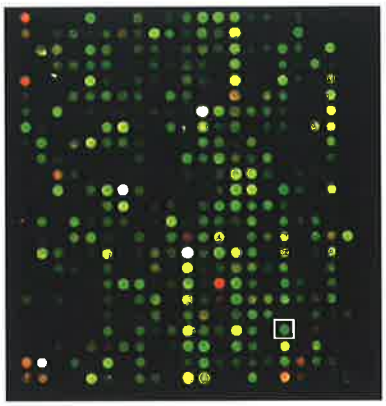
As shown in Figures 3.13 and 3.14, a large amount of data is obtained from one cDNA array. However as suggested in Chapter 1, relatively few changes in transcript expression are associated with tumourgenesis and for those that are, they may be highly significant (66). Therefore, statistical analysis is carried out on the data produced by cDNA hybridisation to determine the genes with the most significant difference in expression (i.e. yellow signifies equal gene expression in tumour and normal samples, and red or green fluorescence signifies gene expression in one tissue sample and not in the other).

Potential candidate tumour-specific molecular markers identified included Dipeptidase-1 (*DPEP-1*), Human axis inhibitor *2 (Axin2)*, Melanoma cell adhesion molecule *(MCAM)* and Glypican-5. Table 3.5 demonstrates the National Centre for Biotechnology Information (NCBI) sequence identification numbers for each of the potential candidate genes, the primer sequences used and whether they have previously been reported to be over-expressed in colorectal carcinoma.

Table 3.5 Glass Slide cDNA Array (AMC)

Gene Name	NCBI sequence ID	Previously reported in CRC	Primer Sequences Used 5' - 3'	Product size
Dipeptidase-1 (*DPEP-1*)	nm_004413	Buckhaults *et al.*, (2001)	F- CCT GAG GCT GGT GAA ACA GAC A R- GGG ACT CTG GTC TCC CAG GTT T	502bp
Human Axis Inhibitor 2 (*Axin2*)	nm_004655	Lammi *et al.*, (2004)	F1- CCG GTG GAC CAA GTC CTT ACA C R1- TTG GCG AAA GTT TGC ACT TGA A F2- CCG GTG GAC CAA GTC CTT ACA C R2- CTG CGA TGC ATT TCT CTC TGG A	511bp 549bp
Melanoma Cell Adhesion Molecule *(MCAM)*	nm_006500	No Reports	F1-CAC ACA GAC AGA CAC ACA CAC C R1- GGA TCA CGT AAA ACT GAA AGG C F2- TCC AGC TCC GCG TCT ACA AAA GC R2- GAC CCC GTT GTC GTT GGT TGT C	213bp 532bp
Glypican-5	nm_004466	No Reports	F- GCT CGC CAG GAT ATG CAG CAG T R- AGC AGG GAC CTC CCC ATC TGT C	421bp

A



B

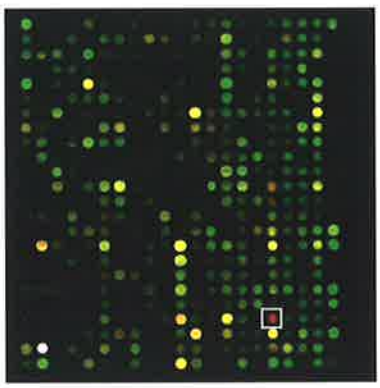


Figure 3.15 Identification of over-expression of the *DPEP-1* gene in two patients. A; patient 015, colon tumour total RNA labelled with Cy3 (green), normal mucosa labelled with Cy5 (red). B; patient 010, colon tumour total RNA labelled with Cy5 (red), normal mucosa labelled with Cy3 (green). Samples fluorescent labelling was reversed for two of the arrays to verify that labelling did not effect expression of genes.

3.5 *Potential Candidate Genes Identified*

3.5.1 Dipeptidase-1 (*DPEP-1*)

As demonstrated in Figure 3.15 *DPEP-1* was over-expressed in 2/4 patients. It was listed as the 3rd highest in patient 015 and the 9th highest up-regulated gene in patient 010, as determined by statistical analysis. Over-expression was validated in matched patient tumour and normal mucosa samples as published (112). Results for this gene marker will be described in detail in Chapter 4.

3.5.2 Human Axis Inhibitor (*Axin2*)

Axin2 is located on chromosome 17q23-q24 (113) and has been reported to be a gene that may be responsible for hereditary colon cancer as investigated by Lammi *et al.,* (2004) (114). Previously, it was reported by Liu *et al.,* (2000) (115) that *Axin2*, encoding a Wnt-signalling component, is mutated in 11 of 45 CRC with defective DNA mismatch repair (MMR), however, the mutations were not detected in MMR proficient tumours. Their results have also provided evidence that mutant *Axin2* activates TCF-dependent transcription which may be a causal link for tumour development. They suggest that *Axin2* is over-expressed in CRC and that *Axin2* mutations effect β-catenin regulation and the activation of β-catenin/T-cell factor signalling. The study demonstrates that mutations in the *Axin2* are involved in the development of colorectal cancer by elevating the level of β-catenin, a protein known to be associated with the development of colorectal cancer (116).

Axin2 interacts with *APC*, *GSK3β* and *β-catenin* and is therefore a potential mutational target for CRC. Anderson *et al.*, (2002) have reported that while investigating mutations in the *APC* tumour suppressor gene they found that expression patterns for *APC* and Axin co-localise in the nucleus and at lateral cell borders, with *Axin2* being identified as limited to the nucleus. The study of intact human colon crypts, adenomatous polyps, and adenocarcinomas looked at subcellular localisation of some key components of the β-catenin regulatory complex formed by APC and associated proteins including Axin and Axin2 (117).

In this study *Axin2* was identified as the 2nd highest up-regulated gene in patient 010. Initial primers were designed to amplify a 511 base pair (bp) product. However, these primers also amplified a product from genomic DNA samples. A second set of primers, amplifying a 549 bp product were designed to overcome this.

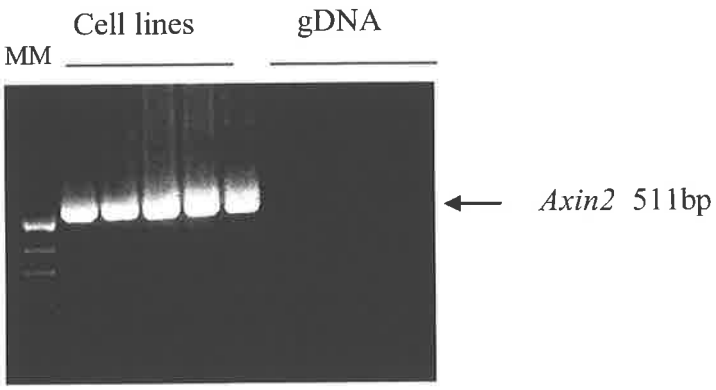


Figure 3.16 Expression of *Axin2* in colon cancer cell lines and DNA samples. MM; molecular marker, pUC19 (lane 1), colon cancer cell line SW48 (lane 2), LIM 2405 (lane 3), SW480 (lane 4), LIM 2412 (lane 5), LIM 1863 (lane 6), gDNA samples (lane 7-10) and negative (no template) control (lane 11).

Axin2 was expressed in 5 out of 5 colon cancer cell lines as shown in Figure 3.16. Expression was also examined in RNA samples collected from white blood cells.

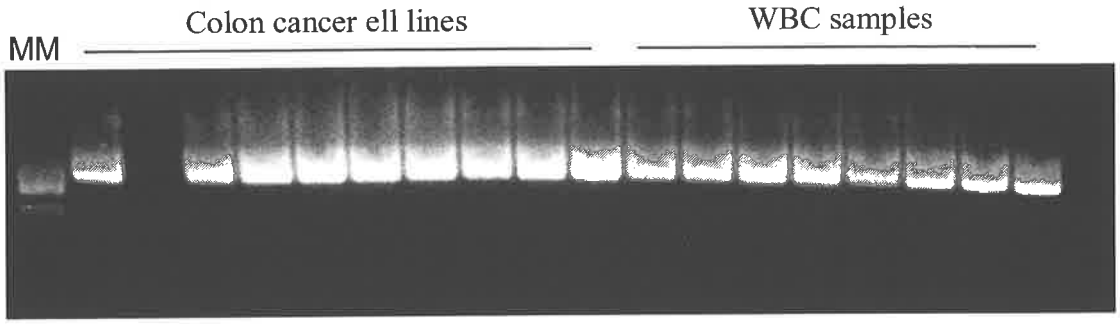


Figure 3.17 Expression of *Axin2* in colon cancer cell lines and WBC mRNA samples. MM; molecular marker (lane 1), colon cancer cell lines (lanes 2-11), WBC mRNA samples (lanes 12-19), negative (no target) control. Lane 3, LIM 2412.

Axin2 was excluded from further analysis as a potential candidate tumour-specific molecular marker as expression was positive in 8/8 WBC mRNA samples, shown in Figure 3.17.

3.5.3 Melanoma Cell Adhesion Molecule (*MCAM*)

In this study *MCAM* was identified as the highest up-regulated gene overall, determined by statistical analysis carried out by AMC. It was the 8th highest up-regulated gene in patient 010, the 11th highest in patient 016 and the 5th highest in patient 017.

MCAM, also known as *MUC 18*, was originally identified as a melanoma associated antigen (118) involved in tumour progression and the development of metastasis in

human malignant melanoma. The gene spans approximately 14 kb and contains 16 exons. *MCAM* is a cell surface glycoprotein and member of the immunoglobulin superfamily with the greatest sequence similarity to a group of neural cell adhesion molecules which are expressed during organogenesis (119).

The expression of *MCAM/MUC 18* in normal adult tissues appears to be limited to vascular smooth muscle. Lehmann *et al.*, (1987) used mouse antibodies targeted towards MUC18, however no staining was found in a large variety of surgically removed normal and tumour tissues except for smooth muscle cells of the blood vessel wall and hair follicles. This finding has led to the suggestion that the expression of this molecule by melanoma cells may contribute to their interaction with elements of the vascular system, an essential step in the dissemination of tumour cells (119). Therefore this may also be the case for colorectal cancer cells.

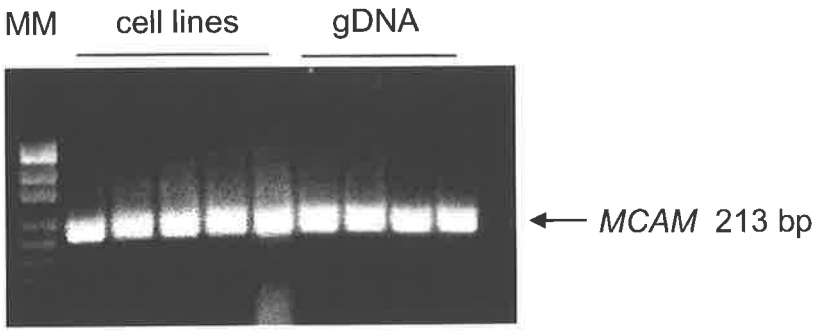


Figure 3.19 Expression of *MCAM* in colon cancer cell lines and DNA samples. MM; molecular marker, pUC19, colon cancer cell lines SW48 (lane 2), LIM 2405 (lane 3), SW480 (lane 4), LIM 2412 (lane 5), LIM 1863 (lane 6), gDNA samples (lanes 7-10) and negative (no template) control (lane 11).

The first set of primers designed to amplify the *MCAM* product also amplified a product from genomic DNA samples making them inappropriate for use with the immunobead technique, therefore a second set of primers were designed with a new product size of 513 base pairs.

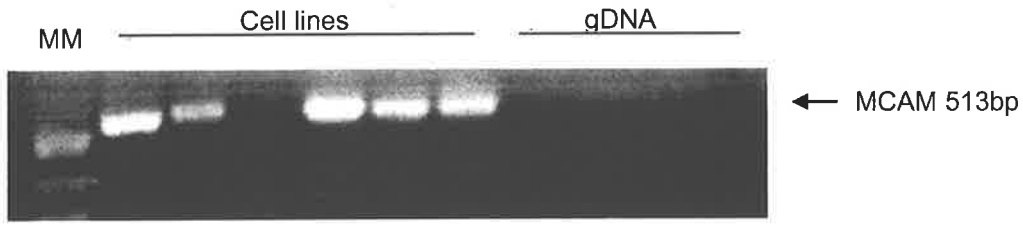


Figure 3.21 *MCAM* expression in colon cancer cell lines and gDNA samples.

Further analysis of *MCAM* to determine if it is a potential candidate gene has been carried out in the laboratory. However, it was not used for the remainder of this study.

3.5.4 Glypican-5

Glypicans are a family of glycosyl-phosphatidylinositol anchored cell surface heparan sulphate proteoglygans implicated in the control of cellular growth and differentiation (120). There are currently 6 members of this family that have been identified to date. Glypicans are expressed predominantly during development and are thought to play a role in morphogenesis (121).

Glypican-1 has previously been identified as over-expressed in breast cancer by northern blot analysis (120). However, they found that Glypican-2 and -5 were not expressed in tumour or normal breast tissue. Overall, their research indicated that Glypican-1 may play

a pivotal role in the ability of breast cancer cells to exhibit a mitogenic response to multiple heparin-binding growth factors and may contribute to disease progression in this malignancy.

Veugelers *et al.,* (1997) used fluorescent *in situ* hybridisation to map the Glypican-5 gene to chromosome 13q32 (122). Genomic alterations within or around this region have been implicated in malignancies such as small and non-small cell lung cancers, squamous cell carcinoma of the head and neck (123) and B-cell lymphoma (124). Northern blots of mRNA from various human tissues identified an approximate 3 kb message in fetal brain, lung and liver, and adult brain. However, it was not detected in adult heart, placenta, lung, liver, skeletal muscle, kidney, or the pancreas (122).

In this study Glypican-5 was identified as the highest up-regulated gene in patient 015. Sequence alignment with other members of the glypican family enabled primers to be designed that would only amplify the Glypican-5 product.

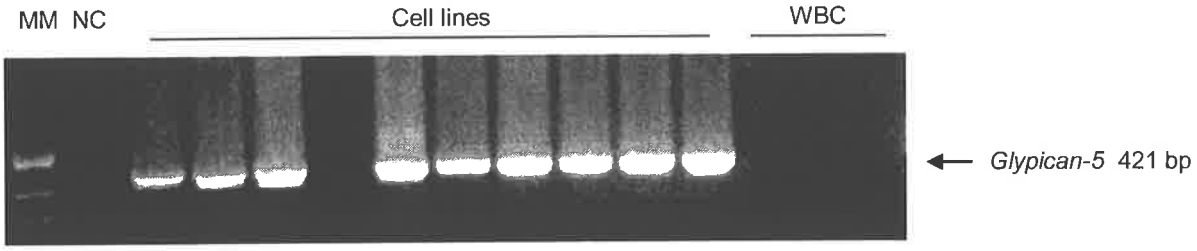


Figure 3.22 Expression of Glypican-5 in colon carcinoma cell lines and white blood cell cDNA samples. Lane 1; pUC19 molecular marker, lane 2; PCR (no template) negative control, lanes 3-12; colon carcinoma cell lines, lanes 13-15; patients' WBC cDNA samples.

As demonstrated in Figure 3.22 Glypican-5 was expressed in 9/10 cell lines and not white blood cell samples. The linear range was also examined, as shown in Figure 3.23, to determine the most appropriate cell cycle number for Relative RT-PCR.

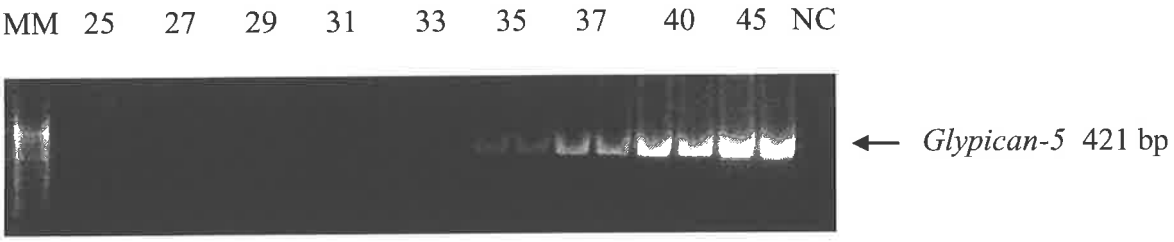


Figure 3.23 Glypican-5 expression linear range. Lane 1; pUC19 molecular marker, lane 2-19; cell line cDNA removed from PCR at varying cycle numbers, lane 20; negative (no template) control.

Glypican-5 expression was also examined in patients' tumour and matched normal colon tissue samples, as demonstrated in Figure 3.24.

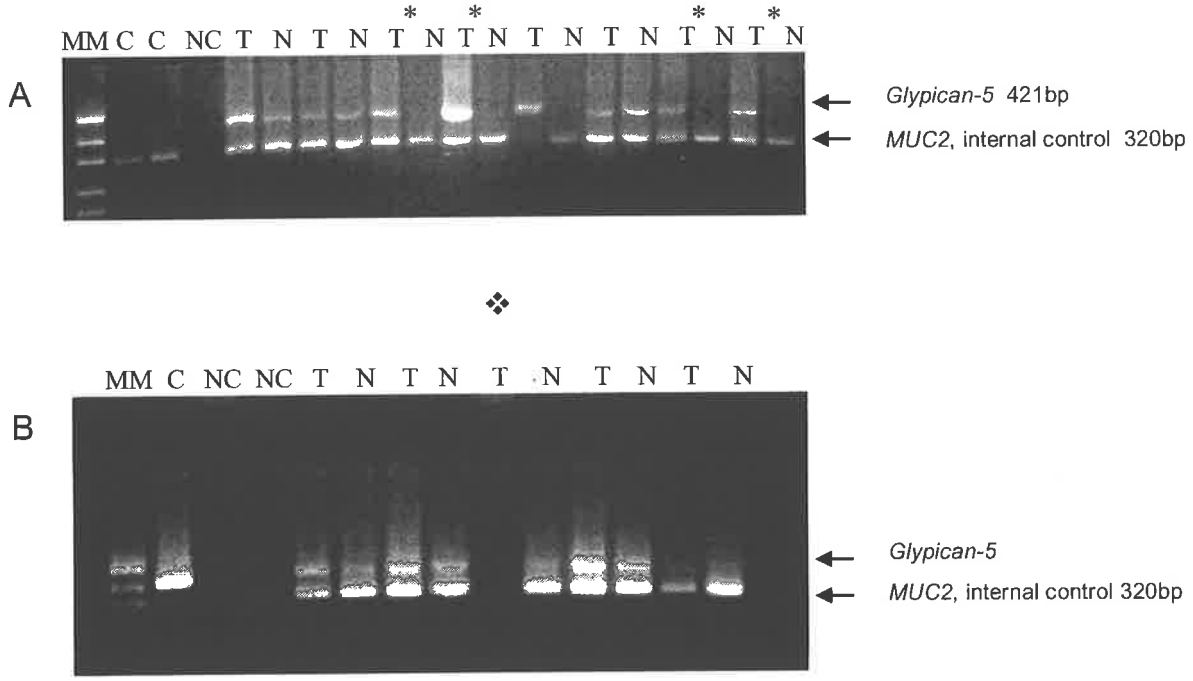


Figure 3.24 Expression of Glypican-5 in patient matched tissue samples. A; 4/8 patients, were identified as over-expressing Glypican-5 (identified by *). B; 1/4 patients identified as over-expressing Glypican-5, 1 patient was excluded (identified by ❖) from analysis as no expression was detected in the tumour sample due to degradation. MM; Molecular Marker, C; Cell Line Control, NC; Negative Control, T; Tumour, N; Normal.

Over-expression of Glypican-5 was detected in 15/27 (55.5%) patients' tumour samples, as demonstrated in Table 3.4. This gene was therefore excluded from further analysis as a majority of samples (greater than 70%) were required to be over-expressed for use in the immunobead RT-PCR technique for this study.

Table 3.4 Net Intensity and Ratio of Glypican-5 Expression in 27 Patients' matched Tumour and Normal Colon Samples.

Patient ID	Stage	Net Intensity of Expression-Tumour	Net Intensity of Expression – Normal Colon	Ratio T:N
026	C	2.10	1.60	1.3:1
033	C	0.93	1.50	0.62:1
061	C	1.40	no expression	>100X:1
006	B	1.90	0.45	4.2:1
039	C	0.40	0.38	1.1:1
065	A	0.54	no expression	>100X:1
063	B	1.77	0.19	9.3:1
064	B	2.20	no expression	>100X:1
062	B	0.40	0.70	0.6:1
060	B	0.52	no expression	>100X:1
027	B	0.78	0.10	7.8:1
028	D	0.90	0.40	2.25:1
029	A	0.70	0.60	1.2:1
014	B	0.90	0.60	1.5:1
066	C	no expression	no expression	0:0
023	B	0.40	0.30	1.3:1
013	B	1.40	no expression	>100X:1
024	B	0.60	0.29	2.1:1
067	A	no expression	no expression	0:0
068	A	1.80	no expression	>100X:1
034	C	0.50	0.50	1:1
025	B	0.90	0.80	1.13:1
019	C	1.00	0.23	4.3:1
037	B	0.30	no expression	>100X:1
047	B	13.60	0.30	45:1
050	A	0.75	0.40	1.9:1
069	B	0.66	no expression	>100X:1

3.6 Potential Candidate Markers Chosen

For the purpose of this study only *DPEP-1*, identified from the cDNA arrays, was used for further analysis. Two candidate markers previously identified from the literature were also chosen for this study, the gamma-2 chain of Laminin-5 (*LAM-γ2*) and Matrilysin (*MAT*). Analysis of these potential candidate markers and further characterisation on their functional roles will be described in Chapters 4, 5 and 6.

Chapter 4

Identification of DPEP-1 as a potential candidate marker and as a therapeutic target for colorectal cancer

4.1 Introduction

The development of techniques for the accurate staging of colorectal cancer (CRC) may enable clinicians to make more appropriate decisions about treatment options. To date, the majority of staging systems rely on histological examination of the tumor and surrounding lymph nodes. Staging systems based on these variables are not always reliable for the prediction of CRC patients' outcomes, particularly as patients diagnosed within the same stage may have markedly different disease outcomes (21).

The detection of tumor cells in peripheral blood and intra-peritoneal wash samples has the potential to identify early stage CRC patients at risk of recurrent or metastatic disease. Tumor cells have been detected in blood samples from various carcinomas including breast (125), prostate (126), and colorectal (127). Previously, Hardingham *et al.*, (1993) developed a sensitive technique, immunobead RT-PCR, to detect minimal numbers of tumor cells in blood. This technique uses immuno-magnetic beads for the enrichment of epithelial cells and RT-PCR for the epithelial-specific markers *MUC 1, MUC 2, CK 19* and *CK 20* (32, 38-40). Circulating tumor cells were detected in pre-operative peripheral blood samples from 19/94 CRC patients. Those patients in whom marker positive cells were detected had a significantly shorter overall survival ($P<0.0001$) than patients who were negative. However, positive expression of these markers was also found in 4/34 blood samples from patients with benign inflammatory bowel disease (40). Castells *et al.*, (1998) also found positive expression for carcinoembryonic antigen (*CEA*) in 5/9 blood samples from patients with benign inflammatory bowel disease (46). The current study aimed to identify a tumor-specific marker for use in immunobead RT-PCR.

In this study 8K human cDNA micro-arrays were used to identify genes with the greatest differential expression between colon tumour and matched normal mucosa. Findings were confirmed by using the digital gene expression displayer (DGED) tool from the Cancer Genome Anatomy Project (CGAP) website (73) and relative RT-PCR. From these analyses Dipeptidase 1 (*DPEP-1*) was identified as a potential tumor-specific molecular marker. *DPEP-1* has been previously found to be a zinc-dependent metalloprotease (128), however its role in colon cancer is yet to be determined. Here we describe the over-expression of *DPEP-1* in a large cohort of primary colorectal tumours compared to matched normal colonic mucosa. This finding suggests that *DPEP-1* expression may be appropriate for use as a molecular marker to identify disseminated tumor cells in CRC patients at risk of developing metastatic disease.

4.2 Results

4.2.1 Over-expression of *DPEP-1*

Micro-array Analysis

The cDNA micro-array analysis identified *DPEP-1* to be the third and tenth highest differentially expressed gene respectively in 2/4 patients (as shown in Chapter 3, Figure 3.15). Over-expression of *DPEP-1* in colon tumours was confirmed using the Digital Gene Expression Displayer (DGED) tool from the CGAP website. A virtual northern tool, vNorthern, showed expression of *DPEP-1* in colon tumor tissue, pancreatic cancer tissue and in normal brain and kidney.

Verification of DPEP-1 Over-expression by Relative RT-PCR

Using relative RT-PCR, *DPEP-1* was found to be expressed in all 10 colon cancer cell lines tested. The primers were designed so as not to amplify a genomic product from DNA samples in the PCR conditions used. *DPEP-1* was found to be over-expressed in colon tumor tissue samples compared to matched normal mucosa in 56/68 (82%) patients by two fold or greater (ratio range 1:1 - >100:1), with 50/68 (74%) showing a ratio of ≥ 100:1 (Table 4.1). Where there was no detectable expression in the normal tissue, a ratio of > 100:1 was assigned.

Table 4.1 Patient characteristics and normalized *DPEP-1* expression in colon tumour and normal mucosa

Patient ID	Age (yrs.)	Sex	Stage	Tumour (ng)	Normal (ng)	Tumour: Normal
084	90	M	A	116.23	0	>100:1
537	81	M	A	16.9	0	>100:1
570	69	F	A	65.97	0	>100:1
245	71	M	A	93.98	0	>100:1
512	50	M	A	36.27	0	>100:1
151	45	M	A	71.43	0	>100:1
375	71	F	A	76.65	0	>100:1
376	81	F	A	82.46	0	>100:1
716	86	F	A	92.01	0	>100:1
762	76	M	A	48.29	0	>100:1
081	55	N	A	156.76	0	>100:1
197	87	F	A	0	0	>100:1
798	83	M	A	62.8	0	>100:1
438	86	M	B	0	0	>100:1
951	74	F	B	193.31	0	>100:1
429	86	F	B	82.03	0	>100:1
512	68	M	B	11.14	0	>100:1
798	80	M	B	21.17	11.12	2:1
680	83	M	B	56.48	0	>100:1
421	74	M	B	49.09	0	>100:1
134	72	F	B	26.03	0	>100:1
001	73	M	B	99.42	104.85	1:1
186	78	F	B	51.44	47.42	1:1
427	84	M	B	271.75	0	>100:1
728	78	F	B	114.12	82.73	2:1

877	79	M	B	234.97	0	>100:1
534	92	F	B	33.47	0	>100:1
473	64	M	B	18.38	0	>100:1
047	67	M	B	77.72	0	>100:1
969	71	M	B	60.64	0	>100:1
798	55	M	B	139	0	>100:1
584	79	F	B	113.6	0	>100:1
958	86	M	B	90.5	0	>100:1
321	59	M	B	99.29	0	>100:1
008	58	M	B	85.94	0	>100:1
596	77	F	B	57	1.4	41:1
426	67	M	B	96.13	0	>100:1
309	50	M	B	73.64	0	>100:1
878	80	F	B	74.59	0	>100:1
142	57	M	B	127.29	0	>100:1
741	74	F	B	26.2	1.9	14:1
295	41	M	B	0	0	>100:1
105	85	F	B	19.16	0	>100:1
847	83	F	C	92.52	0	>100:1
476	73	M	C	0	0	>100:1
694	51	F	C	5.5	0	>100:1
688	55	F	C	8.4	0	>100:1
444	89	M	C	54.75	0	>100:1
581	78	F	C	87.93	0	>100:1
684	69	M	C	0	0	>100:1
484	68	M	C	68.24	0	>100:1
367	61	F	C	0	0	>100:1
605	55	M	C	0	0	>100:1
744	74	M	C	95.08	0	>100:1
456	64	M	C	142.52	10.67	13:1
033	69	F	C	84.47	0	>100:1
423	67	M	C	99.26	32:39	3:1
590	47	M	C	89.46	0	>100:1
634	71	F	C	83.88	0	>100:1
411	66	M	D	0	0	>100:1
908	70	M	D	48.98	33.1	1.5:1
214	61	M	D	93.64	59.74	1.5:1
862	74	F	D	107.3	0	>100:1
741	54	M	D	90.32	0	>100:1
711	74	M	D	95.01	0	>100:1
479	77	M	D	113.76	0	>100:1
268	59	M	D	90.76	0	>100:1
961	78	M	D	83.08	0	>100:1

Abbreviation NE: no expression of *DPEP-1* in either tumour or normal

In five cases there was no detectable expression of *DPEP-1* in either tumour or normal mucosa, despite expression of MUC2. A representative gel is shown in Figure 4.1.

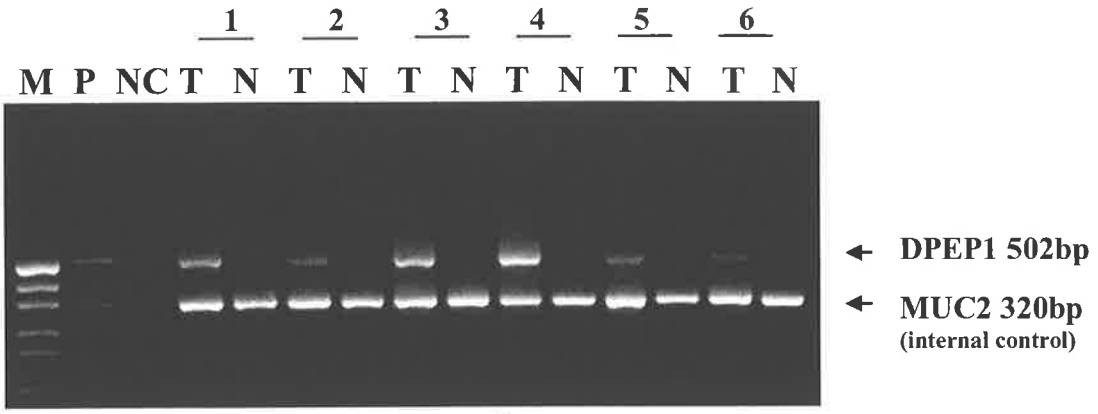


Figure 4.1 Relative RT-PCR of matched tumor/normal tissue samples from 6 patients. M, molecular weight marker; P, positive cell line control (LIM 2099); NC, negative control; T, tumor; N, normal.

Over-expression of *DPEP-1* was found amongst all stages of disease with 12/13 (92%) stage A, 26/30 (86%) stage B and 18/25 (72%) late stage (C and D) patients showing a two fold increase or greater (Figure 4.2).

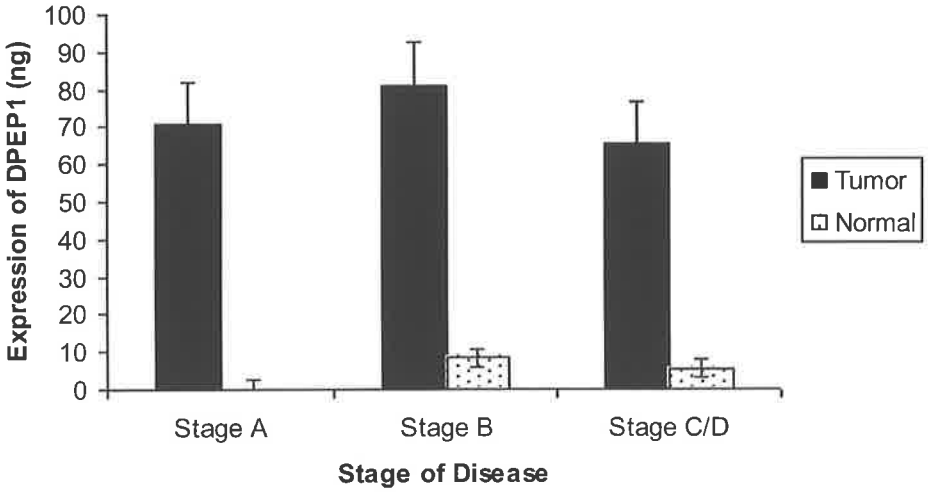


Figure 4.2 Comparison of mean *DPEP-1* expression between stages of disease.

The median expression of *DPEP-1* , for stage A was 71 ng (range 0-157 ng), stage B 74 ng (range 0-272 ng) and stage C and D 84 ng (range 0-143 ng). The difference in mean expression of *DPEP-1* between colon tumour and normal mucosa was highly significant (P= 3.3 x 10^{-15}) (Students t-test). There was, however, no statistical difference in mean expression of *DPEP-1* in colon tumour between the stage of disease (P=0.65).

4.2.2 Investigation of *DPEP-1* as a potential therapeutic target

It was also investigated whether *DPEP-1* plays a role in the ability of colon tumour cells to invade basement membranes. Using a Matrigel™ coated 2-chamber transwell assay (invasion assay), I investigated the up-regulation of *DPEP-1* in transfected HT29 cells and their capacity to migrate through the basement membrane-like matrigel™ compared to untreated HT29 cells. RNA interference was used to knockdown *DPEP-1* expression and to examine the effect on metastatic colon carcinoma cells, LIM 2099, in the invasion assay.

Recently the crystal structure of this gene has been determined (129), as shown in Figure 4.3. The structure of this gene may, in future, be used to identify suitable target regions for knock-down therapies. The location of *DPEP-1* expression was also examined in colon tumour tissue using fluorescent immunohistochemistry.

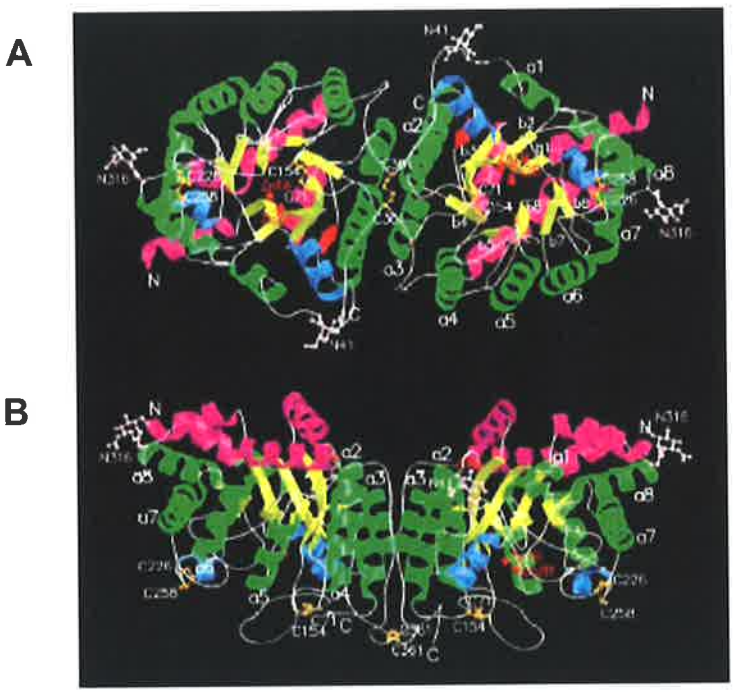


Figure 4.3 Crystal structure of *DPEP-1*, determined by Nitanai *et al.*, 2002, viewed from the membrane-binding side (A) where the active sites are located and the dimer viewed from a 90° rotation along the long axis of the dimer (B). The α-helices and β-strands composing $(\alpha/\beta)_8$ barrels are shown in green and yellow, respectively. The α-helices capping the barrels are shown in magenta. Zinc ions are drawn as red spheres.

4.2.3 Xi-Clone™ Conversion and Directional Cloning

To investigate the effect of *DPEP-1* over-expression in colon tumour cells Xi-Clone Conversion technology was used. The Xi-Clone™ PCR Cloning Technology (Gene Therapy Systems Inc., San Diego, USA) allows rapid, efficient, and directional cloning of PCR products. Using the conversion kit, a Xi-Clone-ready vector does not require the use of a ligase or multiple restriction digests for cloning experiments. The technology simplifies directional cloning by using only a single restriction digest in the multiple cloning site (MCS) of the vector of choice, in this instance the pIRES-EGFP vector.

Briefly, primers were designed following the manufacturer's instructions. Sense and anti-sense primers were designed 5' of the start and 3' of the stop codon of *DPEP-1*, as shown in Figure 4.4, to amplify the open reading frame of the gene. The 5' PCR primer contained 28 nucleotides overlapping with the 5' end of the linear vector plus 20 nucleotides that were gene specific sequence. The 3' PCR primer contained 28 nucleotides that were complementary to 3' end sequence of the linear vector plus 20 nucleotides that were gene specific sequence (Table 4.2). The PCR product was amplified, using Expand Long Template PCR System (Roche, Mannheim, Germany) containing a proofreading polymerase, according to the manufacturer's instructions.

Table 4.2 Xi-Clone PCR Primer Sequences 5′-3′

DPEP-1 (Xi-clone) sense
CGGACTCAGATCTCGAGCTCAAGCTTCGAGGTCCCCGGGGACCCCACC
DPEP-1 (Xi-clone) anti-sense
GCCCCGCGGTACCGTCGACTGCAGAATTGGGGACTCTGGTCTCCAGGTT

The pIRES-EGFP vector was linearized using Eco-RI restriction enzyme (MBI Fermentas, Hanover, MD, USA) to convert the plasmid into a Xi-Clone™-ready vector according to the manufacturer's instructions (Gene Therapy Systems) as described in Chapter 2. Verification was carried out by sequencing to ensure that the amplified sequence was correct (Appendix I). One base pair substitution was identified at position 425 (C→T). However, this substitution did not result in an amino acid change and therefore the amino acid sequence was conserved. The resulting PCR product was transfected into a pIRES-EGFP vector for further analysis.

Figure 4.4 Xi-Clone primer locations on the NCBI Genebank sequence (nm_004413) for *DPEP-1*. Boxed sequence; Sense and Anti-sense primers. Shaded sequence; start and stop codons. Underlined sequence; open reading frame.

HT29 cells transfected with either the pIRES/EGFP-*DPEP-1* vector construct or pIRES/EGFP vector only were incubated with 2500 μg/mL of G418 antibiotic. Stable transfectants were then used in the invasion assay. CHO cells were also transfected with either pIRES/EGFP-*DPEP-1* or pIRES/EGFP vector only as a positive transfection control. HT29 cells were transfected with 75% efficiency (Figure 4.5).

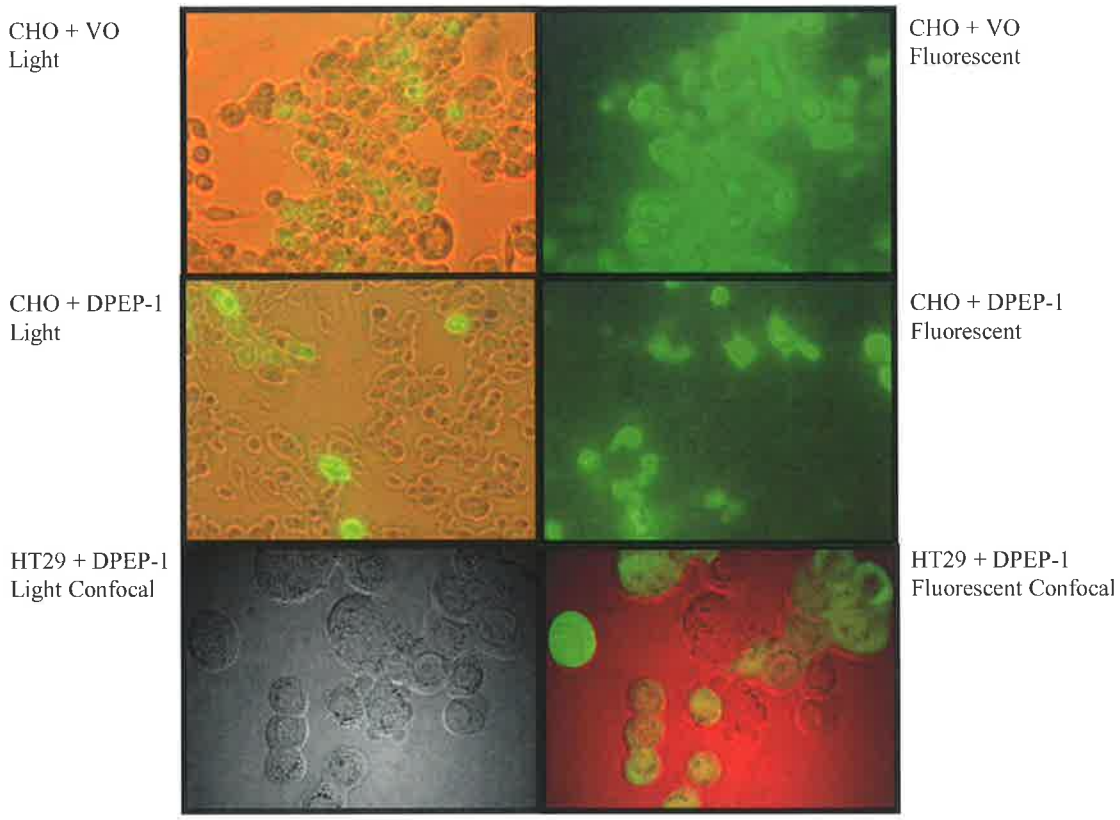


Figure 4.5 Immunofluorescence of pIRES-EGFP transfected HT29 cells and CHO cells (positive transfection control) under fluorescent and light conditions. pIRES-EGFP only transfected CHO cells (x400) using fluorescent microscopy. *DPEP-1* transfected CHO cells (x400) using fluorescent microscopy and *DPEP-1* transfected HT29 cells using confocal microscopy.

As previously mentioned, all 10 available colon cancer cell lines expressed *DPEP-1*. Therefore, HT29 colon carcinoma cells were chosen for invasion assay experiments due to reports that the cells have a reduced capacity to migrate through an invasion assay compared to a range of other colon carcinoma cell lines (130). HT29 cells were originally derived from a well-differentiated early stage colon carcinoma explaining their low capacity to invade through the basement membrane-like Matrigel™. These cells were

therefore chosen as suitable to investigate the role of *DPEP-1* in colon tumour cell migration.

RNA was extracted and 2 μg was reverse transcribed from transfected HT29 and untreated HT29 cells. *DPEP-1* expression was quantified using real-time RT-PCR. The amount of *DPEP-1* expression in untreated HT29 cells and vector only control cells was 0.8 ng for both, compared to 3.3 ng in *DPEP-1* transfected cells.

After 72 hours invasion assay filters, seeded in duplicate, were fixed and stained. Nine fields of view were taken from each filter and the number of cells in each field counted (Table 4.3), the average cell counts of the duplicates were recorded for each experiment.

Table 4.3 *DPEP-1* transfected Invasion Assay Summary Cell Counts

	HT29 untreated	*DPEP-1* transfected	Vector Only
Mean	107	305.25	196.75
Median	107.16	325.5	204.25
SD	33.87	53.24	16.63
Range	77-136	168-402	153-225

N=4

HT29 cells transfected with the *DPEP-1* construct migrated through the Matrigel™ basement membrane-like substance in greater numbers than untreated cells (Figure 4.6). Invasion of pIRES/EGFP-*DPEP-1*, pIRES/EGFP only and HT29 untreated cells were determined to be significantly different by ANOVA (P=0.007, Figure 4.7).

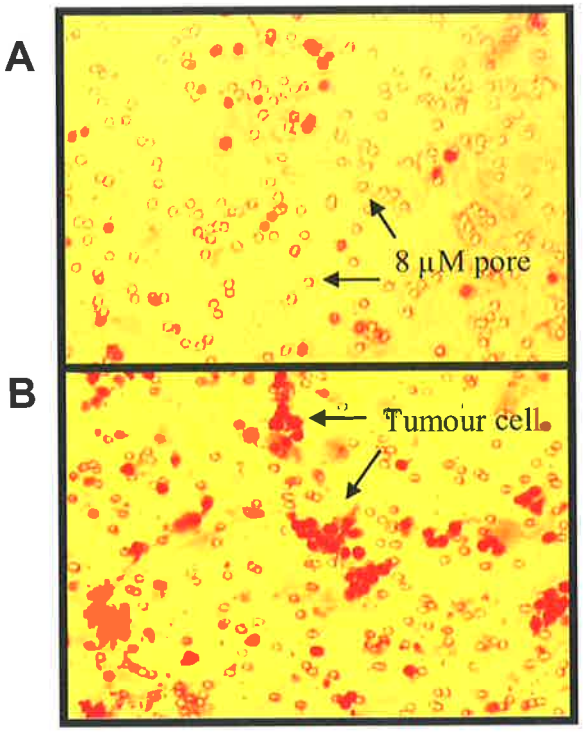


Figure 4.6 Invasion assay filters at 100X magnification. A; HT29 untreated cells, B; *DPEP-1* transfected HT29 cells.

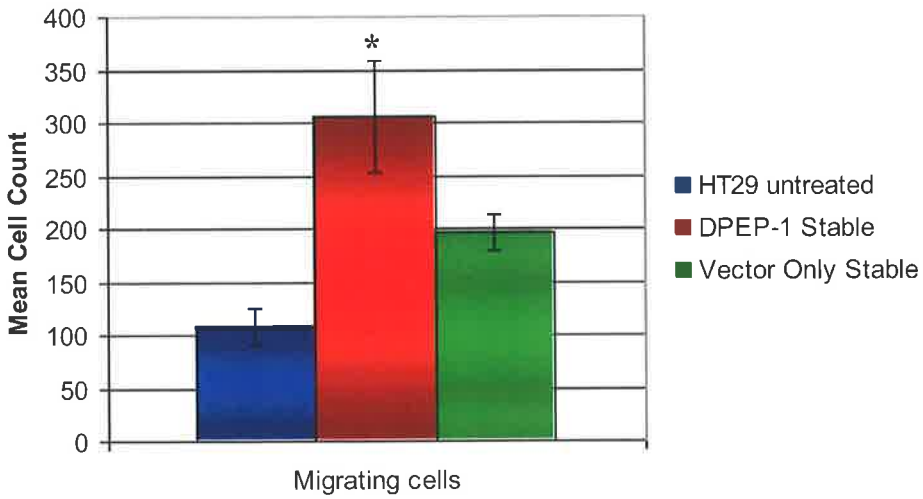


Figure 4.7 Mean number of cells migrating though the Matrigel™ coated invasion assay (N=4). There was a significant difference in the mean number of cells migrating through the assay (*P=0.007) determined by ANOVA.

To determine if *DPEP-1* expression could be knocked-down in colon carcinoma cells RNA interference experiments were conducted using siRNA sequences as shown in Chapter 2, Table 2.4. LIM 2099 cells are derived from a metastatic deposit in the liver of colon carcinoma origin (131). Therefore the cells have migration and invasion abilities and were deemed the most appropriate cell line cells to use for these experiments. Real time RT-PCR showed that the amount of expression of *DPEP-1* was reduced in siRNA treated LIM 2099 cells (0.06 ng) compared to mock transfected (0.09 ng), a 33% knockdown and siRNA control (0.08 ng) a 25% knockdown.

LIM 2099 cells either treated with siRNA, mock transfected or untreated were seeded into the top chamber of the invasion assay. As shown in Figure 4.8, cells treated with *DPEP-1* siRNA migrated through the assay in reduced numbers when compared to untreated, mock transfected or scrambled siRNA treated cells (summary Table 4.4).

Table 4.4. Summary siRNA invasion assay mean cell counts

	MT	Scrambled	*DPEP-1*
Mean	1100.5	613.5	328
SD	303.35	201.53	28.28
Range	886-1315	471-756	308-348

N=2

A students T-test identified a significant difference in migration capacity between the mock transfected (MT) cells when compared to *DPEP-1* siRNA treated cells (P=0.034). However, this result was not determined to be statistically significant by ANOVA (P=0.075).

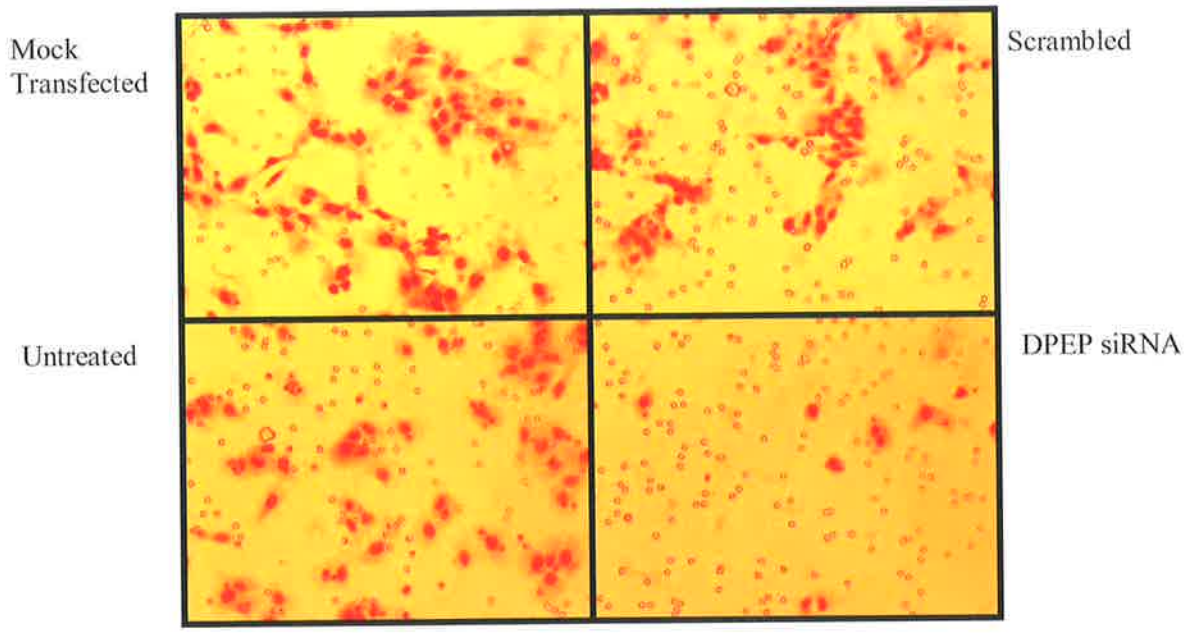


Figure 4.8 LIM 2099 cells and the four different treatment conditions in the Matrigel™ invasion assay. Cells were treated with no siRNA (mock transfected), scrambled siRNA (negative control sequence), untreated LIM 2099 cells or *DPEP-1* siRNA.

As shown in Figure 4.9, LIM 2099 cells treated with scrambled siRNA also demonstrated a reduced capacity to migrate through the invasion assay. This is in keeping with the knockdown effect of the scrambled siRNA on *DPEP-1* expression. This effect may be avoided by better selection of a negative control sequence rather than a scrambled sequence; such sequences are now commercially available. Due to the mock transfected cells (cells treated with transfection reagent but no siRNA) migrating at similar rates to the untreated cells it is unlikely that the transfection reagent itself was having an effect on the cells ability to migrate through the assay.

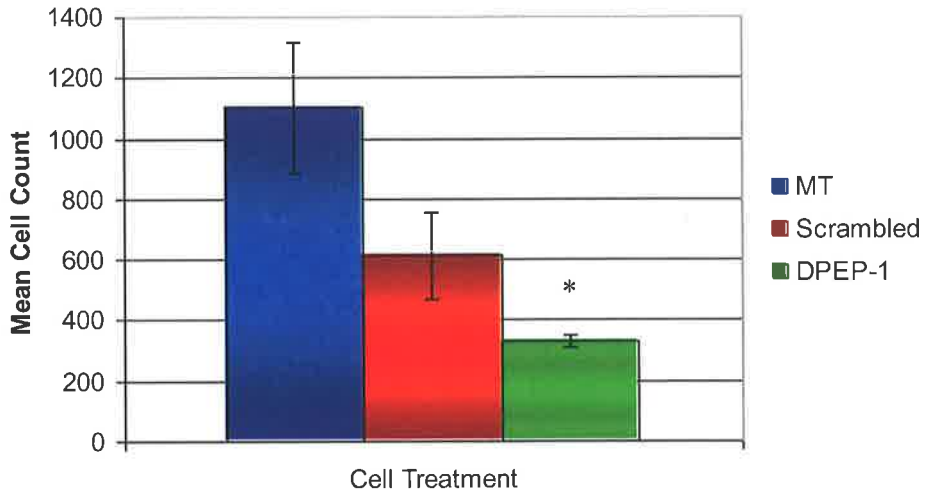


Figure 4.9 Mean cell counts for the different treatment conditions used in the Matrigel™ invasion assay (N=2). A students T-test identified a significant difference between mock transfected and *DPEP-1* siRNA treated cells (P=0.034). No significant difference was found between the other cell treatments.

Immunohistochemistry has not been reported on colon tumour tissue to date and this may be due to the recent identification of its over-expression in this tissue. However, it has previously been conducted on porcine pancreatic tissue (132, 133). Here I used fluorescent immunohistochemistry to identify *DPEP-1* expression in the crypts of colon tumour tissue from a formalin-fixed paraffin-embedded tissue section. This colon tumour tissue sample was chosen for immunohistochemical analysis as *DPEP-1* was identified to be over-expressed in this patient by RT-PCR. Initially frozen tissue sections were used on tissue samples also identified to be over-expressed, however, inconclusive results were obtained. A large amount was found in one crypt in particular, with diffuse staining in all other surrounding crypts within the colon tissue (Figure 4.10). The tissue section was also

stained with *LAM-γ2* to determine if there was co-localisation with *DPEP-1*. No co-localisation was observed using these antibodies.

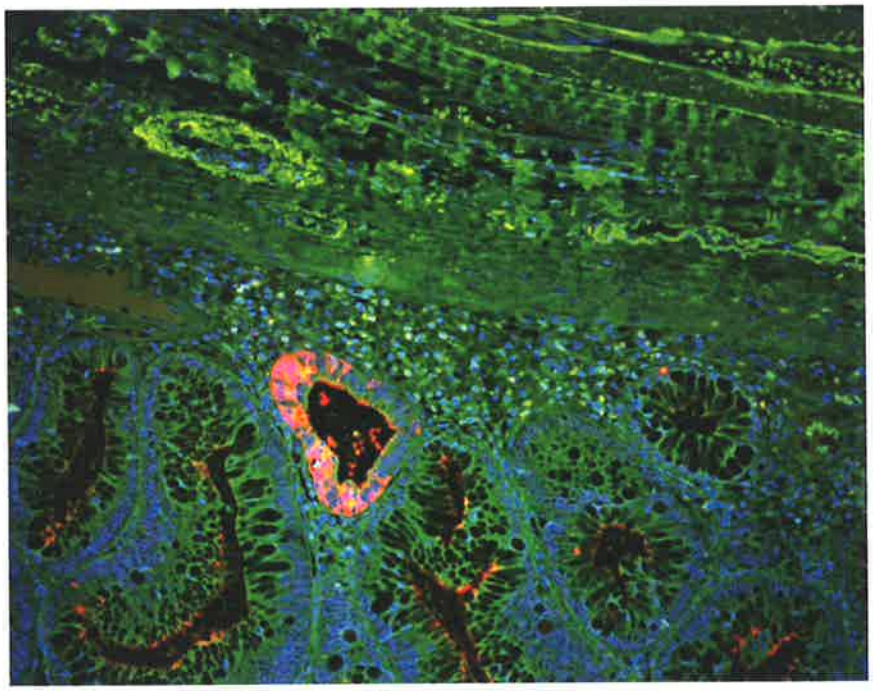


Figure 4.10 Fluorescent immunohistochemistry of human colon tumour from a formalin-fixed paraffin-embedded tissue section at 400X magnification. *DPEP-1* (Cy5-red fluorescence), DAPPI (blue fluorescence, used to identify cell nuclei) and basement membrane laminin-5 (gamma2 chain, Cy3-green fluorescence).

4.3 Discussion

The most important property of malignant cells is invasive growth (130). This ability enables cancer cells to leave the compartment in which they are restricted by the basement membrane, and gain access to the extracellular matrix and the vasculature to enable them to travel to distant sites and establish a secondary (metastatic) deposit.

Current molecular markers used to detect circulating colon tumor cells such as *CK 20*, *CK 19*, and *CEA*, are epithelial-specific rather than tumor-specific. Therefore, this study aimed to identify potential tumor-specific molecular markers to detect disseminated tumor cells using immunobead RT-PCR. *DPEP-1* was found to be over-expressed in colon tumours compared to matched normal mucosa by micro-array analysis in 2/4 CRC patients, and this result was verified by semi-quantitative RT-PCR in 56/68 (82%) CRC patients.

DPEP-1, also known as membrane dipeptidase, microsomal dipeptidase or renal dipeptidase, is a zinc-dependent metallopeptidase that hydrolyses a variety of dipeptides and has been implicated in the metabolism of glutathione and leukotriene D4 (128). The encoded protein has been found to have a highly hydrophobic sequence located at its carboxyl terminus and is anchored to the membrane by a covalently attached glycosyl-phosphatidylinositol moiety (134, 135). The enzyme has also been found to hydrolyse not only various dipeptides, including glycyldehydrophenylalanine, but also some β-lactam antibiotics (136).

DPEP-1 was originally identified at band q24 of chromosome 16 (137) and later localised to the region of 16q24.3 (138). Interestingly, chromosome 16q is a region reported to be frequently involved in allele loss in several tumours including breast and prostate carcinomas (139). Further, *DPEP-1* was identified as a potential tumor suppressor gene due to its decreased expression in Wilms tumor when compared to

normal kidney (140). In contrast, this study has shown this gene to be over-expressed in colorectal tumours compared to normal mucosa.

Previous expression studies have shown membrane dipeptidase to be expressed by one of two human colon carcinoma cell lines tested (141) in kidney (142), in cultured human tracheal epithelial cells (143), pancreas, lungs (144) and on the brush border membranes of the kidney and intestines (145). More recently, renal dipeptidase was identified from SAGE analysis to be expressed by colon adenomas and carcinomas but not in normal colonic mucosa and this finding was verified by comparing expression in matched colorectal neoplasms and normal mucosa (146). However, only a small number of patient samples were tested.

In the current study *DPEP-1* was found to be over-expressed in the majority (82%) of colon tumours compared to normal mucosal samples, with 74% of patients showing a differential expression ratio of > 100:1. However, there was no statistical difference in expression between the stages of the disease (Figure 4.2). These findings suggest that *DPEP-1* would be useful as a tumor-specific marker of disseminated cells.

To date, the role of *DPEP-1* in cancer progression is essentially unknown. However, due to it being a membrane bound dipeptidase, it may be involved in the degradation of surrounding extracellular matrix components. Here it is suggested that this mechanism would aid in the ability of tumour cells to migrate from the primary site through the

extracellular matrix. Studies carried out by Rajotte *et al.*, 1999 found that membrane dipeptidase (MDP/*DPEP-1*) is a receptor for the peptide GFE-1 and is involved in tissue-specific homing. They also found that the GFE-1 peptide can inhibit MDP activity and suggest that the tendency of peptides to bind to functionally important regions of their target proteins differs from antibodies and may be an advantage in some situations (147). Further investigations could look at peptide inhibition of *DPEP-1* in colon cancer cells to determine whether there is a relationship with tumour cell migration.

HT29 cells derived from an early stage carcinoma have previously been reported to migrate poorly through a Matrigel™ -coated invasion assay (130). HT29 cells have an epithelioid morphology and the percentage of migrating cells were found to be less than 13 percent. These cells were also found to not invade confluent layers of skin fibroblasts, instead the cells grew in compact colonies on the surface of the fibroblast cells. HT29 cells were therefore chosen to be transfected with *DPEP-1* to investigate the role of this gene in migration capacity of colon tumour cells.

Here it has been demonstrated that over-expression of *DPEP-1* does significantly increase the migration capacity of these cells (P=0.007) when compared to HT29 untreated cells. However, a significant difference in migration was also found between vector-only "control" transfected cells. The reason as to why the expression vector alone increased the migration of cells compared to the untreated cells is unknown and I can only speculate that the transfection of the vector itself influenced the expression of genes that may be involved in the ability of these cells to migrate.

Here it has also been demonstrated that RNA interference of *DPEP-1* can affect the migration capacity of a colon carcinoma cell line LIM 2099. This cell line was originally derived from a liver metastasis that originated from colon cancer. siRNA directed at the *DPEP-1* coding sequence reduced the migration capacity of LIM 2099 cells when compared to mock transfected (no siRNA) cells (P=0.034). This result is further evidence that *DPEP-1* plays a significant role in the ability of colon cancer cells to migrate through the basement membrane-like Matrigel™ invasion assay.

To date, immunohistochemistry on colon tumour tissue has not been performed to identify the location of *DPEP-1* in the tissue. Immunofluorescence microscopy has recently been conducted on Madin-Darby canine kidney (MDCK) cells to investigate the signals required for targeting of the naturally N-glycosylated and glycosyl-phosphatidylinositol (GPI) –anchored membrane dipeptidase, that are involved in the mediation of the apical sorting of proteins in polarised epithelial cells (148). Previously, Grondin *et al.*, (1999) used immunohistochemistry on porcine pancreatic tissue to localise the membrane dipeptidase protein exclusively to the islet of Langerhans, observed within the content of secretory granules and therefore would be released into the interstitial space as the granules undergo exocytosis (133). Here, using fluorescent immunohistochemistry, *DPEP-1* was identified in the crypts of colon tumour tissue in a Stage B patient (Figure 4.10). Further immunohistochemistry would ascertain if this staining pattern was consistent across all stages of colon carcinoma.

Chapter 5

Identification of LAM-γ2 as a potential molecular marker and therapeutic target for colorectal cancer

5.1 Introduction

Laminin gamma-2 (*LAM-γ2*) is one of three chains comprising the laminin-5 molecule (also known as kalinin, epiligrin or nicein). It was first described by Kallunki *et al.*, (1992) who localised it to chromosome 1q25-q31 and characterised the full-length cDNA clone by *in situ* hybridisation (149). More recently the structure of the human gene has been described and an alternative transcript identified (150).

Eleven different laminins are known to date, named 1 to 11 (151, 152). They are large, complex extracellular matrix (ECM) glycoproteins consisting of a heterotrimeric structure of polypeptide chains (alpha, beta and gamma) which are bound to each other by disulphide bonds. This results in a cross-shaped molecule comprising one long and three short arms with globules at each end, as shown in Figure 5.1 (153). Structural isoforms exist for each subunit, and association of these in different combinations gives rise to different laminins, with laminin-5 consisting of α3, β3 and γ2 subunits. The gamma chain, as with the beta chain, has domains used for forming polymers. In addition to this, gamma chains also bind to entactin and nidogen in the basement membrane. The alpha chains in comparison, have an invariable set of five modules at the c-terminus used for binding to proteoglycans (151, 152).

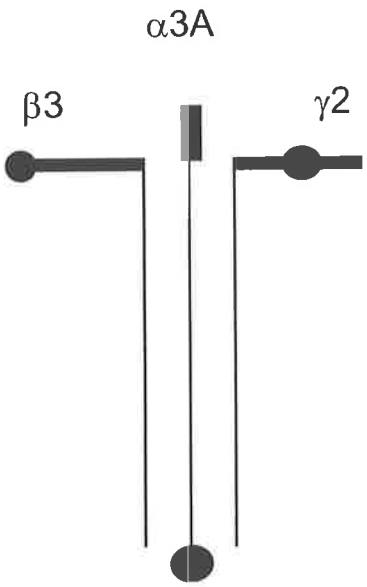


Figure 5.1 A schematic representation of the cross-shaped molecule of Laminin-5 taken from Sasaki (1999).

Laminins have the ability to interact with other laminin isoforms as well as interacting with extracellular matrix (ECM) ligands and with several integrin and non-integrin receptors. These different interactions cause polymerisation into large networks and binding to other basement membrane proteins through their subunits (151). Integrins in particular bind laminins and modulate intracellular signalling in response to this binding. They serve as signal transducing elements that activate chemical signalling pathways responsible for mediating the effect of the laminins on cell behaviour, including controlling cell growth, motility, and differentiation (152, 154, 155). Therefore the biological functions of members of the family of laminins have been implicated in cell growth, differentiation, adhesion and locomotion, as well as being structural, noncollagenous, components of the basement membrane (156).

The gamma-2 chain, which is specific to laminin-5, was originally thought to be a truncated version of the beta chain B2t (149). It is highly homologous to the gamma-1 chain, however it lacks domain VI and domains V, IV and III are shorter (Figure 5.2). Laminin-5 is an integral part of the anchoring filaments that connect epithelial cells to the underlying basement membrane. Due to the epithelial-specific expression of the gamma-2 chain it has been implied that it is an epithelium attachment molecule, and mutations in this gene have been associated with junctional epidermolysis bullosa, a disease characterised by blisters due to the disruption of the epidermal-dermal junction in the skin (157).

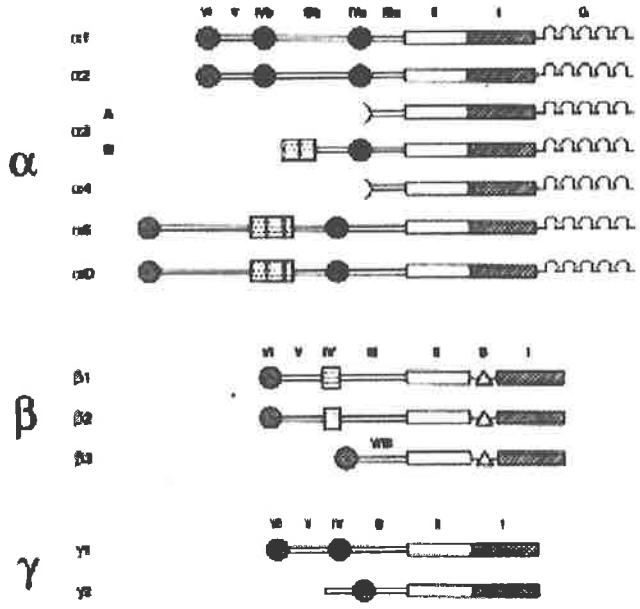


Figure 5.2 Schematic representation of the domain organisation in various laminin chains, originally adapted from E. Engvall (1995) (158).

Due to the indication that *LAM-γ2* played an important role in establishing adhesion contacts between epithelial cells and the basement membrane, Pyke *et al.*, (1994) investigated whether this gene was expressed in cancer tissue (74). *In situ* hybridisation for the detection of *LAM-γ2* mRNA was performed using S-35 anti-sense probes. Malignant cells were found to express *LAM-γ2* in 29/30 carcinomas studied (colon adenocarcinoma, mammary ductal carcinoma, malignant melanoma and squamous cell carcinoma) and the expression was particularly high in cancer cells located at the invasion front. They also found that in colon cancer there was a clear histological correlation between the expression of *LAM-γ2* by the cancer cells and their engagement in tumour budding processes.

More recently Shinto *et al.,* (2005) examined the prognostic significance of *LAM-γ2* expression in different areas of individual CRCs using tissue microarrays, to clarify the optimal areas for prognostic assessment. *LAM-γ2* was found to show high expression in the submucosal invasive front (35%) and subserosal invasive front (30%) of 120 CRCs. By multivariate analysis, high expression in the submucosal invasive front (hazard ratio=2.0, P=0.047) and subserosal invasive front (hazard ratio=2.9, P=0.0033) were independent prognostic factors. In contrast, the grades of *LAM-γ2* expression in the central area and rolled edge did not have a significant impact on patient prognosis (159).

The laminin-5 protein has also been found to be specific for cells of epithelial origin (160). It was found that *LAM-γ2* immunoreactivity occurred in all cases of colon adenocarcinomas and squamous cell carcinomas, but not sarcomas. In colon

adenocarcinomas positive staining was located at the tip of invading malignant epithelium. It was also found that the histological distribution of laminin-5 positive cells was identical to that of the receptor for urokinase-type plasminogen activator in colon adenocarcinomas, suggesting that Laminin-5 is a marker of invading cancer cells. Mizushima *et al.*, (1996) also found that enhanced gene expression of the three subunits of Laminin-5 in carcinoma cell lines could be induced by the tumour promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and epidermal growth factor (EGF) (161). This suggests that *Laminin-5* is associated with growth and migration of cancer cells.

5.2 Validation of Over-expression

In this study the expression of the gamma-2 chain of Laminin-5 (*LAM-γ2*) in a large cohort of matched patient colon tumour and normal mucosal mRNA samples was investigated. First the expression of *LAM-γ2* in colon cancer cell lines and patient white blood cell RNA samples was tested. As shown in Figure 5.3, *LAM-γ2* was expressed in 10/10 cell lines tested.

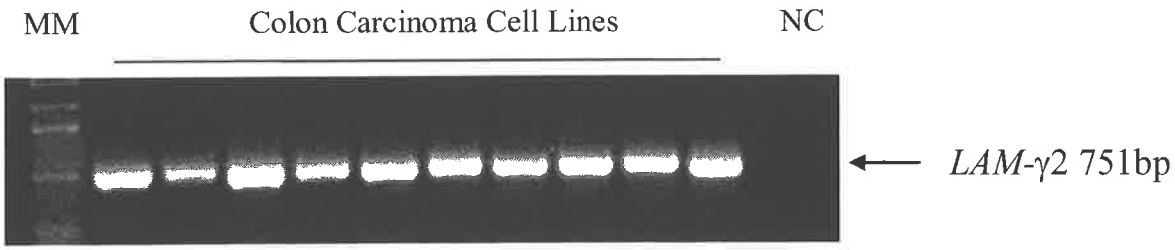


Figure 5.3 Expression of *LAM-γ2* mRNA in 10 colon cancer cell lines. MM; molecular marker SPP1, NC; negative (no RNA target) control.

LAM-γ2 was not expressed in white blood cell RNA samples (Figure 5.4) and therefore further investigations were carried out to determine if it would be a suitable tumour marker for immunobead RT-PCR.

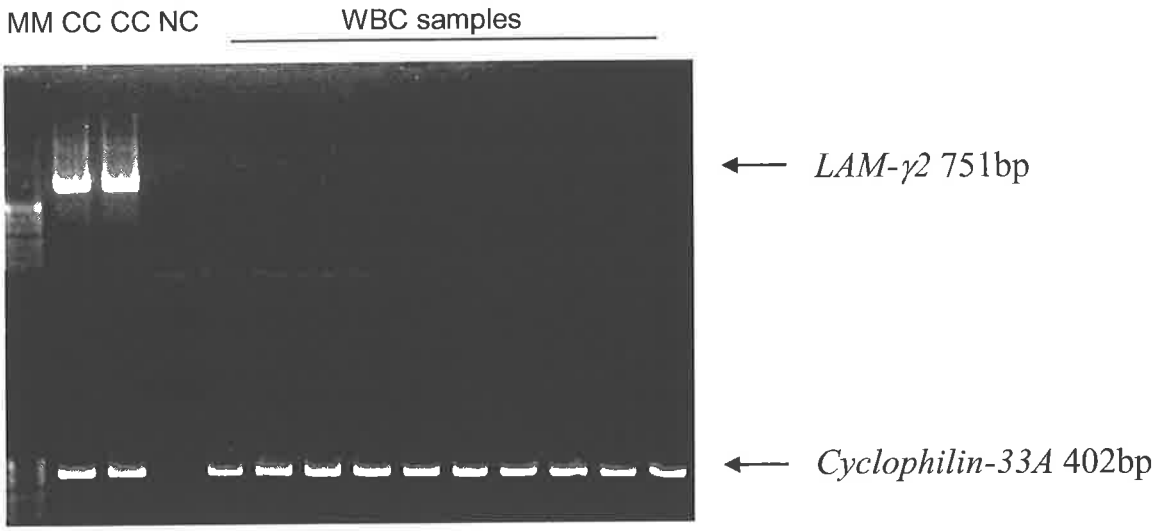


Figure 5.4 Expression of *LAM-γ2* in donor white blood cell mRNA samples. Expression of *Cyclophilin-33A* demonstrates that none of the mRNA samples were degraded and that equal amounts of cDNA were used from each sample. MM; molecular marker, pUC19, CC; cell line control, NC; negative (no target) control.

The expression of *LAM-γ2* in matched patient colon tumour and normal mucosa mRNA samples was investigated. As shown in Figure 5.5, relative RT-PCR identified *LAM-γ2* to be over-expressed by ≥ 1.5 fold in 22/30 (73.3%) patients' tumour mRNA samples compared to normal mucosa mRNA. Of the 30 patients tested (Table 5.1), 9 had no detected expression in normal mucosa samples compared to the matched colon tumour. Of these patients, one was classified as stage A, 5 stage B and 3 late stage C/D, demonstrating consistent over-expression across all stages of the disease.

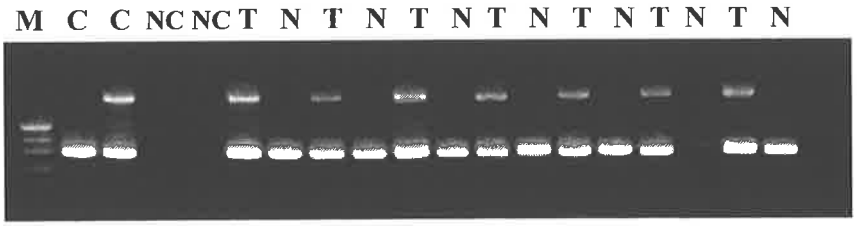


Figure 5.5 Relative RT-PCR representative gel of *LAM-γ2* expression in 7 patients matched colon tumour and normal mucosa mRNA samples. M; molecular marker, pUC19, C; cell line control, NC; negative (no target) control, T; tumour mRNA, N; normal colonic mRNA.

The mean amount of expression of *LAM-γ2* was also examined in each of the stages of the disease. There was a significant difference in expression in tumour compared to normal colon mucosa mRNA in all stages of the disease (P=0.05 Stage A, P<0.05 Stage B, P=0.02 Stage C/D) as shown in Figure 5.6.

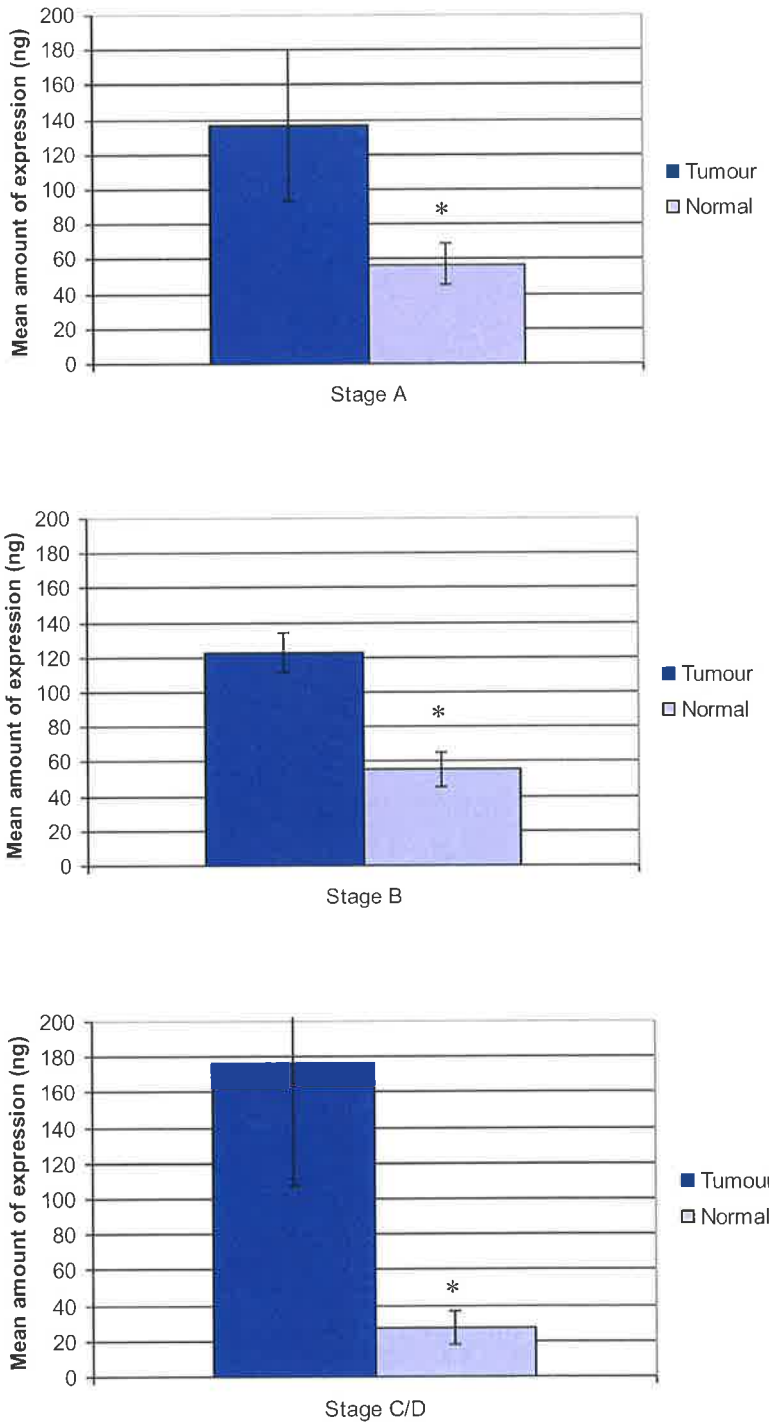


Figure 5.6 Mean expression of *LAM-γ2* in tumour compared to normal mucosa across all stages of the disease (Mean ± SE) * indicates $P \leq 0.05$. Stage A $P=0.05$, Stage B $P=0.0004$, Stage C/D $P=0.02$.

The over-expression of *LAM-γ2* in over 70% of patients' tumour samples and the absence of expression in white blood cells validated it as a suitable candidate marker for the immunobead RT-PCR technique.

Table 5.1 Expression of *LAM-γ2* normalised to an internal control gene *MUC 2* in matched patient tumour and normal colonic mucosa samples.

		Amount of Expression (ng)	Amount of Expression (ng)	
Patient ID	**Stage**	**Tumour**	**Normal**	**Ratio T:N**
001	A	94.04	61.2	1.54:1
002	A	63.56	60.89	1.04:1
003	A	114.7	No expression	>100:1
004	A	119	63.53	1.87:1
005	A	350.3	68.8	5.09:1
006	A	78.73	86.63	0.91:1
007	B	54.44	No expression	>100:1
008	B	73.55	64.54	1.14:1
009	B	139.8	No expression	>100:1
010	B	169	78.68	2.15:1
011	B	91.14	No expression	>100:1
012	B	80.58	No expression	>100:1
013	B	159.1	88.93	1.79:1
014	B	231.7	No expression	>100:1
015	B	106.7	77.26	1.38:1
016	B	132.1	85.83	1.54:1
017	B	79.59	80.42	0.99:1
018	B	160.3	89.44	1.79:1
019	B	142.2	75.8	1.88:1
020	B	128.3	76.07	1.69:1
021	B	120.7	93.63	1.29:1
022	B	96.5	72.67	1.33:1
023	B	80.32	78.38	1.02:1
024	C	93.27	46.36	2.01:1
025	C	572.2	48.79	11.73:1
026	C	114.7	50.98	2.25:1
027	C	54.79	No expression	>100:1
028	D	91.5	44.98	2.03:1
029	D	206.3	No expression	>100:1
030	D	99.7	No expression	>100:1

5.3 *Characterisation of LAM-γ2*

As described earlier, *LAM-γ2* has previously been identified as expressed at the leading edge of colorectal tumour cells (74), it therefore appears to play a significant role in migration. It has also been implicated that *LAM-γ2* plays a role in the ability of tumour cells to migrate through the extracellular matrix and therefore may be an important factor in metastasis (74).

In this study LIM 2099 cells, a colon cancer cell line that was originally derived from a liver metastasis originating from a colon tumour, were used in a 2-chamber invasion assay. LIM 2099 cells were previously identified in optimisation experiments as a cell line that invades and migrates through the invasion assay in large numbers. An antibody directed at *LAM-γ2* was found to significantly reduce the ability for the cells to invade through the assay. Cells were seeded into chambers containing 8 μM pore filters coated with Matrigel™, a basement membrane-like substance (as described in Chapter 2). Cells were either untreated or treated with 5 μg of normal IgG_1 antibody or 5 μg antibody directed at *LAM-γ2*. As shown in Figure 5.7, untreated cells and cells treated with normal IgG_1 migrated through the assay in vast numbers (Table 5.1). However, with anti-*LAM-γ2* treated cells, the number of cells migrating through the assay were significantly reduced in numbers (P=0.0006), as determined by an ANOVA (Figure 5.8, summary Table 5.1). This was a similar result to that found by Salo *et al.*, (1999) who investigated the migration of a mouse squamous cell carcinoma cell line (KLN-205) treated with an anti-*LAM-γ2* antibody (158). They found that when polyclonal IgG against the short arm of the γ2 chain was added to the upper compartment of the chamber containing the cells, the

migration of cells through the filter was decreased up to 50% of that observed with the pre-immune IgG. Here, it has been found that cell invasion can be inhibited by greater than 80% in a metastatic human colon carcinoma cell line.

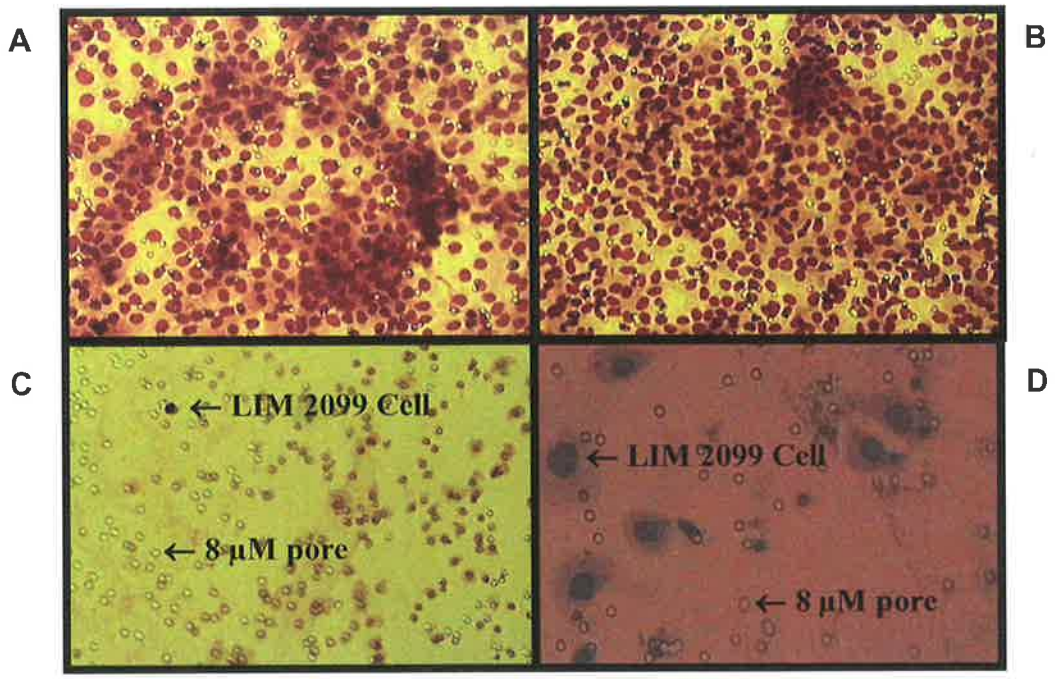


Figure 5.7 Migration assay seeded with untreated and antibody treated cells. A; LIM 2099 untreated (x100), B; LIM 2099 treated with 5 µg Normal IgG_1 (x100), C; LIM 2099 treated with 5 µg anti-*LAM-γ2* (x100), D; LIM 2099 treated with 5 µg anti-*LAM-γ2* (x400).

Table 5.1 Summary of antibody-treated invasion assay mean cell counts

	Untreated	Normal IgG1	anti-LAMγ2
Mean	2556	3116	413.25
Median	3000	3000	255.5
Standard Deviation	1003.13	232	521.46
Range	1056-3000	3000-3464	20-1122

N=4

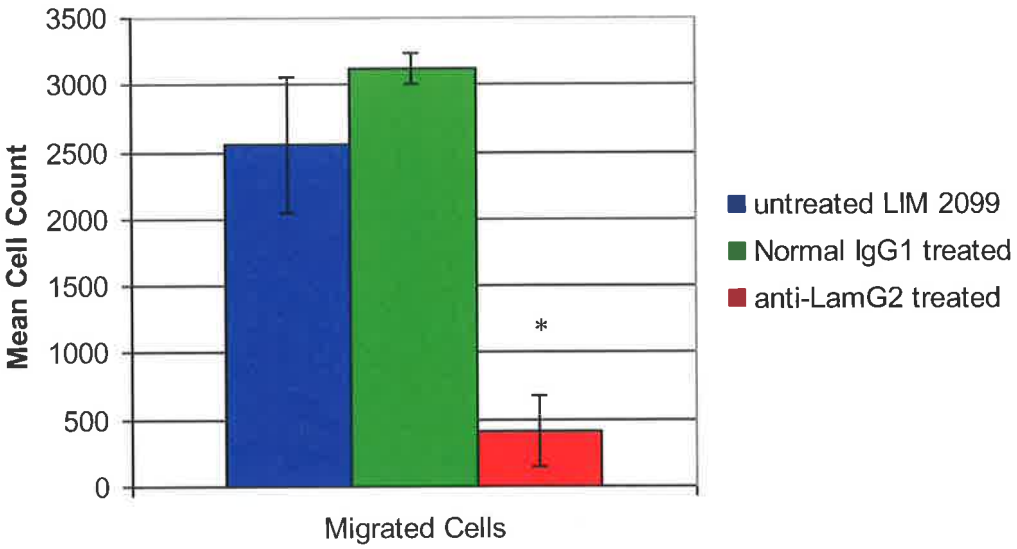
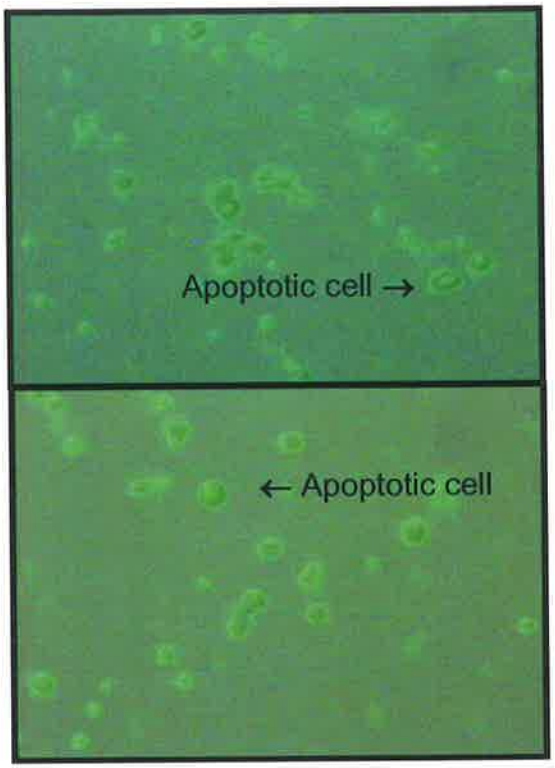


Figure 5.8 Mean number of LIM 2099 cells migrating through the Matrigel™ -coated two-chamber invasion assay (* P=0.0006) N=4. Cells were seeded (1 x 10^5) into the top chamber, as described in Chapter 2, in 100 µL of culture medium and 5 µg of either normal IgG_1 (control) or anti-*LAM-γ2* antibody was added to the chamber. The assay was incubated for 48 hours.

To determine whether the antibody was killing the tumour cells or if it was inhibiting their ability to migrate and invade through the Matrigel™, the rate of cell death was investigated using an Annexin-V apoptosis assay. As shown in Figure 5.9, there was no visible difference in the rate of apoptosis between cells treated with the anti-*LAM-γ2* antibody and LIM 2099 cells that were not treated with the antibody. Annexin-V is a calcium-dependent phospholipid-binding protein which has a high affinity for phosphatidylserine, which is translocated from the cytoplasmic side of the cell membrane

to the extracellular side during apoptosis and therefore detected using fluorescent microscopy (Roche Applied Science Manual).



LIM 2099 untreated cells

LIM 2099 treated with anti-*LAM*-γ2 antibody

Figure 5.9 Annexin V apoptosis assay in untreated LIM 2099 cells (x200) and LIM 2099 treated with 5 μg anti-*LAMγ2* antibody (x400).

This result indicates that antibodies directed at *LAM-γ2* interfere with the tumour cells ability to migrate and invade through the extracellular matrix. This is consistent with the theory suggested by Salo *et al.*, (1999) that the γ2 chain is probably involved in the process of cell locomotion. They also provided further evidence that *LAM-γ2* is involved in cell migration by identifying a *cis*-acting element that was active in migratory epithelial cells of healing wounds in mice.

More recently, Seftor *et al.*, (2001) found matrices conditioned by aggressive melanoma cells could induce poorly aggressive melanoma cells to form tubular networks (162). Their results suggest that instructional information is deposited into the extracellular matrix and since aggressive cells express significantly higher levels, compared with poorly aggressive cells of the basement membrane ECM component Laminin, specifically the γ2 chain, this component could serve as a putative molecular target for therapeutic intervention.

The indication that *LAM-γ2* plays an important role in cell locomotion and that it may contribute to instructional information deposited into the extracellular matrix, lead to the investigation of RNA interference (RNAi) as a potential therapeutic intervention. As described in Chapter 1, RNAi can be used as a molecular trigger to mediate changes in gene expression known as RNAi-mediated gene silencing. As described in Chapter 2, Table 2.4, two siRNA sequences were designed according to the instructions outlined by a publicly available siRNA template design tool (www.ambion.com).

In this study LIM 2099 cells were transfected with 10 nM siRNA, specifically designed to target the coding region of the *LAM-γ2* gene as shown in Figure 5.10. The amount of expression of *LAM-γ2* was examined using real-time RT-PCR. It was found that the siRNA directed at the *LAM-γ2* gene was able to significantly reduce *LAM-γ2* expression when compared to mock transfected and control siRNA treated cells (P=0.012). As shown in Figure 5.11, the siRNA was able to reduce expression of *LAM-γ2* by almost 80%.

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1     gaccacctga tcgaaggaaa aggaaggcac agcggagcgc agagtgagaa ccaccaaccg
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121   cctgcgctct ggctgggctg ctgcctctgc ttctcgctcc tcctgccccgc agccccgggcc
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301   ggcattcact gcgagaagtg caagaatggc ttttaccggc acagagaaag ggaccgctgt
361   ttgccctgca attgtaactc caaaggttct cttagtgctc gatgtgacaa ctctggacgg
421   tgcagctgta aaccaggtgt gacaggagcc agatgcgacc gatgtctgcc aggcttccac
481   atgctcacgg atgcggggtg cacccaagac cagagactgc tagactccaa gtgtgactgt
541   gacccagctg gcatcgcagg gccctgtgac gcgggccgct gtgtctgcaa gccagctgtt
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5101 caccttggct gggaagacta tggtgctgcc ttgcttctgt atttccttgg attttcctga
5161 aagtgttttt aaataaagaa caattgttag atgccaaaaa
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Figure 5.10 The sequence for the *LAM-γ2* gene. Underlined sequence represents the open reading frame of the gene. Blue box represents the mRNA target sequence (1) for siRNA. Green box represents the mRNA target sequence (2) for siRNA.

Figure 5.11 Real-time PCR expression of *LAM-γ2* in LIM 2099 cells treated with either siRNA directed at the *LAM-γ2* sequence, mock transfected (MT, transfection reagent only) or scrambled (nonsense siRNA control) sequence. **P*=0.012, N=4.

These results indicate that siRNA directed at the *LAM-γ2* mRNA sequence can induce gene silencing in LIM 2099 cells. The biological significance of this was determined by using the Matrigel™ invasion assay as shown in Figure 5.12.

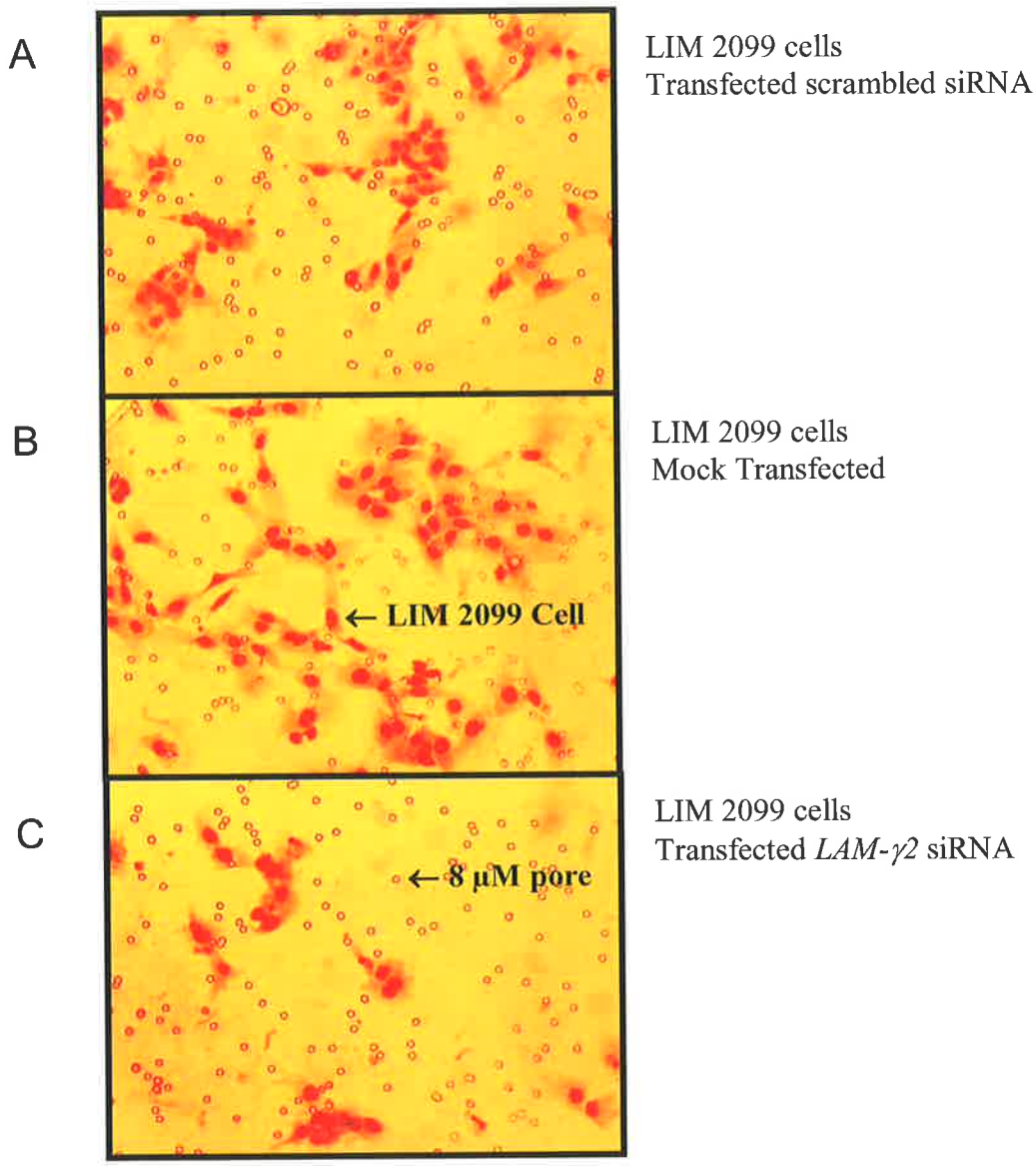


Figure 5.12 Matrigel™ invasion assay of siRNA transfected LIM 2099 cells. A; LIM 2099 cells transfected with scrambled (control) siRNA, B; LIM 2099 cells mock transfected (no siRNA), C; LIM 2099 cells transfected with siRNA directed at *LAM-γ2* gene expression.

As shown in Figure 5.13, siRNA-mediated gene silencing of *LAM-γ2* significantly reduced (P=0.007) the number of cells migrating through the Matrigel™ invasion assay.

However, the mean number of cells transfected with the nonsense siRNA were also reduced (summary Table 5.2). The significance of this result is unclear, but since the real-time RT-PCR results have demonstrated that *LAM-γ2* expression in the nonsense and the mock transfected is similar, my conclusion is that the nonsense sequence is interfering with other mRNA sequences causing non-specific gene-silencing, which in turn is having an effect on the migration of the LIM 2099 cells.

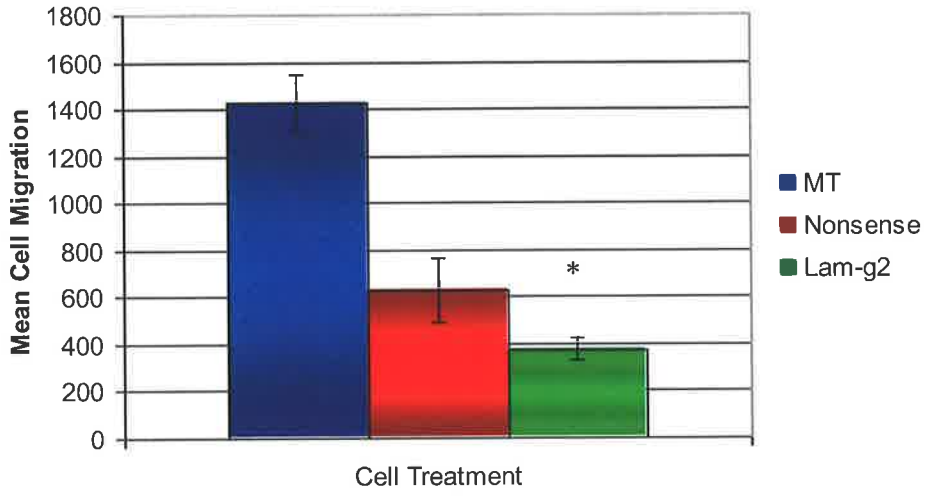


Figure 5.13 Matrigel™ coated invasion assay of LIM 2099 cells either transfected with siRNA directed at the *LAM-γ2* gene, control siRNA or mock transfected (no siRNA). N=2. Significant reduction in mean cell migration shown by *(P=0.007) when compared to the mock transfected cells.

Table 5.2 Summary of siRNA invasion assay mean cell counts

	MT	*LAM-γ2*	Scrambled
Mean	1428.5	377.5	627.5
Standard Deviation	170.4	67.17	187.38
Range	1308 - 1549	330 - 425	495 - 760

N=2

A limitation of this study has been the reduction in the mean number of cells migrating through the Matrigel™ coated invasion assay when transfected with a nonsense siRNA sequence. Work by Bridge *et al.,* (2003) found that although siRNAs are thought to be too short to induce interferon expression, a substantial number of short hairpin RNA vectors can trigger an interferon response (94). They also report that many commonly used tumour cells have a defective interferon response which may explain why these effects have not previously been reported. Moss and Taylor (2003) also explain that while the activation of the interferon system in these experiments by Bridge *et. al.*, (2003) was modest, a point can be made that a non-specific response can be triggered by the use of siRNAs that is unrelated to the specific mRNA targeted for down-regulation (93).

Here it has been demonstrated that *LAM-γ2* is expressed by 1.5 fold or greater in 73% of colon tumour tissue compared to normal mucosa. I have also shown that *LAM-γ2* plays a significant role in the ability of colon carcinoma cell lines to migrate through an artificial basement membrane. Experiments using siRNA, a new technique with the potential for gene-specific therapeutic intervention of many solid tumours including colon cancer, has been able to reduce the expression of *LAM-γ2* in LIM 2099 cells and this has resulted in a reduction in the numbers of cells migrating on and through the Matrigel™ barrier.

Chapter 6

Identification of Matrilysin (MAT) as a potential molecular marker for colorectal cancer

6.1 Introduction

Matrilysin (*MAT*), also known as Matrix Metalloproteinase-7 (*MMP-7*), is a member of the matrix metalloproteinase (MMP) family of zinc metallo-endopeptidases secreted by human cells, which are responsible for much of the turnover of matrix components. The MMP family currently consists of 26 members, all of which share a common catalytic core with a zinc molecule in the active site. *MAT* is the smallest member of the MMP family as it has only a signal, pro-peptide and catalytic domain (75). It is distinct from other MMP's in that it is expressed in epithelial-derived rather than mesenchymal-derived cells (163, 164). *MAT* mRNA has been detected in human adenomas, as well as carcinomas and adenocarcinomas of the breast and colon (165).

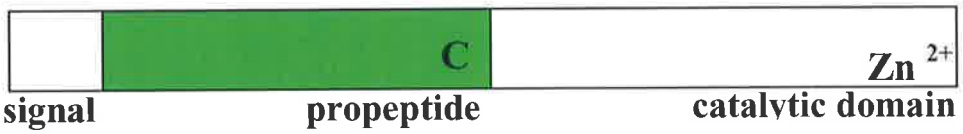


Figure 6.1 Schematic diagram of the structural domains of *MAT* derived from Wilson & Matrisian (1996).

MMP's have long been associated with metastasis, and they are major functional contributors to the metastatic process. Recent evidence suggests that MMP's play a much broader role in metastasis than previously believed, and that the action of MMP's at steps both before and after the breakdown of the apparent physical barriers to metastasis may be of even greater importance. MMP's and their inhibitors appear to be important regulators of the growth of tumours, both at the primary site and as metastases (166).

Muller *et al.*, (1988) were the first to isolate the cDNA encoding human *MAT* (1078 bp) from a mixed tumour library (reviewed in Wilson & Matrisian, 1996). The gene, of approximately 9.65 kbp, was characterised in Wilson and Matrisian's laboratory using genomic clones obtained from placental genomic DNA libraries. It is composed of 6 exons, the first five are organised in a manner homologous to other MMP family members for which the structure is known, exon 6 however does not have a corresponding homolog to other MMP genes (75).

Matrilysin is one of the MMP's that has a critical role in tumour invasion and is often expressed in gastrointestinal cancers. Adachi *et al.,* (1999) examined the relationship between *MAT* expression and Dukes' staging by immunohistochemistry and found that *MAT* expression is more common in Dukes' C and D colorectal cancers than in type A or B, as well as in nodal metastases. This result indicates that there is a correlation between *MAT* expression and the progression of colorectal cancer and formation of metastases that are derived from it (166).

Adachi *et al.*, (1999) also examined the *in vivo* invasive and metastatic potential of colon cancer cells transfected with *MAT* cDNA by subcutaneous injection into SCID mice. Matrilysin transfectants formed invasive tumours and multiple liver metastases in these mice. Casein zymography also demonstrated that invading and metastatic tumours showed large amounts of *MAT* activity, which correlated with the number of metastatic lesions. They suggest that the proteolytic degradation of the extracellular matrix by MMP's is one of the most important mechanisms in tumour cell invasion of basement

membranes and the stromal matrix and that *MAT*, after being activated, has a broad proteolytic activity against a variety of extracellular matrix substrates, including collagens, proteoglycans, elastin, laminin, fibronectin and casein (166).

As MMP's have classically been implicated in basement membrane destruction associated with late-stage tumour cell invasion and metastasis, Wilson *et al.*, (1997) analysed *MAT* expression in benign intestinal tumours from mice heterozygous for the Apc^{Min} allele (*Min*/+) to confirm recent findings that *MAT* is expressed in a high percentage of early stage human colorectal tumours. Their study found that *MAT* mRNA was indeed induced in the majority of these adenomas. They concluded from their findings that *MAT* is a suppressor of the *Min* phenotype, a mouse model of intestinal neoplasia, possibly by functioning in an independent manner to matrix degradation. The study did not investigate the possible mechanisms by which *MAT* is able to promote tumour development in this model, however, the reported reduction in tumour diameter and other preliminary data suggest that *MAT* contributes to tumour growth rate.

Zeng *et al.*, (2002) (167) examined the expression of *MAT* in human colorectal cancer liver metastases and normal liver tissue. All of the investigated liver metastases (44/44) over-expressed *MAT* mRNA and protein compared to normal liver tissue. They suggest that although *MAT* is highly regulated at the gene level, over-expression is insufficient for the promotion of invasive behaviour, because most MMP's are secreted as latent precursors. Therefore not only did they study *MAT* proenzyme but also the activated form which was expressed at high levels constitutively in the liver metastases, while being

absent in normal liver tissue. They conclude that the detection of the activated form of *MAT* in liver metastases supports the hypothesis that the activation of pro-MMP-7 is one of the critical steps in ECM breakdown, facilitating tumour invasion and metastasis. They also found that *MAT* is localised to the cytoplasm of tumour cells, as identified by immunohistochemistry, and that the strong signal is concentrated in the tumour front areas.

6.2 Identification of MAT as a potential candidate marker

In the present study, *MAT* was considered as a potential candidate molecular marker due to the previous reported studies that it was over-expressed in colorectal carcinoma tissue and colorectal derived liver metastases.

Expression of *MAT* was identified in 10/10 colon cancer cell lines including HT29, derived from an early stage cancer and LIM 2099, derived from a CRC liver metastasis. Expression in WBC RNA samples was also examined, as demonstrated in Figure 6.2.

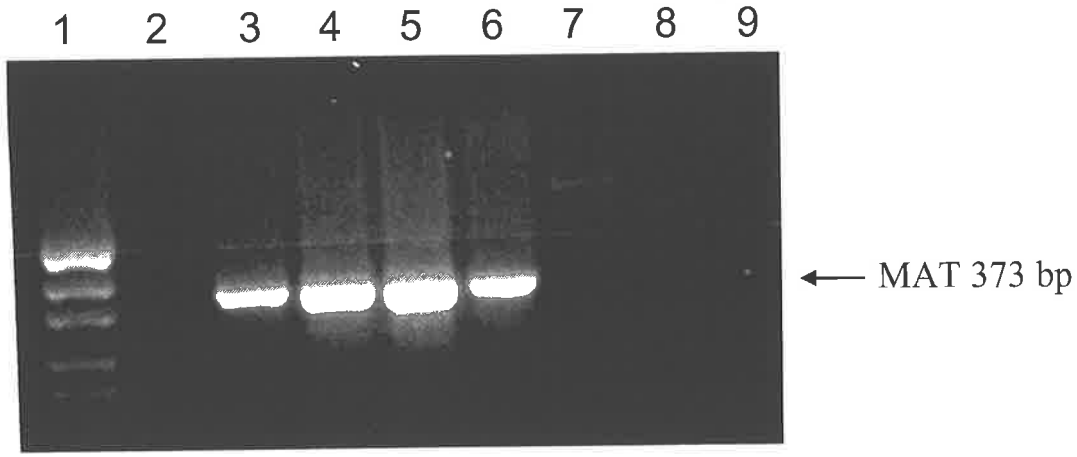


Figure 6.2 Expression of *MAT* in colon carcinoma cell lines and WBC RNA samples. Lane 1; pUC19 molecular marker, lane 2; PCR negative (no template) control, lane 3; SW480, lane 4; LIM 2412, lane 5; LIM 2099, lane 6; HT29, lane 7-9; patient WBC cDNA samples.

Expression of *MAT* was also examined in matched patient samples. A representative gel of 8 patients is shown in Figure 6.3. Overall fifty two patients' matched tumour and normal colonic mucosal samples were tested for *MAT* over-expression.

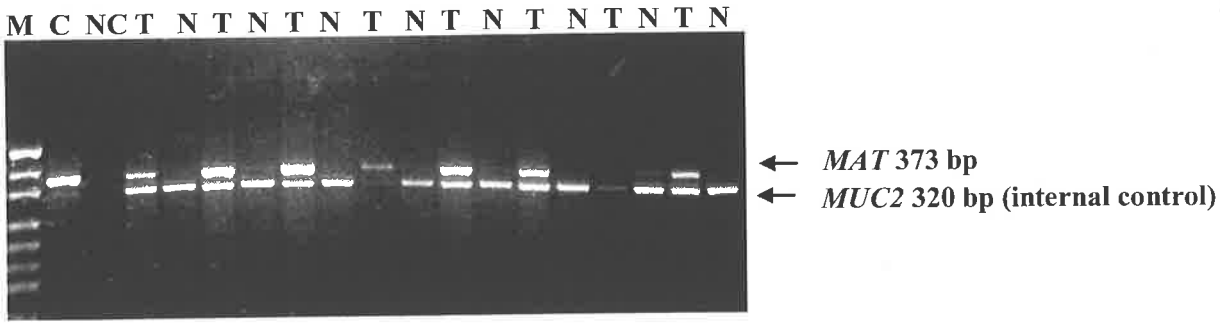


Figure 6.3 Expression of *MAT* in 8 patients' matched tumour and normal colonic samples. M; molecular weight marker, C; cell line control, HT29, NC; negative control (no template), T; tumour cDNA sample, N; normal colonic cDNA sample.

As shown in Table 6.1, the net intensity of Ethidium bromide stained PCR products for each of the patient samples was determined and normalised to the net intensity of the internal control. *MAT* was over-expressed in tumour samples by two fold or greater in 47/53 (88.7%) CRC patients. Therefore *MAT* was deemed an appropriate marker for colorectal cancer cells to be used in immunobead RT-PCR to detect circulating tumour cells in CRC patients.

Expression of *MAT* was examined in patient samples from a range of CRC stages of disease. *MAT* was expressed in tumour tissue in a ratio of ≥ 2:1 in 6/7 (85.7%) patients with early (stage A) stage CRC and for 5/7 of these patients the ratio was ≥ 100:1, which was statistically significant (P=0.00003). For patients with Stage B CRC, 27/30 (90%) showed over-expression in tumour tissue by ≥ 2:1 (P=0.0004). Fourteen of sixteen (87.5%) patients with more advanced stage C/D colorectal tumours over-expressed *MAT* in tumour tissue samples by ≥ 2:1 (P=0.04). These results suggest that *MAT* over-expression may be involved in both tumour growth and metastasis due to its expression throughout all stages of colorectal tumour samples.

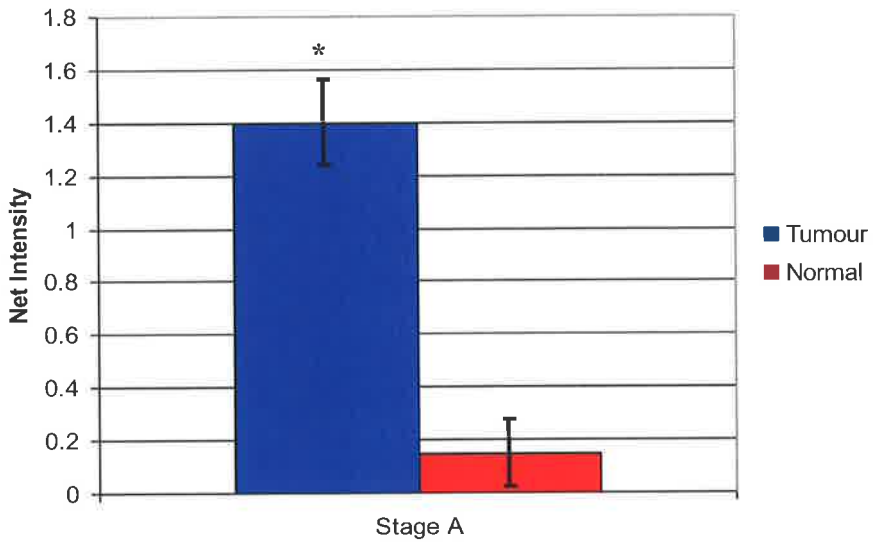
Table 6.1 Net Intensity of *MAT* expression in patient matched tumour and normal colonic mucosal. Expression normalised to the internal control.

Patient ID	Stage	Normalised colorectal tumour (net intensity)	Normalised normal colon (net intensity)	Ratio T:N
004	B	no expression	no expression	0:0
007	B	1.1	no expression	>100X:1
009	A	1.8	no expression	>100X:1
011	B	5.96	0.33	18.06:1
012	B	0.32	no expression	>100X:1
013	B	0.79	no expression	>100X:1
014	B	1.3	no expression	>100X:1
017	C	0.87	no expression	>100X:1
019	C	no expression	no expression	0:0
020	C	1.2	no expression	>100X:1
023	B	0.7	no expression	>100X:1
024	B	1.33	0.18	7.4:1
025	B	1.5	no expression	>100X:1
026	C	0.69	no expression	>100X:1
027	B	0.9	no expression	>100X:1
028	D	1.6	no expression	>100X:1
029	A	1.58	no expression	>100X:1
030	C	26.08	0.24	>100X:1
031	B	0.38	no expression	>100X:1
032	B	0.55	no expression	>100X:1
033	C	0.67	0.07	9.5:1
034	C	0.98	no expression	>100X:1
035	A	1.14	0.93	1.2:1
036	C	0.58	no expression	>100X:1
037	B	0.53	no expression	>100X:1
038	B	1.2	no expression	>100X:1

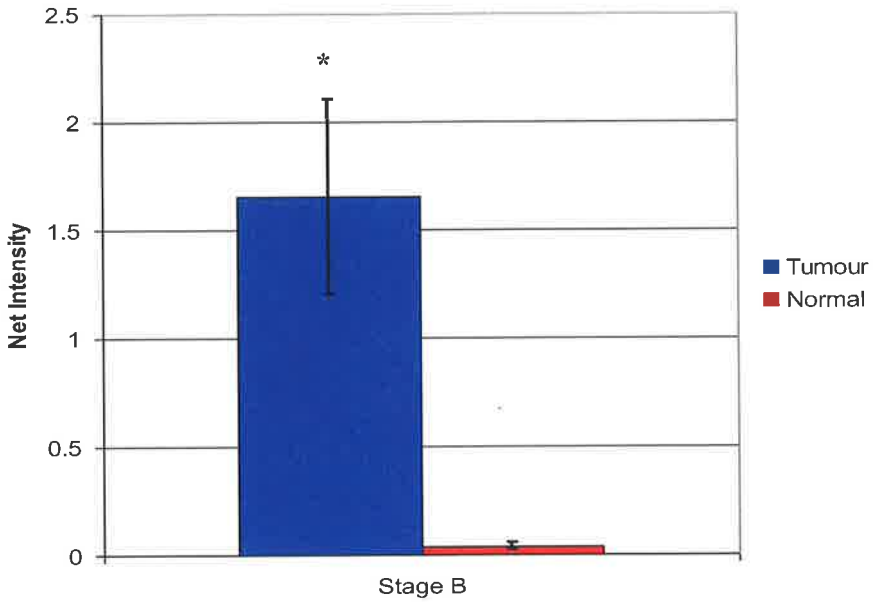
039	C	0.57	no expression	>100X:1
040	B	0.53	no expression	>100X:1
041	B	1.27	no expression	>100X:1
042	A	1.1	no expression	>100X:1
043	C	0.69	no expression	>100X:1
044	B	0.46	0.28	1.6:1
045	B	0.55	no expression	>100X:1
045	D	0.95	no expression	>100X:1
046	B	13.4	no expression	>100X:1
047	B	0.23	0.15	1.5:1
048	B	0.45	no expression	>100X:1
049	B	1.6	no expression	>100X:1
050	A	2.1	no expression	>100X:1
051	B	2.1	no expression	>100X:1
052	C	8.5	no expression	>100X:1
053	A	1.1	0.1	11:1
054	B	0.23	no expression	>100X:1
055	B	1.3	no expression	>100X:1
056	C	no expression	no expression	0:0
057	C	0.45	no expression	>100X:1
058	A	0.96	no expression	>100X:1
059	B	1.98	no expression	>100X:1
060	B	1.5	no expression	>100X:1
061	C	1.2	no expression	>100X:1
062	B	2.2	0.2	11:1
063	B	1.45	no expression	>100X:1
064	B	3.8	no expression	>100X:1

Expression was examined in patient samples from each of the stages of CRC. Net intensity of expression was significantly greater in tumour tissue samples across all stages of the disease, as shown in Figure 6.4.

A



B



C

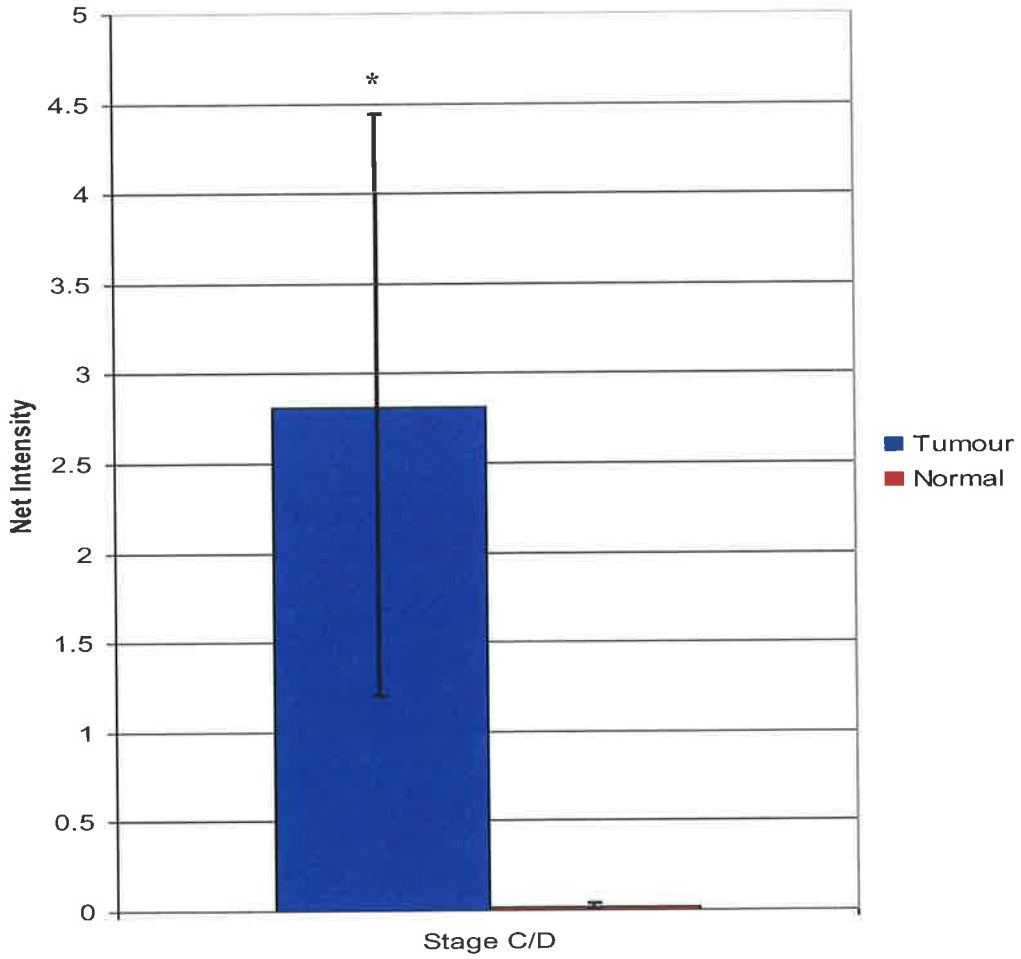


Figure 6.4 Net Intensity of Expression of *MAT* in different stages of CRC. *MAT* was significantly over-expressed in all stages of disease. A: Stage A; *P*=0.00003 (n=7), B: Stage B; *P*=0.0004 (n=30) and C; Stage C/D; *P*=0.04 (n=16).

6.3 MAT expression and Immunobead RT-PCR

The identification of *MAT* as over-expressed in 47/53 (88.7%) patient matched tumour samples compared to normal mucosa determined this gene marker to be suitable for use in immunobead RT-PCR, the results of which are presented in Chapter 7.

Chapter 7

Immunobead RT-PCR

7. Immunobead RT-PCR

7.1 Introduction

One of the most challenging aspects of CRC for clinicians is the management and treatment of recurrent and metastatic disease, and the selection of patients who would benefit from adjuvant therapy. It has been well documented that a significant number of patients with early stage CRC who undergo "curative" resection, will suffer recurrent and metastatic disease within 5 years. As best stated by Burchill and Selby (2000), for all cancers "the accurate detection of low-level disease in patients with cancer is essential to improve the staging of the disease and consequently to define appropriate treatment strategies" (168). The earlier CRC can be detected and removed, and with accurate staging, the better the outcome for the patient.

7.2 Detection of circulating tumour cells

Over the past decade, many laboratories including ours have reported a number of molecular studies that have demonstrated that micrometastases and disseminated tumour cells can be detected not only in lymph nodes that had appeared tumour free on conventional histology, but also in such body compartments as bone marrow, the peritoneal cavity and blood (reviewed in Tsavellas *et al.,* 2001). Vogel *et al.,* (2001) concluded that conventional staging most likely under-estimates the true tumour stage (30). This is supported by Tsavellas *et al.,* (2001) who also concluded from their review of 25 RT-PCR studies investigating the detection of circulating tumour cells, that conventional staging methods for colorectal cancer in particular are imprecise (127). This

is also evident in the fact that up to 30% of early stage (A and B) CRC patients do not survive beyond 5 years post-resection (169).

In the early nineties our laboratory developed a technique termed immunobead-PCR to aid in the detection of circulating tumour cells, a potential mechanism for the development of metastatic disease (32, 38). Subsequently the technique was applied to RT-PCR (immunobead RT-PCR) (39, 40). This technique uses magnetic beads coated with an epithelial-specific antibody to enrich for tumour cells from peripheral blood or intra-peritoneal lavage samples, while removing other blood cells, particularly lymphocytes and macrophages. The subsequent PCR or RT-PCR is carried out specifically on DNA or RNA from the tumour cells. As described in Chapter 1, Chelly *et al.,* (1989) demonstrated that gene transcription even at very low levels, may be detected in non-specific cells, i.e. a general phenomenon of basal transcription of any gene in any cell type. By using the immunobead technique, there is removal of other potentially contaminating cells which in turn alleviates the potential for detection of low-level illegitimate expression. Immunobead RT-PCR also has another advantage in that the use of nested PCR is not required, a technique which is used to increase sensitivity. Ficoll-Hypaque separation gradients are also not required, reducing the potential loss of tumour cells in the procedure.

In the past, the viability of circulating tumour cells has been questioned. In 1984 Umpleby *et al.,* found viable tumour cells (as assessed by their characteristic morphology and ability to exclude trypan blue) shed into the intestinal lumen in 52 of 74 specimens

from 49 patients with carcinoma of the large bowel (60). Fehm *et al.,* (2002) also investigated whether circulating epithelial cells in patients, including those with early stage tumours are aneusomic and whether their aneusomic patterns match those from the primary tumour indicating common clonality. This work determined using cytogenetic evidence, that circulating epithelial cells in patients with carcinoma are in fact malignant (170).

In 1995, Hardingham *et al.,* (32) reported using the immunobead PCR technique for the detection of tumour cells bearing mutations in codon 12 of the *K-ras* gene. Peripheral blood samples were collected peri-operatively and tumour cells were detected in a third of the patients investigated (9 of 27), 5 patients were positive in both pre and post-operative blood samples, 1 patient was positive in the pre-operative sample only and 3 patients were positive in the post-operative sample only. The results demonstrated a strong association with reduced disease free survival using Kaplan-Meier analysis (P=0.0001). However due to low patient numbers, they were unable to show whether the detection of tumour cells in peripheral blood samples was independent of stage as a prognostic indicator. More recently immunomagnetic beads have been used to isolate tumour cells in patients with colon (40, 112, 171), gastric (172), breast (173) and other epithelial (174) cancers.

In 2000, Hardingham *et al.,* used the immunobead RT-PCR technique to identify 19 of 94 (20%) patients positive in peripheral blood samples collected before surgery for CRC. A panel of epithelial markers were used including *CK 19*, *CK 20*, *MUC 1* and *MUC 2*. They

found four patients were positive for more than one marker, six patients were positive for *CK 19* only, six patients were positive for *MUC 2* only, three patients were positive for *CK 20* only, and one patient for *MUC 1* only. These results demonstrate that patients' tumour cells may vary in the expression of particular molecular markers and therefore using a panel (more than one molecular marker) would be more accurate in the detection of tumour cells in patients with varying stages of the disease. This study also found that 3 of 30 patients with adenomas and 4 of 34 patients with benign inflammatory bowel disease were also positive using these markers. This suggests that the molecular markers chosen may be epithelial-specific but not tumour-specific.

As previously described (Chapter 1), the immunobead technique is highly sensitive and has the ability to detect gene expression from 10 tumour cells in 10 mls of blood. In 2002, Raynor *et al.,* reported optimising the RT-PCR technique for use in a multi-marker assay in breast cancer. Tubes containing 100 mononuclear cells only were included to determine specificity of the chosen markers *ELF3*, *EpCam*, *EphB4*, *EGRF*, *CEA*, *MUC1* and *MGB1*. The study demonstrated that the specificity of markers needs to be tested as mononuclear cells may be retained in the immunomagnetic enrichment of epithelial cells and therefore could give false positive detection of tumour cells (175).

DPEP-1 has also been used to detect circulating tumour cells in peripheral blood and intra-peritoneal lavage samples using the immunobead technique. Fifteen of 38 patients were identified to have positive expression for this molecular marker (112). More recently our laboratory has identified 41/125 (32.8%) early stage patients who were

positive for disseminated tumour cells using the immunobead RT-PCR technique and the tumour-associated markers *CEA*, *LAM-γ2*, *EphB4*, *MAT*, and *CK20* (171).

In this study the molecular marker *DPEP-1* was identified by cDNA array analysis, and markers *LAM-γ2* and *MAT* were identified in the current literature as over-expressed in colon tumour compared with normal mucosa as described in Chapters 4, 5 and 6. They were determined to be suitable for use in the immunobead RT-PCR technique.

7.3 Patients and controls

One hundred and seventeen patients (64 males and 53 females, median age 73.5 years, range 43 - 95 years) diagnosed with early stage (stage A or B) primary CRC were recruited into the study between January 1999 and June 2003. Samples were also collected from twenty-nine stage C and twenty-two stage D patients (males 25 and females 26, median age 69 years, range 52-88 years). Informed consent was obtained from all patients and the research protocol was approved by The Queen Elizabeth Hospital Ethics of Human Research Committee.

Patients with non-malignant surgical colorectal disorders were used as negative controls to assess the tumour-specificity of the candidate markers. This group consisted of 48 patients admitted for resection for inflammatory bowel disease (Crohns Disease, Ulcerative Colitis and Diverticulitis), fifteen patients with other non-malignant bowel conditions and twenty-nine patients with adenomas. Adenomas (polyps), however are

considered to have the potential to develop into colon carcinomas and therefore may not be a true negative control.

Saline lavage samples for all patients undergoing surgery were collected from the pelvic floor (PF) and tumour bed (TB) region of the abdominal cavity before and after surgical removal of the colon tissue. For patients with benign disease and adenomas the lavage sample was collected from the pelvic floor region only. Patient peripheral blood and intra-peritoneal lavage samples were collected and processed as described in Chapter 2. As further negative controls for the panel of markers, peripheral blood samples from patients undergoing venesection for polycythemia vera were collected.

7.4 Results

7.4.1 Specificity of markers

To determine specificity of each marker, expression was examined in peripheral blood samples from healthy patients not undergoing surgery for CRC (negative control samples). Expression was not detected in 57 samples for *MAT*, 57 for *LAM-γ2* and 15 samples for *DPEP-1*.

7.4.2 CRC Patients

Overall, 82 of 168 (48.8%) patients were positive for one or more marker in at least one sample, a summary of which is shown in table 7.1. This group comprised 14 Stage A, 32 Stage B, 17 Stage C and 19 Stage D patients. Of patients who were positive for one or

more marker in any sample, 41 suffered disease relapse (recurrence) or death resulting from the cancer progression within the follow-up period. Tables 7.5 to 7.7 show all patients that were positive for each of the 3 molecular markers and the patients' current survival status. Currently a range of markers are being tested in the laboratory and therefore not all patients' samples were tested with the same panel of markers.

Forty-eight patients were positive for *MAT* and 48 were positive for *DPEP-1*, however *LAM-γ2* expression was identified in the least number of patients. Of the 115 patients tested with this marker, only 16 had positive expression in one or more sample (13.9%).

Table 7.1 Summary of samples positive for each marker in each sample type for all CRC patients.

Marker	**CRC samples**				**No. patients positive (%)**
	Pre-op PB	Post-op PB	Pre-op lavage	Post-op lavage	
MAT	5	6	26	41	48/141 (34%)
DPEP-1	8	3	24	34	48/102 (47%)
LAMγ2	1	1	4	13	16/115 (13.9%)

Statistical analysis was conducted on the early stage (stage A and B) CRC patients. As shown in Tables 7.5 to 7.7, samples from patients with CRC were collected over a number of years that this study was conducted and therefore the follow-up period for each of these patients was not equal. To take this into account, Kaplan-Meier survival analysis was used. Four patients were excluded from statistical analysis: two patients received radiotherapy or chemotherapy prior to surgery, which may have affected tumour cell viability and RNA integrity and 2 patients died post-surgery due to complications

arising from the operation. Fourteen of 41 (34.1%) stage A and 32 of 72 (44.4%) stage B patients were positive for one or more molecular marker.

Twenty-nine early stage patients were positive in one or both of their post-operative lavage samples compared to 17 who were positive in their pre-operative lavage samples. Five were positive in their pre-operative peripheral blood sample and four were positive in their post-operative peripheral blood sample.

Kaplan-Meier survival analysis was conducted on 113 early (A and B) stage patients. As shown in Figure 7.1, patients who were positive for any marker in any sample had significantly shorter disease-free survival (P=0.026).

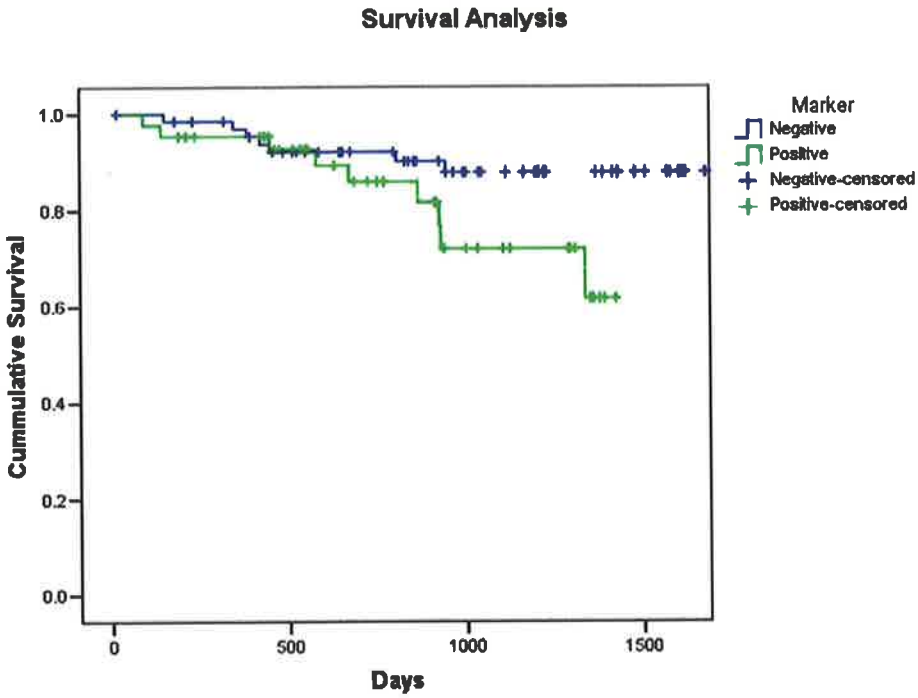


Figure 7.1 Kaplan-Meier survival curve of early stage (A and B) patients positive or negative for one or more molecular marker.

Further analysis revealed that patients who were positive for any marker in their post-operative lavage samples also had a significantly shorter disease-free survival than patients who were negative (P=0.038), as shown in Figure 7.2.

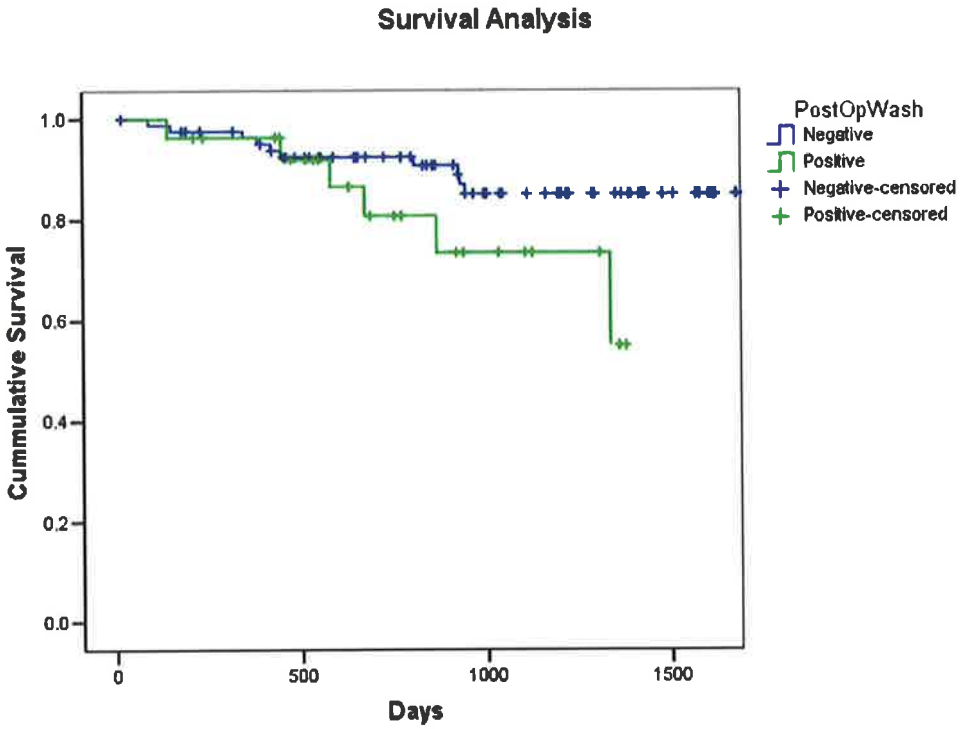


Figure 7.2 Kaplan-Meier survival curve of early stage (A and B) patients positive or negative for one or more molecular marker in post operative lavage samples.

A multivariate analysis was performed (Cox proportional hazards regression model) to test whether positive detection of disseminated tumour cells with any marker in any sample was an independent prognostic factor for disease relapse. Other clinico-pathological variables tested included site of tumour (colon or rectum), stage of tumour and age. The analysis showed that detection of disseminated tumour cells with any molecular marker remained significant (P=0.015, hazard ratio 3.459, 95% CI 1.272-9.410) and was independent of other risk factors as a prognostic marker of disease

relapse, therefore patients that were positive for any marker were 3.5 times more likely to suffer relapse than patients that were negative.

7.4.3 Non CRC Patients

Eleven of 48 (23%) patients with benign inflammatory bowel disease were positive for one or more molecular marker in any of their samples as shown in Table 7.2.

Table 7.2 Benign inflammatory bowel disease patients positive for one or more molecular marker.

Disease	Marker	Sample positive
Diverticulitis	*MAT*	Post PF
Diverticulitis	*LAM-γ2*	Post PB
Diverticulitis	*LAM-γ2*	Post PF
Diverticulitis	*LAM-γ2*	Post PB
Diverticulitis	*DPEP-1*	Pre PF
Diverticulitis	*DPEP-1*	Pre PF
Diverticulitis	*DPEP-1*	Pre PF
Diverticulitis	*DPEP-1*	Post PB
Diverticulitis	*DPEP-1*	Pre PF
Crohns disease	*LAM-γ2*	Pre PF
Caecal ulcer	*DPEP-1*	Post PF

PB; Peripheral Blood, PF; Pelvic Floor.

Five of 15 (33%) patients with other non-malignant bowel conditions were also positive as shown in Table 7.3. No patients' sample was positive for *LAM-γ2*.

Table 7.3 Patients with other non-malignant bowel conditions positive for *MAT* or *DPEP-1*.

Disease	Marker	Sample positive
benign disease	*MAT*	Pre PF + Post PF
pelvic abcess	*MAT*	Pre PF + Post PF
idiopathic constipation	*MAT*	Pre PF + Post PF
bowel obstruction	*DPEP-1*	Post PF
sigmoid volvulus	*DPEP-1*	Post PF

PB; Peripheral Blood, PF; Pelvic Floor. NB No patients positive for *LAM-γ2*

Ten of 29 (34%) patients with adenomas were positive for one of the three molecular markers as shown in Table 7.4. Patients were diagnosed with either tubulovillous adenoma (TVA) or Familial Adenomatous Polyposis (FAP).

Table 7.4 Patients with adenomas positive for *MAT*, *DPEP-1* or *LAM-γ2*.

Disease	Marker	Sample positive
FAP	*DPEP-1*	Post PF
TVA	*MAT*	Pre PF
TVA	*MAT*	Pre PF + Post PF
TVA	*LAM-γ2*	Pre PF + Post PF
TVA	*DPEP-1*	Pre PF
TVA	*DPEP-1*	Pre PF
TVA	*DPEP-1*	Post PF
TVA	*MAT*	Post PF
TVA	*MAT*	Post PF
FAP	*MAT*	Post PF

PB; Peripheral Blood, PF; Pelvic Floor.

A Fishers exact test found that the proportion of CRC patients positive for markers compared to benign cases was significantly different (P=0.002). However, there was no difference in the proportion of cases positive between CRC and adenoma cases (P=0.16) indicating that adenoma is not a suitable control group for CRC.

7.5 Discussion

Seven early (A & B) stage CRC patients were positive in their pre-operative peripheral blood sample for either *MAT* or *DPEP-1*. As suggested in Chapter 1 identification of circulating tumour cells in the peripheral blood before surgery, could indicate that the tumour is more advanced and the patient is therefore at risk of recurrence or developing metastasis. Of these patients, 2 (1 Stage A and 1 Stage B) experienced tumour recurrence (both positive for *DPEP-1*), 1 patient (stage B) experienced a wound recurrence and subsequently died of the disease (positive for *MAT*).

One theory that has been put forward is the possibility of whether or not manipulation of the bowel during colorectal surgery can aid in the dissemination of tumour cells into the peritoneal cavity, hence a risk for local tumour recurrence and metastases. Overall, 39 patients were positive for one or more markers in either one or both of their post-operative lavage samples, but were not positive in pre-operative samples. This suggests that tumour cells had been shed as a result of surgery. Fifteen patients were positive for *DPEP-1* in either one or both of their post-operative lavage samples, while 12 patients were positive for *MAT* and 6 patients were positive for *LAM*-γ2, as shown in Tables 7.5 to 7.7. Of these patients 14 died of disease (1 Stage A, 5 Stage B, 1 Stage C and 7 Stage D) and 7 developed disease recurrence (5 Stage B and 2 Stage C).

Six early stage (Stage A and B) patients with positive expression in their post-operative lavage samples only for one of the three markers died of colorectal disease, indicating

that surgical manipulation of the bowel may in fact enable viable tumour cells to gain access to the peritoneal cavity and circulation. This result is supported by the findings of our study using an extended panel of markers (171) in which we found that early stage patients who were marker positive for disseminated cells in post-resection lavage samples showed a significantly poorer prognosis (hazard ratio 6.2, 95% CI 1.9-19.6, P=0.002) and was independent of other risk factors. Of the 125 early stage patients (median follow up 42.3 months), 12% suffered disease relapse within the follow up period which comprised of 1/43 (2.3%) stage I and 14/82 (17%) stage II patients.

Seven patients were positive in their post-operative peripheral blood sample, 3 were positive for *MAT*, 3 were positive for *DPEP-1* and one was positive for *LAM-γ2*. Only 2 of these patients were early stage (Stage A or B) patients. Five of the 7 were stage C or D suggesting that as the tumour becomes more advanced the cells may gain access to the circulation more easily.

Here it has been demonstrated that tumour cells can be detected in the peripheral blood and lavage samples of patients undergoing colorectal surgery for cancer using the immunobead technique and three molecular markers *MAT*, *DPEP-1* and *LAM-γ2*. Early stage patients who were marker positive for disseminated cells showed a significantly poorer prognosis (hazard ratio 3.459, 95% CI 1.272- 9.410, P=0.015) than those who were marker negative. This was also found to be independent of other risk factors. Statistical analysis showed that patients who were positive for any marker were 3.5 times more likely to suffer relapse or death than patients who were negative.

This indicates that recurrence and distant metastasis in early stage patients may reflect dissemination of tumour cells during the surgical process rather than developing prior to surgical removal, as was also found in our study in which we used an extended marker panel (171). In this study, 21 patients were positive post-resection that were negative pre-resection. Ten of 21 (46.7%) relapsed post surgery, 9/10 being positive for tumour markers in peritoneal lavage samples and 1/10 in peripheral blood. Five patients developed distant metastases and 5 developed local recurrence.

An interesting finding was the rate of detection of circulating tumour cells with *LAM-γ2*. A possible explanation for this could be that this gene is up-regulated at the budding edge by tumour cells to enable the cells to migrate through the extracellular matrix. However, having achieved that step of the metastatic cascade, expression of this gene may be down-regulated to a level below the sensitivity of detection in the circulating cell.

Positive detection in samples collected from patients with benign bowel conditions may indicate that these markers are also epithelial-specific rather than tumour-specific. Even though the initial testing in tumour and normal colonic tissue samples showed that the markers were over-expressed in tumours in the majority of matched tumour-normal pairs (see chapters 4, 5 & 6), the markers were not proven to be 100% tumour-specific. To determine if these markers are detecting pre-malignant cells, further patient follow-up would be required to determine if these patients develop malignant colorectal carcinomas.

Table 7.5 Positive Tumour Cells Detected with *MAT* in CRC Patient Samples

Year of Surgery					Peripheral Blood		Tumour bed		Pelvic Floor		
	Patient No.	Age	Sex	Stage	Pre-Op	Post-Op	Pre-Op	Post-Op	Pre-Op	Post-Op	Current Patient Status
2003	1	65	F	A			POS	POS	POS	POS	Alive
	2	71	F	B		POS		POS		POS	Alive
	3	70	M	B				POS			Alive
	4	65	F	D					POS		Liver Metastasis
	5	68	F	B				POS		POS	Alive
	6	47	F	A			POS		POS	POS	Alive
	7	83	M	B					POS		Alive
	8	72	M	C		POS					Died CRC
	9	65	F	B			POS		POS	POS	Alive
	10	70	M	D	POS		POS				Died CRC
	11	78	F	D			POS	POS			Liver Metastasis
2002	16	81	F	C	POS						Died CRC
	17	61	F	B				POS		POS	Alive
	18	52	F	C					POS		Alive
	19	77	F	A				POS		POS	Alive
	20	75	F	D		POS					Died CRC
	21	59	F	D			POS		POS	POS	Died CRC
	22	76	M	B				POS		POS	Alive
	23	61	M	D			POS				Died CRC
	24	78	F	B				POS		POS	Alive
	25	70	M	A			POS	POS	POS	POS	Alive
	26	77	M	C			POS	POS			Died CRC
	27	60	M	A			POS				Alive

2001	39	82	F	A			POS	POS			Alive
2000	54	69	F	A		POS					Alive
	55	64	F	B						POS	Hyperplastic Polyp
	56	65	M	A						POS	Died Other Causes
	57	81	F	B			POS		POS		Alive
	58	50	M	B				POS		POS	Recurrence
	59	66	M	B						POS	Alive
	60	79	M	A						POS	Died complications
	61	79	M	B				POS		POS	Died CRC
	62	83	M	B				POS			Died CRC
	63	80	M	B					POS		Alive
	64	45	M	B				POS		POS	Alive
	65	86	M	D						POS	Died CRC
	66	85	F	B	POS						Alive
	67	84	F	B				POS		POS	Alive
1999	78	88	F	B	POS						Died CRC
	79	68	F	C		POS					Died Other Causes
	80	59	M	C						POS	Died CRC
	81	74	F	B		POS					Recurrence - Adenoma
	82	64	M	B						POS	Died CRC
	83	75	M	C	POS				POS		Died CRC
	84	71	F	D			POS				Died CRC
	85	85	F	B				POS	POS		Recurrence - Adenoma
	86	85	F	B						POS	Died Other Causes
	87	86	F	B					POS		Died Other Causes

Table 7.6 Positive Tumour Cells Detected with *DPEP-1* in CRC Patient Samples

Year of Surgery					Peripheral Blood		Tumour bed		Pelvic Floor		
	Patient No.	Age	Sex	Stage	Pre-Op	Post-Op	Pre-Op	Post-Op	Pre-Op	Post-Op	Current Patient Status
2003	4	65	F	D			POS	POS	POS	POS	Liver Metastasis
	7	83	M	B				POS		POS	Alive
	11	78	F	D			POS		POS	POS	Liver Metastasis
	12	72	F	B				POS			Alive
	13	64	F	D						POS	Died CRC
	14	65	M	B					POS		Alive
	15	85	M	C				POS		POS	Alive
2002	17	61	F	B				POS			Alive
	28	61	F	A					POS		Alive
	29	55	M	A	POS						Recurrence
	30	65	F	C				POS		POS	Alive
	31	62	M	C				POS			Liver Metastasis
	22	76	M	B				POS			Alive
	32	66	F	A			POS				Alive
	33	82	F	A	POS						Alive
2001	40	66	F	B			POS				Died CRC
	41	60	M	D	POS		POS		POS		Recurrence
	42	85	F	C					POS		Alive
	43	71	F	A				POS			Alive
	44	81	F	A	POS						Alive
	45	80	F	C			POS				Alive
	46	65	M	D		POS		POS			Died CRC
	47	77	F	D				POS			Alive

	48	76	M	C	POS			POS			Alive
	49	79	F	B		POS					Recurrence - polyps
	50	60	M	D			POS		POS		Died CRC
	51	70	F	A						POS	Alive
	52	77	F	C	POS						Alive
2000	54	69	F	A		POS					Alive
	55	64	F	B			POS			POS	Hyperplastic polyp
	57	81	F	B			POS				Alive
	58	50	M	B						POS	Recurrence
	61	79	M	B				POS		POS	Died CRC
	62	83	M	B						POS	Died CRC
	66	85	F	B	POS						Alive
	68	86	M	B				POS		POS	Alive
	69	74	M	B						POS	Recurrence
	70	89	M	C						POS	Died complications
	71	78	F	B				POS			Recurrence - Adenoma
	72	80	M	D				POS			Died CRC
	73	71	M	D			POS		POS		Recurrence
	74	58	M	B					POS		Alive
	75	77	F	B			POS		POS		Died Other Causes
1999	88	76	M	D			POS	POS	POS	POS	Died CRC
	89	83	M	B					POS		Died CRC
	90	54	M	D						POS	Died CRC
	91	64	M	C						POS	Recurrence
	92	50	M	B	POS						Recurrence

Table 7.7 Positive Tumour Cells Detected with *LAM-γ2* in CRC Patient Samples

Year of Surgery					Peripheral Blood		Tumour bed		Pelvic Floor		
	Patient No.	Age	Sex	Stage	Pre-Op	Post-Op	Pre-Op	Post-Op	Pre-Op	Post-Op	Current Patient Status
2003	**0/5 positive**										
2002	18	52	F	C						POS	Alive
	20	75	F	D			POS	POS		POS	Died CRC
	21	59	F	D				POS			Died CRC
	23	61	M	D				POS			Died CRC
	34	80	M	A				POS			Alive
	35	54	M	D		POS					Died CRC
	36	59	M	C				POS			Alive
	37	85	F	B						POS	Alive
	38	82	M	A				POS			Died CRC
2001	41	60	M	D	POS						Recurrence
	44	81	F	A					POS		Alive
	46	65	M	D				POS		POS	Died CRC
	50	60	M	D			POS				Died CRC
	53	86	F	C				POS			Alive
2000	76	81	F	C			POS				Alive
	77	85	F	B						POS	Alive
1999	**0/16 positive**										

Chapter 8

Discussion & Future Directions

8. Discussion

8.1 Identification of Potential Markers

This thesis describes the identification of three candidate tumour-specific molecular markers *DPEP-1*, *LAM-γ2* and *MAT*, for the detection of disseminated tumour cells in blood and intra-peritoneal lavage samples from patients undergoing surgical resection for CRC. Further characterisation was also carried out on *DPEP-1* and *LAM-γ2* to identify their potential roles in the migration and invasion of colon tumour cells through the extracellular matrix.

Previous work in the area of detection of circulating tumour cells identified that commonly used epithelial markers *CK 19*, *CK 20*, *MUC 2* and *CEA* may not be appropriate as tumour-specific markers for detecting circulating tumour cells in the blood due to their ability to detect non-tumour cells (40, 46). Here I have used a cDNA array approach to identify potential colon tumour-specific molecular markers for use in immunobead RT-PCR, a technique which has previously been shown to be highly sensitive in detecting circulating tumour cells in patients' peripheral blood samples collected prior to surgery (40).

DPEP-1 was previously identified by Buckhaults *et al.*, (2001) using serial analysis of gene expression on benign and malignant tumours. By *in situ* hybridisation they also identified that *DPEP-1* was localised exclusively to epithelial cells of colorectal tumours. However, validation of over-expression in tumour compared to normal mucosa was only carried out on 5 patients. Using cDNA microarrays I also identified *DPEP-1* as over-

expressed (112). Validation of this as a suitable molecular marker for immunobead RT-PCR was carried out in 68 patients' colorectal tumours, in which over-expression compared to matched normal mucosa was found in 82% (56/68). Over-expression was also found to be consistent across all stages of the disease.

Further investigation into the functional characteristics of *DPEP-1* found that HT29 cells, transfected with the *DPEP-1* construct to induce over-expression, migrated through the Matrigel-coated invasion chamber in greater numbers than untreated cells (P=0.007). The knockdown of *DPEP-1* expression using siRNA-mediated gene silencing determined by qRT-PCR, resulted in cells migrating through the invasion assay with significantly less capacity than mock transfected cells (P=0.034). However, the invasive capacity of *DPEP-1* siRNA treated cells was not significantly different from scrambled siRNA treated cells, suggesting that the interferon response had been invoked in these controls cells. This may have arisen due to a particular feature of the negative control sequence. To determine if this was the case, alternative negative controls which are more appropriate would need to be used, for example, biotechnology companies now have available suitable negative controls which have been optimised for use in conjunction with their siRNA products. Any future experiments would therefore use these controls.

LAM-γ2 and *MAT* have both been previously described in colorectal cancer, although neither had been previously used as a molecular marker to detect circulating tumour cells. Here I have shown that *LAM-γ2* is over-expressed in CRC tissue compared to adjacent normal colonic mucosa in a majority of CRC patients (73.3%). This pattern of expression

was consistent across all stages of disease. *MAT* was also found to be over-expressed in a majority (88.7%) of CRC patients and therefore was deemed to be an appropriate molecular marker for use with the immunobead RT-PCR technique.

I have also demonstrated that the migration capacity of anti-*LAM-γ2* antibody treated LIM 2099 cells was significantly reduced compared to untreated and normal isotype control IgG_1 treated cells (P=0.0006). This result showed that the anti-*LAM-γ2* antibody inhibited cell migration and invasion by greater than 80%, which is consistent with previous findings (158). An Annexin-V apoptosis assay indicated that there was no significant difference in the rates of cell apoptosis or necrosis between the treated cell groups.

At the time of commencing these studies, there were no reports in the literature of RNA interference to knockdown *LAM-γ2* expression and the effect on migrating tumour cells due to the relatively new technology of RNA interference. Here I have shown that the expression of *LAM-γ2* was significantly reduced using this technique, as determined by qRT-PCR when compared to *LAM-γ2* expression in mock transfected and control siRNA treated cells (P=0.012). siRNA-mediated gene silencing of *LAM-γ2* expression also resulted in a significant reduction of cell migration when compared to mock and control transfected treated cells (P=0.007). However, there was also a difference in cell migration between the mock transfected (transfection agent alone) and the negative control (transfection agent and scrambled siRNA treated) cells. This indicates once again that the negative control sequence (commercially obtained) would need to be redesigned.

8.2 Immunobead RT-PCR

In this thesis I have shown that tumour cells can be detected in the peripheral blood and lavage samples of patients undergoing colorectal surgery for cancer using the immunobead technique and three molecular markers *MAT*, *DPEP-1* and *LAM-γ2*. Overall, 82 of 168 (48.8%) patients were positive for one or more marker in at least one sample. This group comprised 14 Stage A, 32 Stage B, 17 Stage C and 19 Stage D patients.

Statistical analysis was carried out on 110 early stage (A and B) patients and it was found that those patients who were marker positive for disseminated cells showed a significantly poorer prognosis (hazard ratio 3.459, 95% CI 1.272- 9.410, P=0.015) than patients who were negative. This was also found to be independent of other risk factors. This indicates that patients that were positive for any marker were 3.5 times more likely to suffer relapse or death than patients who were negative.

Although each marker was tested in tumour and normal mucosa tissue samples for specificity, none of the 3 markers showed over-expression in all tumours. This indicates that an expanded panel of markers is required to increase the sensitivity of this technique. Here I used three potential molecular markers, however, a panel of five markers would be more sensitive, which was used by Lloyd *et al.*, (2006).

In this study, expression was also detected in patients with benign inflammatory bowel disease. However, a Fishers exact test found that the proportion of CRC patients positive

for markers compared to benign cases was significantly different (P=0.002). This was not the case for patients with adenomas. There was no difference in the proportion of cases positive between CRC and adenoma cases (P=0.16), indicating that adenoma is not a suitable control group for CRC. All patients with benign disease will be followed-up to determine whether CRC develops. As mentioned earlier, although markers were sought that were tumour-specific no marker identified was 100% over-expressed when tested on patient matched tumour and normal mucosal tissue samples. This may be a result of the diversity of expression of genes in an individual's tumour and therefore an exclusively "tumour-specific" molecular marker may not exist. This also demonstrates the importance of the use of a panel of markers for use with this technique, enabling a greater proportion of patients to be tested.

8.3 Problems and Pitfalls

The use of macro-dissected tissue for RNA extraction was not ideal, however at the time that this study was conducted the technology was not available to our laboratory to perform laser capture micro-dissection (LCM) on the tissue, as used by Kitahara *et al.*, (2001). Therefore there was the potential for contamination from other cells types in the microarray study. With the use of LCM technology a more specific pool of suitable candidate genes for use as markers in immunobead RT-PCR could have been identified.

One of the major problems encountered in this research project involved the hybridisation of the nylon cDNA microarrays. Many optimisation experiments were conducted, however obtaining good hybridisation and array images from the phosphoimager was

quite difficult. According to the manufacturer's instructions, the arrays could be hybridised, stripped and re-probed, unfortunately this delivered inconsistent results. Therefore, when the opportunity arose to use the services of the new Adelaide Microarray Facility (Adelaide University) to hybridise samples, it was decided that this approach would produce better results. The glass slides were also spotted with a greater number of cDNAs (8,000 compared to 1,400) giving us a greater number of potential genes to investigate.

Due to the relatively new siRNA-mediated gene silencing technology introduced to the laboratory, the siRNA component of this project required a vast amount of optimisation with new developments and molecular products rapidly becoming available. Future research would use fluorescent-tagged siRNA to enable the determination of transfection efficiency using fluorescence microscopy or flow cytometry. However, the results obtained particularly for *LAM-γ2,* look very promising for the use of this technique to characterise the functional effects of specific gene knockdown.

8.4 Future Research

The natural progression of this project to identify candidate tumour-specific molecular markers would be to investigate differential amounts of protein found in LCM tumour and normal colonic tissue samples. Using LCM and 2D protein gel electrophoresis, the potential for contamination by other cell types will be alleviated and the protein product may be a better indicator of over-expressed genes than cDNA microarrays. More recent studies have indicated that proteomics may be a better technique to identify biomarkers

with clinical significance. Proteins are the functional molecules in the cell and the relationship between gene expression measured at the mRNA level and the corresponding protein level is not linear (176). Changes in the proteome of colon mucosal cells accompany the transition from normal mucosa via adenoma and invasive cancer to metastatic disease (177). Yu *et al.*, (2004) used proteomics on serum samples from patients with colorectal cancer, adenoma and healthy patients (178). They found that the diagnostic pattern combined with 7 potential biomarkers could differentiate carcinoma patients from those with adenomas with a specificity of 83%, sensitivity of 89% and a positive predictive value of 89%. The diagnostic pattern combined with 4 potential biomarkers could also differentiate carcinoma patients from healthy patients with a specificity of 92%, sensitivity of 89% and a positive predictive value of 86%.

Future work would also investigate the ability of siRNA to modulate *LAM-γ2* and *DPEP-1* expression in an *in vivo* mouse model to determine whether delivery of gene specific siRNA into a solid tumour has the ability to reduce tumour size or prevent metastatic spread. An alternative animal model would be to use a chick embryo assay to examine the effects of siRNA treatment *in vivo* compared to the *in vitro* assays described in this study. The chick embryo model could be used first as a screening test as it is a cheaper model and results could be obtained in a shorter period of time.

Immunobead RT-PCR is also proving to be an important tool in the identification of patients at risk of developing recurrent or metastatic disease. We used five markers on 125 early stage CRC patients and found that patients with positive expression of 1 or

more markers, particularly in post-operative peritoneal lavage samples, were 6.2 times more likely to develop recurrent or metastatic disease than patients who were negative for marker expression (P=0.002) (171). These results demonstrate that this technique may be an appropriate tool for use by clinicians to identify "at risk" patients or to improve the initial staging of the patients' disease. A clinical trial with a panel of suitable molecular markers would determine if this technique is appropriate for use in clinical practice to aid clinicians in selecting 'high risk' early stage patients for adjuvant therapy.

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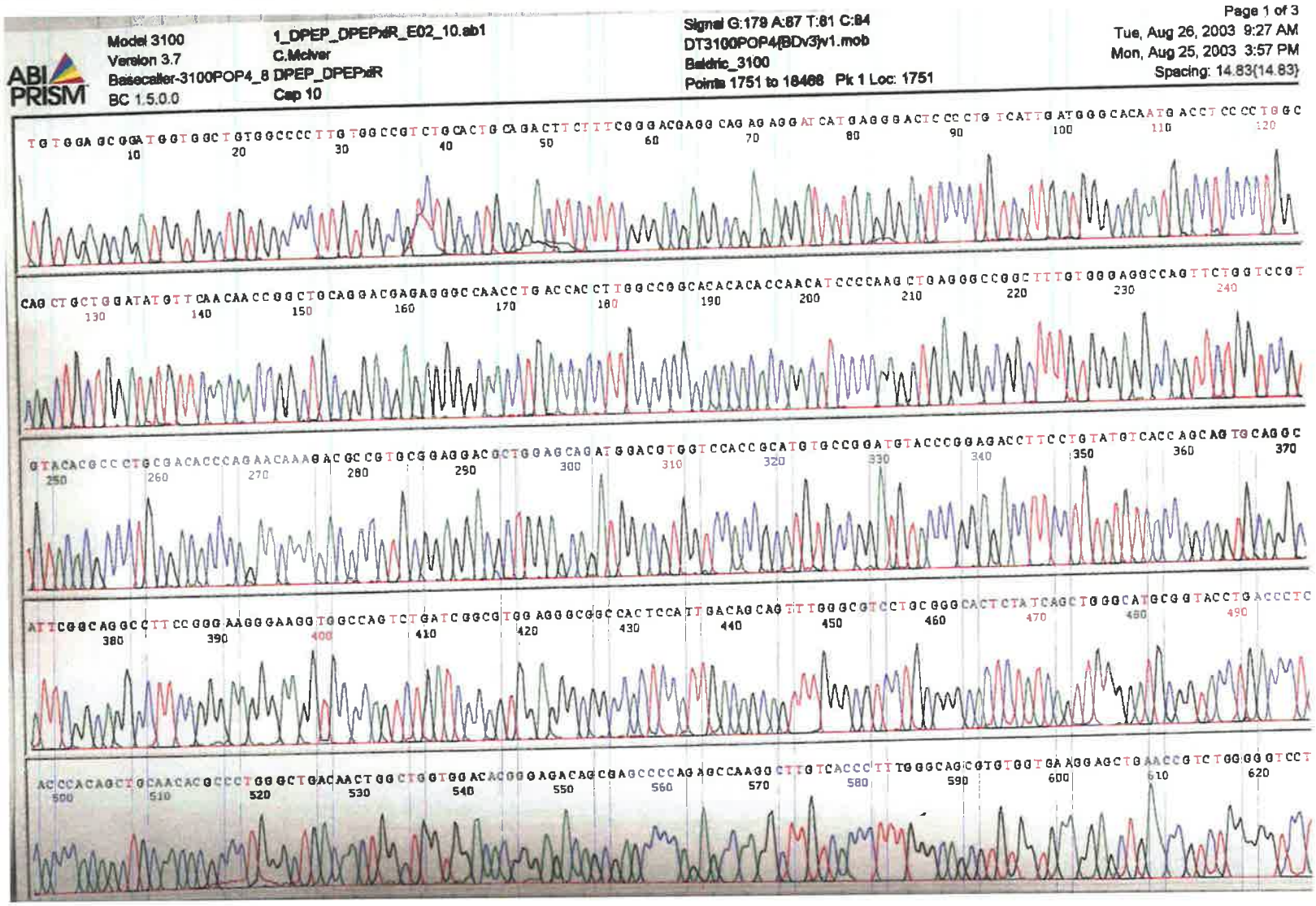
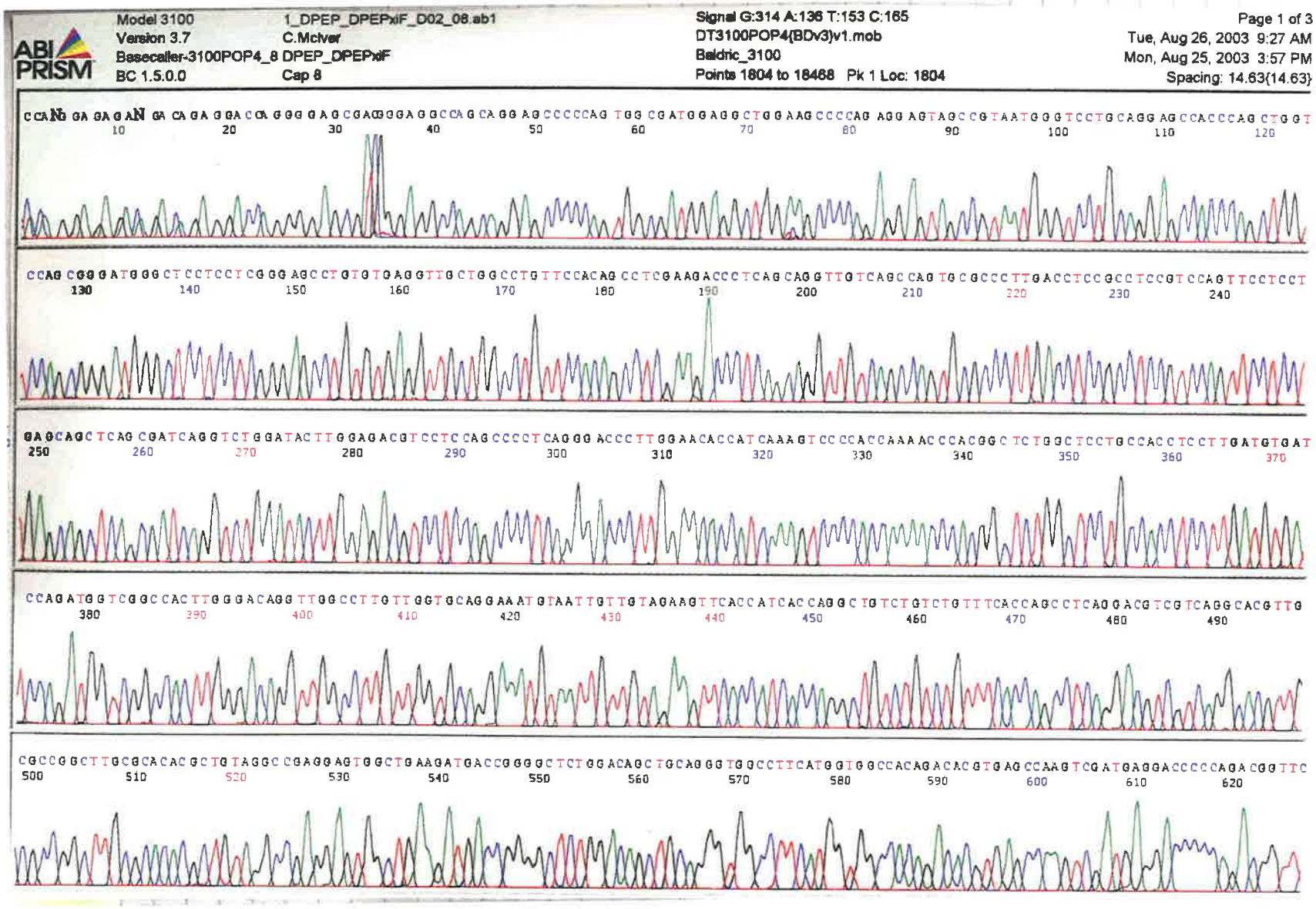
Appendix I: *DPEP-1* sequence chromatography

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C.McIver
DPEP_DPEPxiF
Cap 8
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Baldric_3100
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Mon, Aug 25, 2003 3:57 PM
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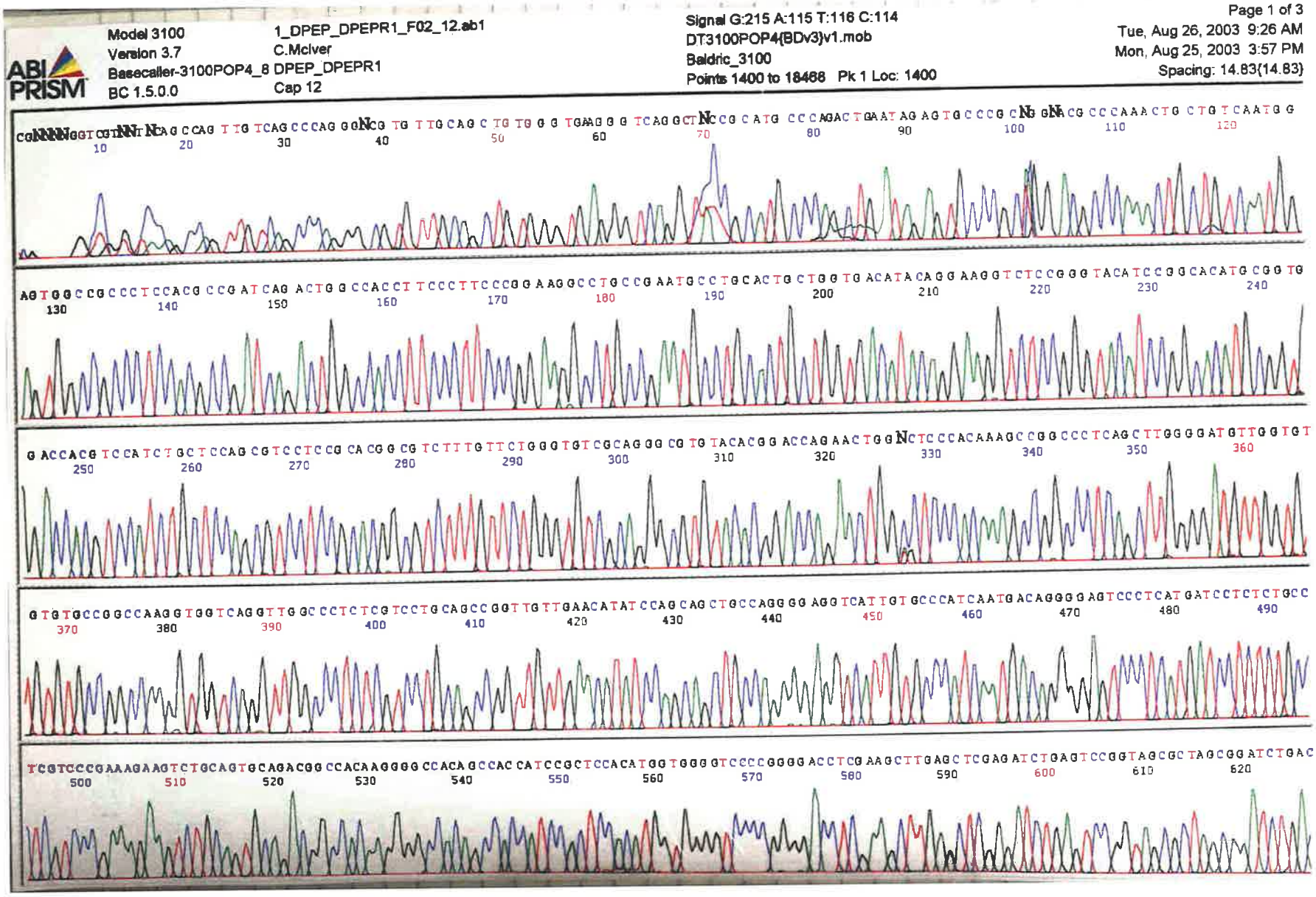
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C.McIver
DPEP_DPEPR1
Cap 12
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Baldric_3100
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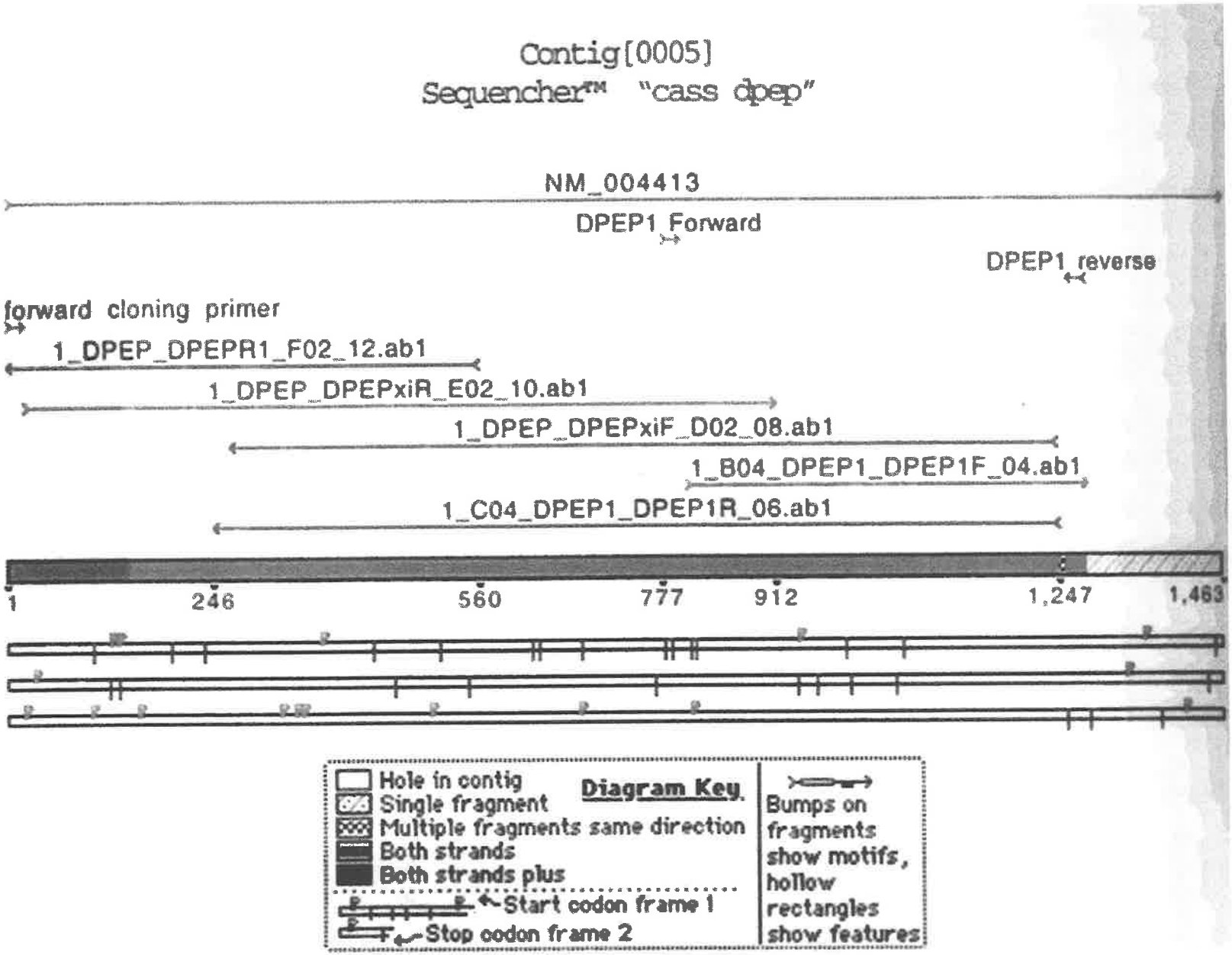
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1
246
560
777
912
1,247
1,463
Diagram Key
Hole in contig
Single fragment
Multiple fragments same direction
Both strands
Both strands plus
Start codon frame 1
Stop codon frame 2
Bumps on fragments show motifs, hollow rectangles show features

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Cancer Letters, v. 2O9 (1) pp. 67-74

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