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Immunocytochemistry Of Rectal Cancer Circumferential Margins In The Assessment Of Micrometastatic Disease

By

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Thesis Submitted For The Degree Of Master Of Philosophy

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Pontypridd

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Declaration

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This thesis is the result of my own investigation, except where otherwise stated. Other sources are acknowledged by giving explicit references.

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Summary

Introduction

Micrometastatic disease has been shown in the literature to exist in the lymph nodes, peripheral circulation and bone marrow of colorectal cancer patients. Furthermore, micrometastatic disease has been shown to be associated with recurrence of the disease. However, there is no evidence of investigation into the micrometastastic disease at the circumferential margins of rectal cancers. Therefore, this study was aimed at the assessment of micrometastatic disease in the mesorectum and circumferential margins of rectal cancers.

Methods

Data on rectal cancer patients who had a mesorectal excision with a curative intention were extracted from patient case notes. This included clinico-pathological information including recurrence and mortality related to cancer. All palliative resections, laparoscopic resections, polyp cancers and non-cancer mortality cases were excluded. We then retrospectively analysed rectal cancer specimens for the presence of micrometastasis (MM) and isolated tumour cells (ITC) in the mesorectum and at the circumferential margins by using automated immunocytochemistry (ICH) with MNF 116 and CK5D3 monoclonal antibodies. The International Union Against Cancer (IUAC) criteria were applied objectively to classify the micrometastatic disease in to MM and ITC. This was compared with Haematoxylin and Eosin (H&E) staining method to assess any difference in the rate of detection of MM and ITC. An association of Micrometastatic disease with local recurrence and survival was studied.

Results

Immunocytochemistry was superior in the detection of ITC in the mesorectum compared with H&E staining (p=0.05) with a non-significant improvement in the detection of MM and ITC at the CRM using ICH. ITCs in the mesorectum and at the CRM were significantly associated with tumours larger than 4 cms in size. MM in the mesorectum was significantly associated with tumours with vascular invasion (p=0.000), lymph nodal involvement (p=0.004), Dukes stage (p=0.001), and nodal stage p= (0.000). Systemic recurrence was associated with MM (p=0.001) and ITC (p=0.001) at the CRM and MM in the mesorectum (p=0.05), but not by the ITC in the mesorectum (p=0.97). Interestingly, local recurrence was neither influenced by ITCs in the mesorectum (p=0.66) or CRM (p=0.66), nor by the MM in the mesorectum (p=0.66) or CRM (p=0.66). However, mesorectal MM (p=0.003), MM at the CRM (p=0.04), and ITC at the CRM (p=0.003) significantly affected the survival.

Conclusions

We have demonstrated the existence of micrometastases and isolated tumour cells at the circumferential margins and in the mesorectum of rectal cancers both by ICH and H&E. The immunocytochemistry was superior in the detection of both MM and ITC compared to H&E. Furthermore, MM and ITC have been shown to be associated with systemic recurrence of the disease and reduced survival. Potential implication of this of this research is that the use of adjuvant therapy may be extended in patients who are positive for micrometastatic disease at the circumferential margins and the mesorectum on immunocytochemistry to improve survival. Furthermore, it can be concluded that the immunocytochemistry may be applied routinely in the assessment of micrometastatic disease of rectal cancer circumferential margins.

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List Of Abbreviations

ACPGBI	Association of Coloproctology of Great Britain and Ireland
CEA	Carcinoembryogenic antigen
CK	Cytokeratin
DNA	Deoxy-Ribonucleic acid
FDG-PET	Fluoro Deoxy Glucose Positron emission tomography
H&E	Haematoxylin and Eosin
ICH	Immunocytochemistry
ITC	Isolated tumour cells
IF	Intermediate filaments
IUAC	International Union Against Cancer
K Da	Kilo Dalton
MM	Micrometastases
MASA	Mutant allele specific antigen
NHS	National Health Service
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase chain reaction
RNA	Ribonucleic acid
TNM	Tumour, node and metastases classification

INTRODUCTION

Introduction

1.1 General introduction and incidence

Colorectal cancer is the second most frequent cause of cancer deaths in the UK, and is one of the most common malignant conditions in the United Kingdom with an annual incidence of approximately 34,000 cases per year (Cancer Research UK, 2003). Although, there have been many advances in diagnosis and treatment modalities in recent years; there has been only a moderate improvement in survival (Mella 1997). The median survival remains static at 40-50% despite reduction in mortality rates by 27% in men and 43% in women (Maxwell- Armstrong 2003, NHS Direct 2003). The prognosis of colorectal cancers depends on the TNM stage of the disease and in particular, lymph nodal status (Rotto 1998) with a good prognosis for early stage tumours. However, between 25-30% of patients in spite of apparent curative resection, will develop recurrence eventually (Leifers 1998). This could be due to the presence of microscopic residual disease in the loco-regional nodes or peritoneal seedling, which cannot be detected by routine histological methods (Leifers 1998). Presumably, this microscopic residual disease subsequently manifests as disease recurrence leading to morbidity and mortality.

1.2 Staging and survival of rectal cancer

Conventionally, rectal cancers are staged based on Dukes' classification (Dukes 1958). Dukes A cancer is a carcinomatous lesion confined to bowel

wall, but in Dukes B stage, carcinoma spreads beyond the bowel wall in to pericolic or perirectal tissues. In Dukes C stage, tumour spreads to lymph nodes and presence of hepatic metastases is classified as Dukes D stage. Although other classification systems such as Jass classification (Jass 1987) have been described, currently TNM staging is accepted as a standard method of evaluating disease extent and forms the basis for any adjuvant therapeutic treatment. Survival of rectal cancers depends on several factors such as the stage of the tumour at presentation, vascular invasion, tumour differentiation and lymph nodal involvement (Broll 1997, Rotto 1998). However, local recurrence of rectal cancers is affected not only by tumour related factors, but also by other factors such as involvement of distal resection margins and circumferential margins, spillage of malignant cells in to the operative field in the pelvis, as in tumour perforation or rupture at the time of operation (Slanetz 1984, Porter 1996, Eriksen 2004).

1.3 Significance of circumferential margin involvement and local recurrence

Tumour involvement of the circumferential margin independently influences both local recurrence and systemic failure (Nagtegaal 2002, Wibe 2002, Adam 1994) in addition to other prognostic factors such as tumour staging (T Stage of the TNM Classification) lymph nodal involvement, vascular invasion and perineural invasion. Local recurrence is a serious problem after rectal cancer surgery usually leading to death, after severe pain and distressing symptoms.

The reported rates of local recurrence in the literature range from 10% (Arbman 1996, Bokey 1999) up to 50% (Hall 1998, Dahlberg 1999, Bolognese 2000).

1.4 Adjuvant therapy

Currently, Total Mesorectal Excision is the standard surgical procedure of choice for operable rectal cancers. Furthermore, adjuvant treatment is recommended for Dukes C stage based on conventional histological staining using Haematoxylin and Eosin. Adjuvant therapy plays a significant role in the treatment of rectal cancers (Swedish Rectal cancer trial 1997, Kapiteijn 2001, Delaney 2002). Neo-adjuvant therapy with radiotherapy is recommended based on the stage of the cancer. Furthermore, rectal tumours with positive resection margins and cases with high risk of recurrence benefit from postoperative adjuvant radiotherapy (Rotto 1997). Adjuvant treatment has been shown to increase recurrence free survival, and overall survival without which there would be an increase in local recurrence and hence decreased survival (Leifers 1998, Rosenberg 2000).

1.5 Micrometastases and detection methods

In some cases, routine histological techniques may fail to detect the presence of malignant cells in the lymph nodes. As a result, more sensitive techniques such as immunocytochemistry have been used to identify malignant cells (Haboubi 1992). Recently, several studies have investigated micrometastases in the lymph nodes using anti-cytokeratin antibodies. It was found that

patients with positive micrometastases in lymph nodes had poor prognosis and increased risk of recurrence (Greenson 1994, Sasaki 1997, Isaka 1999, Yasuda 2001). However, in some other studies, no significant correlation between the presence of micrometastases in the lymph nodes and recurrence or overall survival was noted (Cutait 1991, Jeffers 1994, Adell 1996, Broll 1997, Oberg 1998, Nakanishi 1999). Therefore, there is no agreement whether or not the occult metastases detected in the lymph nodes by immunocytochemical staining, or molecular techniques, significantly affect patient outcomes (Oberg 1998, Isaka 1999, Nakanishi 1999). The continuing efforts on the understanding of micrometastastic disease are limited to research studies only. Furthermore, the search for occult metastases within the NHS setting is associated with financial difficulties and time restrictions. Hence, the routine use of immunocytochemical staining on the lymph nodes is questionable especially as the current literature is uncertain on the significance of these micrometastases in the lymph nodes on the clinical outcome such as survival or recurrence (Isaka 1999).

Immunocytochemistry has been applied to detect malignant cells in the bone marrow (Davidson 1990, Schlimok 1990), peripheral blood (Weitz 1998, Rosenberg 2002) with conflicting results; consequently the significance of routine use of this form of investigative approach in the assessment of micrometastases is unresolved.

Other modalities such as Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) to detect genetic mutations and RNA transcripts for CEA, CK-20 have been recently studied in an attempt to detect micrometastatic disease (Hayashi 1995, Mori 1995). Although, this technique is more sensitive than immunocytochemistry, its availability is restricted to a few centres in view of the cost.

1.6 Rectal cancer assessment of circumferential margins

Rectal cancer spreads locally by radial and axial contiguous growth, vascular invasion and lymphatic spread in addition to spread by neural invasion. Therefore, it could be hypothesized on this basis that the rectal cancer growing radially, towards the circumferential margin may have micrometastases or malignant cells, similar to that observed in lymph nodes. If malignant cells could be missed on routine histological methods in a lymph node, it is possible that involvement of circumferential margins with malignant cells is also misinterpreted using conventional staining methods, resulting in false negative reporting.

Currently, circumferential margins are assessed using the same staining techniques as lymph node staining (Haematoxylin and Eosin). Immunocytochemical staining of Cytokeratins is very sensitive and reasonably specific technique for detection of malignant cells of epithelial origin (Angus 1987). Staining of circumferential margins with immunocytochemistry is a relatively easier technique and less resource

intensive than lymph nodal immunocytochemistry. However, the use of immunocytochemistry to assess the circumferential resection margins of rectal cancers has not been reported to date in the literature. It is an innovative and novel application of immunocytochemistry on the assessment of circumferential margin, which is an important determinant of local recurrence.

1.7 Significance of positive circumferential margins

Positive circumferential margins are associated with significant incidence of local recurrence, which further reduces overall survival and increases morbidity due to chronic pelvic pain (Nagtegaal 2002, Wibe 2002). Therefore, circumferential margin assessment is critical in the routine evaluation of rectal cancers. Furthermore, recently, mesorectal deposits have shown to be significantly associated with decreased survival and increase in local recurrence (Prabhudesai 2003). It is likely that the mesorectal deposits grow from micrometastases in the mesorectum. The purpose of this study was to utilise this approach in the assessment of circumferential margins and mesorectum for micrometastatic disease.

1.8 The Research Problem

Circumferential margin involvement is one of the crucial factors in the determination of local recurrence of rectal cancer. The information on the involvement of circumferential margins helps in the decision making for adjuvant therapy. Therefore, circumferential margin assessment is critical in the routine histopathology reporting of rectal cancers. It is clear from

literature that routine Haematoxylin and Eosin staining is less sensitive than other techniques such as immunocytochemistry in the detection of micrometastases. Therefore, by applying the current staining techniques, it is quite possible that micrometastastic disease could be missed. However, application of sensitive methods such as immunocytochemistry may improve the detection of micrometastatic disease, which may identify patients at increased risk of local recurrence and decreased survival. Furthermore, presence of mesorectal tumour deposits has been shown to affect local recurrence and survival. Similarly, involvement of mesorectum with micrometastatic disease may affect prognosis.

1.9 Aims and Objectives

The aims and objectives of this study were as follows;

1. The analysis of various clinico-pathological factors of the patients with rectal cancers;

2. Staining rectal cancers using immunocytochemical techniques and to assess the presence of malignant cells at the circumferential resection margins and in the mesorectum;

3. Examination of the association between the presence of malignant cells in the mesorectum and their presence at the circumferential resection margins with both local and systemic recurrence of rectal cancer;

4. Examination of the impact of the presence of malignant cells in the mesorectum or the malignant cells at the circumferential resection margins on mortality.

1.10 Hypothesis for the research project

Hypothesis 1: Rectal cancers have malignant cells at the circumferential resection margins and mesorectum. By applying Immunocytochemical methods detection rate of malignant cells is improved compared with routine histological staining.

Hypothesis 2: Presence of malignant cells detected by the immunocytochemistry at circumferential margins or in the mesorectum increases local recurrence rate and decreases overall survival.

1.11 Implications of the current research project

Current literature on the treatment of rectal cancer supports a definitive role of adjuvant therapy. Both Swedish and Dutch rectal cancer trials have shown a significant decrease in local recurrence following radiotherapy (Swedish Rectal cancer trial 1997, Kapiteign 2001). Furthermore, the Swedish trial has shown improvement in survival (Swedish rectal cancer trial 1997).

At present, radiotherapy is offered as a standard practice in patients pre-operatively with locally advanced tumours involving other structures such as urinary bladder or proven or questionable involvement of adjacent structures such as seminal vesicles, prostate or vagina. Conventionally, bulky rectal cancers and cancers that are fixed are given preoperative radiotherapy to down stage prior to surgical treatment

Post-operative chemo-radiotherapy may be offered to patients with positive resection margins and involvement of the circumferential margins based on current histological staining methods. If a significant correlation of positive circumferential resection margin for malignancy on immunocytochemical method and recurrence or poor survival is demonstrated from this study, there is scope, therefore, for expanding indications for adjuvant post operative radiotherapy in a sub-group of rectal cancer patients who are positive for malignant cells at the circumferential margins detected by immunocytochemistry.

1.12 Justification for this research project

There have been no studies documented in the literature to assess the presence of micrometastatic phenomenon at the circumferential margins and in the mesorectum, although micrometastases have been demonstrated in the lymph nodes, peripheral circulation and bone marrow of colorectal cancer patients (Funaki et al 2000, Leinung et al. 2000, Noura 2002, Rosenberg et al. 2002).

Local recurrence in rectal cancers is a serious problem after surgery for rectal cancer and usually leads to painful and distressing symptoms culminating in death. Local recurrence may be explained by many factors such as poorly performed total mesorectal excision, tumour related factors such as locally

advanced tumour, mesorectal invasion, lympho-vascular invasion and poor differentiation.

However, local recurrence in rectal cancers such as Dukes stage A and B is difficult to elucidate. Micrometastatic phenomenon has been demonstrated around hepatic colorectal metastatic deposits suing RT-PCR and ICH (Ambiru 1999). Furthermore, clusters of cancer cells were demonstrated microscopically ahead of the invading margin of the tumour called "tumour budding" were seen in very early primary submucosal tumours (Okuyama 2002, Okuyama 2003). Tumour budding was shown to influence the lymph node metastases even in early cancers such pT1 and Pt2 tumours (Okuyama 2002). This study was performed with an intention to examine the hypothesis that micrometastatic phenomenon may exist at the circumferential margins and in the mesorectum.

METHODS

2.1 Summary

This study was performed retrospectively on rectal cancers identified from two district general hospitals in South Wales. The main objective was to evaluate the clinico-pathological characteristics of the rectal cancer patients and assess local and systemic recurrence patterns and their relationship to the patient and tumour characteristics. The second objective was to conduct immunohistological examination of rectal cancers using anticytokeratin antibodies and assess the presence of micrometastases in the mesorectum and at the circumferential resection margin. The incidence of micrometastases and its relationship to patient and tumour characteristics was studied. A comparison was then performed between routine histological and Immunocytochemical staining methods to assess the effectiveness of the new technique in the evaluation of micrometastases in the mesorectum and at the circumferential margin.

2.2 Ethical approval

This study essentially involved using previously paraffin-blocked rectal cancers from which new sections were obtained. These were stained with more sensitive staining techniques to assess the presence of cancer cells at the circumferential margin and mesorectum. The study did not involve patients or their identity nor did it involve patient participation. The patients were not contacted at any stage of the study. However, the study involved obtaining patients' data from the clinical records. Patients' confidentiality was maintained at every stage during the study.

A formal ethical approval was obtained from the Bro Taf local ethics committee that approves for medical research projects in Wales (Appendix A). Furthermore, research risk review group approval from each hospital was obtained prior to the commencement of the project (Appendix B and C).

2.3 Data Protection and Caldicott Guidelines

Data protection regulations and Caldicott guidelines were followed to maintain the confidentiality of the patients' clinical data. All patient details such as name and date of birth were anonymised to maintain patient confidentiality.

2.4 Collection of clinico-pathological details of rectal cancers

Relevant individual case notes were identified from the medical records department and the patient clinical data were collected on various aspects related to treatment. Rectal cancer patients who had a total mesorectal excision with a curative intent were included in the study. Clinico-pathological data were obtained from patient notes, operative records and pathology reports. The clinical data were collected from each patient as summarised in appendix D.

2.5 Inclusion criteria

Patients were included based on pre-defined criteria as summarized in table2.1 These include rectal cancers who had a surgical resection with a curative intention such as total mesorectal excision either by anterior resection or abdomino-perineal resection. Patients must have had their resection between the years 1997 to year 2000 with a completed follow-up period of 24 months at the time of data collection to assess the local recurrence and systemic disease recurrence. However, patients who died of recurrent cancer from either metastases or local recurrence prior to 24 months were also included in the study.

Table 2.1 Inclusion criteria

Inclusion criteria
• Rectal cancers with curative resection; • Should have had either Anterior resection or Abdomino-perineal resection with a curative intention; • Follow-up for at least 24 months post operatively; • Surgical procedure performed by an open procedure only.

2.6 Exclusion criteria

Some of the patients were excluded from the study at the outset with certain factors, which are likely to affect the study results. The exclusion criteria are summarised in table 2.2. Patients who have not completed 24 months of follow-

up, patients who died post-operatively, and deaths due to any cause other than cancer such as intercurrent illness, trauma, psychological causes or chemo-radiotherapy related deaths were excluded. Patients who underwent resectional surgery in the presence of established hepatic metastases, or any other metastases, including peritoneal disease were also excluded. Furthermore, all cases who had palliative procedures performed were excluded at the outset. Rectal cancers identified in polypectomy specimens, trans-anal cancer excisions such as Trans anal endoscopic microsurgery (TEM) and Trans anal resection of tumour (TART) were excluded. Patients who had inadequate total mesorectal excision, rectal cancers which were operated laparoscopically and patients who were lost during the follow-up with in two years were also excluded from the study.

Table 2.2 Exclusion criteria

- Incomplete follow-up (less than 24 months);
- Lost to follow-up;
- Death due to intercurrent illness, non-cancerous death such as trauma, cardiac, respiratory causes, renal conditions, psychiatric illness, post-operative causes, post chemo-therapy and post radiotherapy;
- Inadequate total mesorectal excision;
- Presence of metastases at the time of surgery;
- Surgical procedure performed laparoscopically;
- Tran-anal excisional procedures such as TEM and TART;
- Rectal cancers identified in polyps;
- Rectal excision in the presence of peritoneal disease;
- Rectal cancers operated in emergency with obstruction or perforation;
- Recto-Sigmoid cancers;
- Upper rectal cancers. *

* In view of minimal mesorectum and difficult to differentiate between recto-sigmoid tumours and upper rectal tumours.

Upper rectal cancers and Rectosigmoid cancer

Tumours arising from upper rectum and rectosigmoid region were excluded for two important reasons. In the case of upper rectal tumours, the extent of mesorectum is limited compared with the mid and lower rectum, as peritoneum covers the rectum as a serosal layer on the majority of the circumference, leaving a small portion where the vascular pedicle travels into the mesorectum (Figures 2.1 and 2.2). Furthermore, it would be difficult to know if the tumour had arisen as a rectosigmoid lesion and subsequently propagated distally into the upper rectum. Although there is no literature to support the view that the biological behavior of these cancers may be different from rectal cancers, we have excluded rectosigmoid cancers.

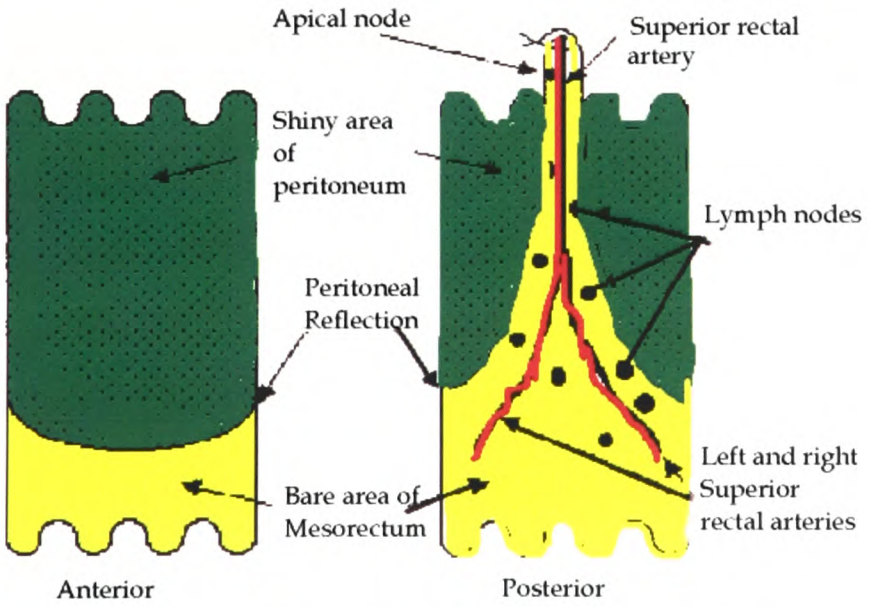


Figure 2.1 Schematic diagram showing mesorectum and peritoneal reflections of the rectum (Adopted from the Royal College of Pathologist's minimum dataset document on rectal cancer)

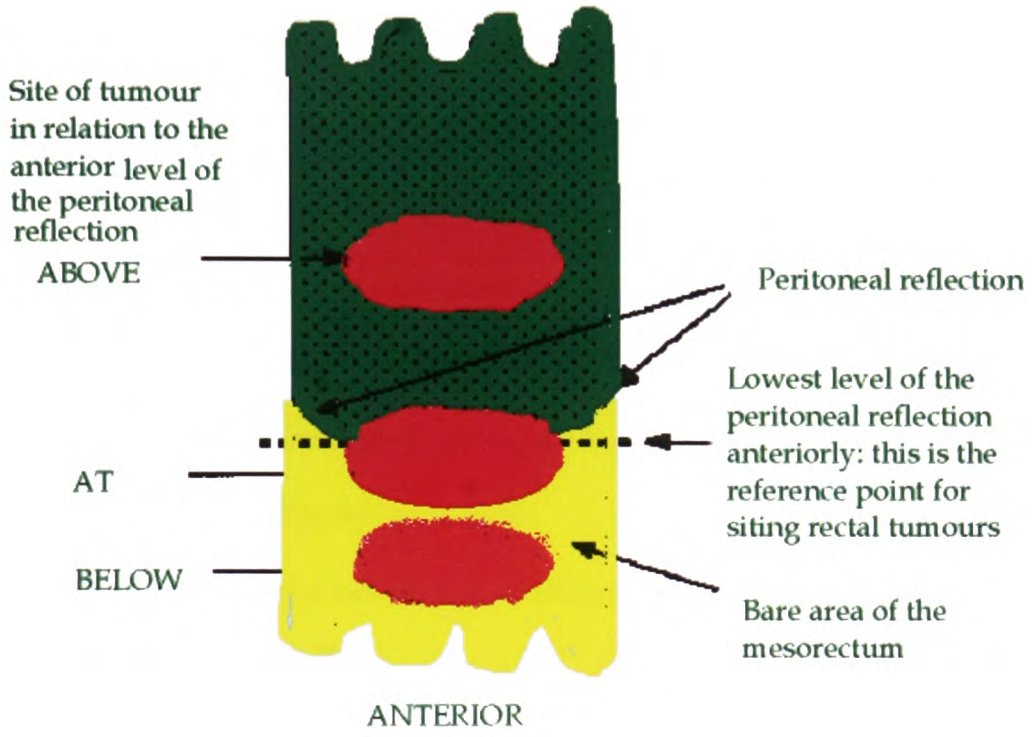


Figure 2.2 Schematic representation of peritoneal reflection on the anterior surface of rectum (Adopted from the Royal College of Pathologist's minimum dataset document on rectal cancer).

2.7 Criteria for micrometastases and Isolated tumour cells

There has been no uniform definition in the classification of minimal residual disease. The presence of minimal residual disease has been variably represented as occult metastases, micrometastases or isolated tumour cells. This has led to difficulty in interpretation of the findings from various studies. Although a universally accepted term for this phenomenon was not found in the literature, a recent publication by the International Union Against Cancer (IUAC 1998) emphasised that isolated tumour cells must be distinguished from

micrometastasis (Harmanek 1999). The differences highlighted by the IUAC between isolated tumour cells and micrometastasis is given in table 2.3. This classification applies to micrometastases and ITC detected in the lymph nodes only. Currently no such classification is available for micrometastases or ITC that exist at the CRM or in the mesorectum. Therefore, in this study, we have followed the classification as suggested in the following table for identification and stratification of micrometastatic disease except the criteria of lymph sinus contact and invasion of lymphatic sinus.

Table 2.3 Differentiation between micrometastasis and isolated tumour cells

Microscopic Feature	**Isolated tumour cell (Disseminated cell)**	**Micrometastasis (Occult metastasis)**
Size	Single cell or small cluster	< 0.2 cm in greatest dimension
Contact with vessel or lymph sinus wall	No	Yes
Invasion and penetration of vessel or lymph sinus wall	No	Yes
Extravascular stromal reaction	No	Yes
Extravascular tumour cell proliferation	No	Yes

It is therefore essential to differentiate micrometastases from isolated tumour cells. Micrometastases occur when there has been arrest and implantation of tumour cells with extravasation, proliferation and often with stromal reaction. Thus, the diagnosis of micrometastases is achieved only by histological

examination. Therefore, in the current study, the criteria recommended by the IUAC (1999) were followed.

2.8 Identification of rectal cancer patients

Rectal cancer patients were identified from the hospital pathology database using the specific codes for rectal cancers (ICD 18-19). From this list of patients with a diagnosis of rectal cancer, only those patients who had formal curative resection were further identified by the full histopathological report. All patient details were further verified in conjunction with MDT documents, surgical department patient data and finally reconfirmed with cancer services coordinator's data.

2.9 Identification of pathology paraffin blocks

Paraffin blocks of the rectal cancer specimens were identified from the pathology specimen number for each patient, which was verified with patient details such as hospital number and date of birth. Of the several paraffin blocks usually made for each patient with rectal cancer, a paraffin block containing the tumour with noticeable inked circumferential margin was selected and further confirmed with pathology records. The paraffin block was then subjected to micro-section for the purposes of Immunohistological staining with anticytokeratin antibodies and Haemotoxylin and Eosin staining.

2.10 Pathology data collection

Routine histopathological data were collected from the formal report on each patient filed in patient case notes.

2.11 Rationale for strict case selection: reasons for pre-defied criteria

Our study was primarily aimed at answering the question of whether micrometastases or isolated tumour cells exist in the mesorectum and at the circumferential margin. The second aim of this study was to examine the relation between the micrometastases and local or systemic recurrence. Therefore, patients who had a full mesorectal excision by either an anterior resection or abdomino-perineal resection with curative intention were included. Patients who had other forms of resection were excluded.

2.12 Sequence of events in the staining process by immunocytochemistry

2.12.1 Identification of paraffin block

Rectal cancer when submitted to the pathology department was routinely examined macroscopically, and various specimens were taken for histological examination. Usually several paraffin blocks were made in each case. These include the lymph nodes, tumour specimens with maximum extension in to the mesorectum with inked circumferential margin and distal and proximal resection margins. The pathology paraffin block intended for micro-section was retrieved from archived blocks using the block key. These blocks were re-examined for the inked circumferential margin and subjected to immunocytochemistry and Haemotoxylin and Eosin staining. As highlighted in

previous studies (Quirke 1986), if multiple sections of circumferential margins were examined, the chance of detection of margin positivity increases by 50%. However, in a retrospective study it would be difficult to precisely select pathology block that was originally intended to study circumferential margin. It would be ideal if several pathology blocks were studied to assess the circumferential margins. We believe that more cases would have micrometastases if multiple blocks were examined. Due to resource limitation, we chose to examine circumferential margins in a block showing maximum tumour penetration in to mesorectum with preserved inked margins.

2.12.2. Micro-Sections

Paraffin blocks were subjected to multiple sections using a microtome. The blocks were levelled to obtain uniform thickness. The sections were of 3 μ thickness as per the standard protocols for the routine histological examination. In the studies reviewed from the literature (Broll 1997, Sasaki 1997, Isaka 1999) micro sectioning varied from 3 to 10 μ thickness, but, in the majority of these studies 4 μ- thickness was utilized. (Adell 1996, Oberg 1998). Interestingly no mention was made on the thickness in some studies (Cutait 1991, Greenson 1994, Jeffers1994, Andreola 2001).

2.12.3 Rationale behind the multiple sections

There is evidence that multiple sectioning and immunocytochemistry (ICH) improves the detection rate of micrometastases (Noura 2002). Furthermore, several studies using single section ICH confirmed a decreased detection rate of

micrometastases resulting in a high false negative rate. There is no consensus on the optimum number of sections that gives a maximum true positive detection of malignant cells (Noura 2002). In a study, the ICH was performed on ten sections of the lymph nodes (Sasaki 1997). However, in our study, we aimed to examine the micro sections at three levels, as there was no firm evidence from the literature on the number of levels to be studied for optimum detection of micrometastases. The majority of the data on this issue was based on lymph node studies only, but not on the cancer specimen.

Current literature has mainly focused on the immunocytochemistry of the lymph nodes. Therefore, the results of these studies cannot be compared to this research project. Further, the circumferential margin extends all along the length of the rectal cancer specimen. It would be impractical to study the circumferential margin along the full length of the rectal cancer due to resource implications.

2.12.4 Haematoxylin and Eosin staining

Paraffin sections intended for routine Haematoxylin and Eosin staining were mounted on an ordinary glass slide and subjected to routine staining. The complete details of the H&E staining module is given in Appendix E.

2.12.5 Method and sequence of tissue sections for staining

Each selected paraffin block was subjected to multiple sections at three micrometers thickness. Paraffin sections were obtained at three different levels. At the first level, two sections were obtained for Immunocytochemical staining

using two different antibodies. A third section obtained was subjected to routine H&E staining and a final section was obtained for a negative control. Hence, at each level, four tissue sections were obtained. We have repeated this procedure at level two and three. Between the levels, arbitrarily, 20 microns thickness of tissue was removed, to maximize the chances of micrometastases detection (see figure 2.3).

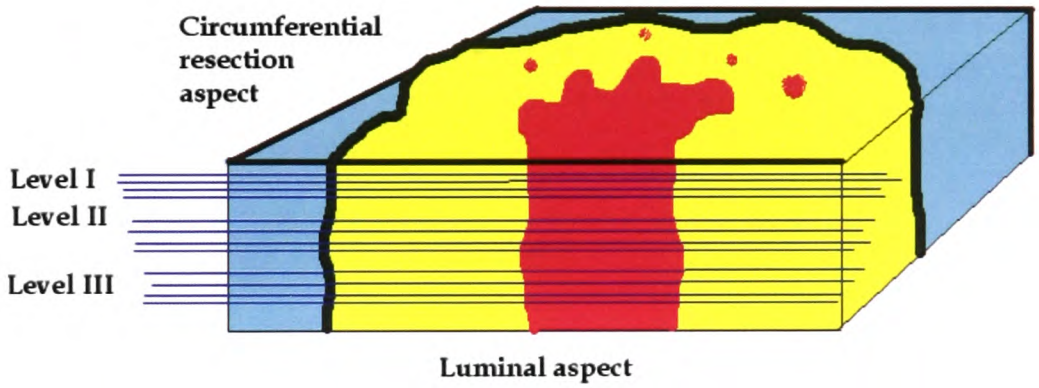


Fig No: 3. Schematic representation of rectal cancer paraffin block: Microsectioning at various levels for the purpose of immunocytochemistry

2.13 Materials

Archived paraffin specimen blocks of rectal cancer were obtained from pathology departments and three-micrometer sections were obtained on a rotary microtome. The paraffin sections of the specimen were then hydrated in a warm water bath with water temperature maintained at 52.8 Celsius and mounted on a Snowcoat X-tra TM (Surgipath®) Richmond, Illinois. Slides with mounted sections were kept dry by storing in an incubator (Lamb-Windsor® incubator). Mounted slides were then immersed in Xylene for 5 minutes three times prior to

final alcohol rinse. Slides were then washed in tap water prior to mounting on Vantana auto-immunostainer (figure 2.4). Mounted slides at this stage should look clear without a foggy or frosty appearance.

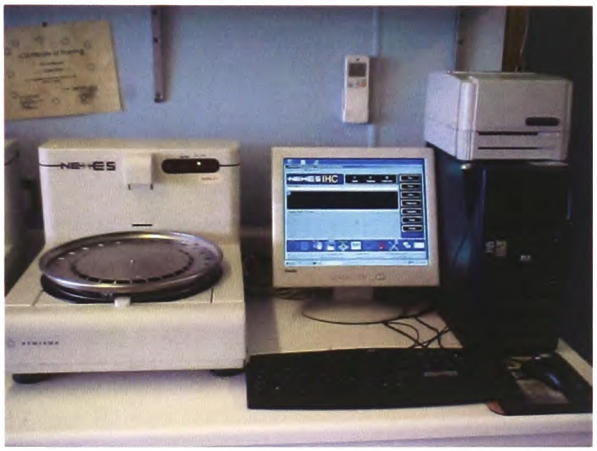


Figure 2.4. Ventana Immuno-stainer with automation through an attached computer system.

Immunocytochemical assay was performed using anticytokeratin monoclonal antibodies. Mouse Antihuman monoclonal MNF116 Isotype IgG 1 (DAKO Ltd) and lyophilized monoclonal anticytokeratin antibody (CK 5D3, Novacostra Ltd) were used in the Ventana Immuno-processor (NEXUS) using automated immuno-histochemical staining module.

2.14 General introduction to Immunocytochemistry

Immunocytochemistry is a sensitive method of investigation where a specific antibody is utilised to target antigens and visualisation of bound antibody by an indirect biotin avidin system coupled to an enzyme product.

Immunocytochemistry of lymph nodes in most studies have yielded a higher rate of detection of metastases than conventional techniques (Sasaki 1997, Noura 2002). This technique is more cost effective than RT-PCR method and is available widely. As emphasized by the IUAC, the diagnosis of micrometastases is a histological demonstration rather than molecular detection of antigens or genetic products. The advantages of immunocytochemistry are summarised in table 2.4.

Table 2.4 Advantages of Immunocytochemistry

- Widely available;
- Feasible to apply this method in most hospitals;
- Less expensive than RT-PCR;
- Technically easier than RT-PCR;
- Less false positive results compared to RT-PCR methods;
- Higher rate of detection of micrometastases.

However, immunocytochemistry is not without problems. Although widely available, it is less sensitive than RT-PCR, and ICH may give a false positive test with any epithelial cell, which expresses cytokeratin such as normal epithelial cells of the mucosa of the rectum. However, this problem can be solved by higher magnification examination of the positive cell. Furthermore, as emphasized by IUAC, by assessing for other criteria for MM (See table 2.3), the

differentiation between MM and benign epithelial cells should be clear. Table 2.5 summarises the disadvantages of immunocytochemistry.

Table 2.5 Problems with Immunocytochemistry

- Less sensitive than RT-PCR;
- Limited reproducibility;
- Loss of antigenic expression in the poorly differentiated tumours;
- CK and epithelial membrane antigen may be positive in non-epithelial cells;
- Lack of standardisation in technique;
- False negative ICH: Tissue fixed in formaldehyde, Destruction of epitope by astringent fixation techniques. True antigen decrease, loss or structural change of the antigen due to poor differentiation or dedifferentiation of the primary tumour and atifactual change due to fixation and processing;
- False positive ICH due to non-malignant cells such as Dendritic cells.

2.15 General introduction to Cytokeratins

Cytokeratins (CK) belong to intermediate filaments, which create a cytoskeleton in epithelial cells. Cytokeratins consists of complex polypeptides with molecular mass ranging from 40 to 68 k Da. So far, 20 different distinct types of CK polypeptides have been found in human epithelia. CK 's are divided into acidic

type A (class I) and basic type B (class II) subfamily (Moll 1994). The fluorescent microscopic appearances of cytokeratin is shown in the figure 2.5.

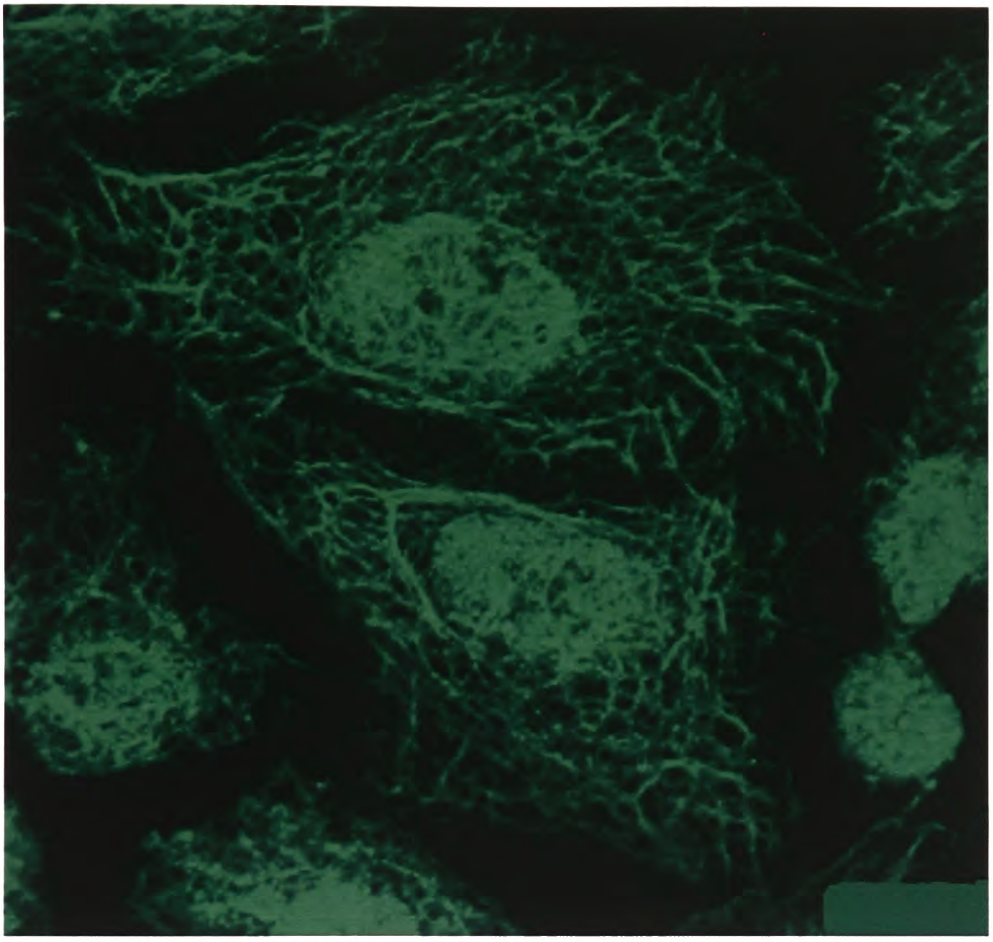


Figure 2.5: Fluorescent micrographic appearances of cytokeratin.

2.16 The Cytokeratin antibodies

Immunocytochemistry involves the identification of a specific epitope of interest on the cell with a highly specific monoclonal antibody and the antigen antibody complex is further identified by biotin streptavidin system coupled to an enzyme product. (Michie 1987, Elias 1989, Gatter 1989, Wold 1989). In this study we used two different panels of antibodies MNF 116 and CK5 D3. Both anticytokeratin

antibodies detect a broad spectrum of cytokeratins including acidic and alkaline class of cytokeratins.

MNF 116 Antibody

The use of MNF-116 monoclonal mouse anti-human cytokeratin clone is intended for detection of cytokeratins. This antibody labels epithelial tissues from simple glandular to stratified squamous epithelium. Therefore, this is useful in the identification of normal and neoplastic cells of epithelial origin (Goddard 1991, Prieto 1996). Mesenchymal cells are generally not reactive with this antibody, however a weak focal labeling of smooth muscle cells is reported. Endothelium, skeletal muscle, cartilage, and lymphoid tissue are not labeled by this antibody (Goddard 1991). MNF 116 is a broad spectrum anti-cytokeratin reagent reacting with intermediate and low molecular weight keratins including cytokeratins 5, 6, 8, 17 and probably 19.

Storage of the antibody

In this study the reagent was stored at the specified recommended temperature to achieve optimum staining (2-8 deg C). This antibody can be used on paraffin preparations fixed in formalin and on fresh frozen preparations. Optimal immunostaining is obtained when using proteinase K for epitope retrieval. However trypsin and pepsin can also be applied successfully to improve the antigen detection. The MNF 116 antibody may be used at a dilution range of 1: 50 to 1:100. However, in our study 1: 50 dilution was used, which was an

established standard practice of the laboratory due to optimal staining of the Cytokeratins at this dilution.

CK5 D3 Antibody

This is another type of antibody that was used in our study to detect malignant cells by Immunocytochemical means. These antibodies belong to Ig G1 class with specific affinity for human cytokeratin 8 and 18 intermediate filaments proteins. This antibody is also effective on frozen section and paraffin wax embedded tissue (Angus 1987).

Application of CK5 D3

NCL 5D3 reacts with cytokeratin intermediate filaments 52.5 k Da and 45 k Da identified as cytokeratins 8 and 18 respectively. Currently, NCL-5D3 is recommended for research purpose only.

2.17 The Immuno- auto stainer: Ventana machine/ DAB detection kit

Vantana medical systems/ VIEW DAB detection kit is an indirect biotin streptavidin system for detecting a specific mouse Ig G, mouse Ig M, or rabbit polyclonal primary antibody. The kit is intended for staining sections of routinely fixed, paraffin embedded tissue and frozen section on the Vantana IHC automated immunohistochemical system. The Vantana medical system /VIEW DAB detection kit utilyses biotinylated secondary antibodies to locate the bound primary antibody followed by the binding of Streptavidin HRP conjugate. The complex is then visualized using hydrogen peroxide and DAB chromogen. The reagents used in the Ventana DAB kit are summarised in the appendix F.

2.18 Principles and conduct of Immunocytochemistry

The Vantana system detects the mouse antibody bound to an antigen in the paraffin embedded tissue sections. In this study the antigen epitope studied was cytokeratin on the cell membrane of the cancer cells in the mesorectum and at the circumferential margins. The specific antigen and antibody complex is located by a biotin conjugated secondary antibody formulation. This step is followed by the addition of Streptavidin enzyme conjugate that binds to biotin present on the secondary antibody. This complex is then visualized using a precipitating enzyme product. Each step is incubated for a precise time and temperature as described in the sequence of events in the immunoassaying procedure Appendix G. At the end of each incubation step the Vantana IHC instrument washes the sections to remove unbound material and applies a liquid cover slip, which minimizes the evaporation of aqueous reagents from the specimen-containing slide.

2.18.1 Preparation prior to mounting the slides on the Vantana autostainer

Each slide was bar coded appropriately specifying the staining recipe and desired specific antibody. The rectal cancer microsection was mounted onto the slides and immersed in two Xylene baths for 5 minutes each. The slides were then soaked for three minutes each in two baths containing 100% ethanol, three minutes in 95% ethanol, three minutes in 80% ethanol and dipped in water ten times.

2.18.2 Immunoassaying procedure

Preliminary procedure for staining was as follows.

Appropriate primary antibody dispenser and the VIEW DAB detection kit dispensers and desired accessory reagents were loaded onto the reagent carousel and placed on the Vantana automated immunohistochemistry system.

The slides were then mounted onto the instrument and treated with APK wash solution to avoid drying of the tissue sections.

Vantana medical systems' VIEW DAB kit has been optimised to work in combination with Vantana medical systems primary antibodies to provide the greatest specific staining to background ratio.

The complete sequence of events carried out by the Vantana automated immunocytochemistry systems is presented in the appendix G

2.18.3 Controls This consists of both positive and negative controls.

Positive controls: Immunoassaying of the cancer itself acts as a positive control in each slide. Rectal cancers with positive circumferential margins on conventional histological staining with Haematoxylin and Eosin will further form a positive control.

Negative controls Tissue sections from each level were taken for negative control. These sections were processed in the Vantana immunostainer without an antibody.

2.19 Pathologist's assessment

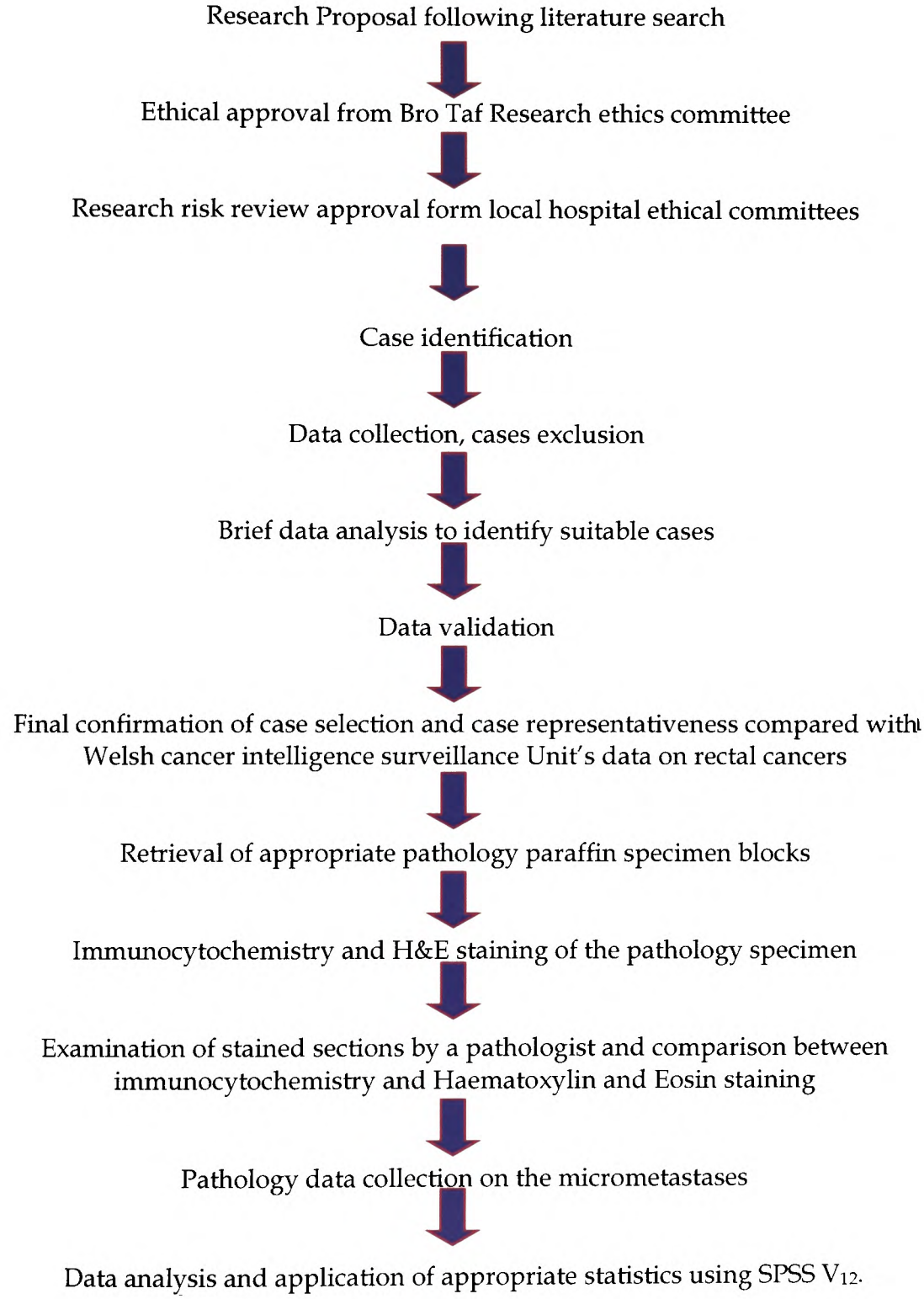
After completion of conventional and immunocytochemistry of the paraffin tissue sections, all the slides were reviewed by an independent pathologist who was blinded to the clinical details of the patients and clinical outcomes such as local and systemic recurrence and mortality. To classify micrometastatic disease, in to MM or ITC, a qualified pathologist followed the classification as recommended by the IUAC. The immunocytochemical and Haematoxylin and eosin staining data were recorded systematically onto a data collection form as mentioned in the appendix H.

2.20 Statistical analysis

The clinical and pathology data were entered in to SPSS Version 12 for the purpose of statistical analysis. The data were examined for the existence of any specific relationships between the recurrence and presence of micrometastases in either mesorectum or at the circumferential margins. Chi-squared test of association was used to study the relation between the clinical and pathological variables. Binary logistic regression was applied to test the significance of micrometastatic disease and recurrence and survival.

The following flow chart simplifies the research method we followed in this study.

2.21 The flow chart summarising the research process



REVIEW OF LITERATURE

3.1 General introduction

Colorectal cancer is one of the most common malignant conditions in the Western world and its incidence is on the rise (Broll et al. 1997, Ratto et al. 1998, Rosenberg et al. 2000, Palma et al. 2003). The prognosis of colorectal cancer depends on the TNM stage of the disease, in particular, lymph nodal status (Broll et al. 1997, Ratto et al. 1998) with a good prognosis for early stage tumours. However, up to 25-30% of patients who have had apparent curative resection will eventually develop recurrence (Sanchez-Cespedes et al. 1999). This is presumably due to the presence of microscopic residual disease in the loco-regional nodes or peritoneal seeding, which cannot be detected by routine histological methods (Sanchez-Cespedes et al. 1999). In an attempt to detect micrometastatic disease, various techniques have been in use since the late 1960's to detect micrometastases including immunocytochemistry (ICH), Reverse Transcriptase Polymerase chain reaction (RT-PCR), and detection of genetic mutation on k-ras and p53 genes. This review was carried out prior to the setting of the research project and was aimed at investigating the current state of micrometastases detection and to evaluate the likely significance that it has on the management of colorectal cancer.

3.2 Literature review method

An up to date literature review was performed using the Medline and Ovid databases on English language publications relating to colorectal micrometastases and its clinical implications. The keywords used to search the database were

colorectal cancer, micrometastases, minimal residual disease, isolated tumour cells, RT-PCR, Immunocytochemistry, p53, k-ras.

3.3 Summary of overall results

Micrometastasis in colorectal cancer is a well recognised, and a frequently occurring phenomenon in the lymph nodes, peripheral blood and bone marrow. ICH against Cytokeratins (CK) or cancer specific proteins is insensitive and not recommended for routine use. RT-PCR for CK 20 or CEA appears to be more sensitive for the detection of micrometastases than the ICH method or DNA based methods. RT-PCR detection of lymph nodal micrometastases was found to be significantly associated with recurrent disease and subsequent mortality.

3.4 Summary of conclusions on the literature review

Lymph node micrometastasis in several studies has shown no correlation with disease recurrence. Micrometastases in peripheral circulation are common, however, there was no significant association with recurrent disease and decreased survival. Bone marrow micrometastases appear significantly to correlate with recurrence and mortality. Radioimmuno-guided surgery (RIGS) may have a limited clinical application. Sentinel node ICH or RT-PCR may have clinical applicability to identify high-risk groups who are likely to develop disease relapse, although this method is associated with a high false negative rate. Although, micrometastatic disease can be detected by various techniques, currently there are no clinical trials of adjuvant chemotherapy or radiotherapy based on the results of these techniques.

3.5 Background for the literature review

Colorectal cancer is one of the most common malignant conditions in the United Kingdom with an approximate annual incidence of 34,000 cases per year and is the second frequent cause of cancer deaths in the UK (Cancer Research UK, 2003). This number is increasing by 1% every year for men although; it is staying the same for women (Cancer screening 2005). Despite progress in the diagnosis and therapy, there is only moderate improvement in the overall prognosis (Rosenberg et al. 2002). In the UK, there has been little improvement in the proportion of patients presenting with advanced disease with only 58% of patients considered to have had curative resection (Mella et al. 1997).

To appreciate prognosis of colorectal cancers, an insight in to the currently accepted staging is essential. American Joint Committee on Cancer (AJCC) system (also called the TNM system), is currently accepted staging system for the evaluation of disease extent and prognostic purposes. (American Joint Committee on Cancer) The AJCC/TNM System describes the extent of the primary Tumor (T), the absence or presence of metastasis to nearby lymph Nodes (N), and the absence or presence of distant Metastasis (M). Tx: No description of the tumour extent is possible because of incomplete information.

Tis: The cancer is in the earliest stage. It has not grown beyond the mucosa (inner layer) of the colon or rectum. This stage is also known as carcinoma in situ or intramucosal carcinoma.

T1: The cancer has grown through the mucosa and extends into the submucosa.

T2: The cancer has grown through the submucosa, and extends into the muscularis propria.

T3: The cancer has grown completely through the muscularis propria into the subserosa but not to any neighbouring organs or tissues.

T4: The cancer has spread completely through the wall of the colon or rectum into nearby tissues or organs.

N categories indicate whether or not the cancer has spread to nearby lymph nodes and, if so, how many lymph nodes are involved.

Nx: No description of lymph node involvement is possible because of incomplete information.

N0: No lymph node involvement is found.

N1: Cancer cells found in 1 to 3 nearby lymph nodes.

N2: Cancer cells found in 4 or more nearby lymph nodes.

M categories indicate whether or not the cancer has spread to distant organs, such as the liver, lungs, or distant lymph nodes.

Mx: No description of distant spread is possible because of incomplete information.

M0: No distant spread is seen.

M1: Distant spread is present

Stage 0: Tis, N0, M0: The cancer is in the earliest stage. It has not grown beyond the inner layer (mucosa) of the colon or rectum. This stage is also known as carcinoma in situ or intramucosal carcinoma.

Stage I: T1, N0, M0, or T2, N0, M0: The cancer has grown through the mucosa into the submucosa *or* it may also have grown into the muscularis propria, but it has not spread into nearby lymph nodes or distant sites.

Stage IIA: T3, N0, M0: The cancer has grown through the wall of the colon or rectum into the outermost layers. It has not yet spread to the nearby lymph nodes or distant sites.

Stage IIB: T4, N0, M0: The cancer has grown through the wall of the colon or rectum into other nearby tissues or organs. It has not yet spread to the nearby lymph nodes or distant sites.

Stage IIIA: T1-2, N1, M0: The cancer has grown through the mucosa into the submucosa *or* it may also have grown into the muscularis propria, and it has spread to 1-3 nearby lymph nodes but not distant sites.

Stage IIIB: T3-4, N1, M0: The cancer has grown through the wall of the colon or rectum *or* into other nearby tissues or organs and has spread to 1-3 nearby lymph nodes but not distant sites.

Stage IIIC: Any T, N2, M0: The cancer can be any T but has spread to 4 or more nearby lymph nodes but not distant sites.

Stage IV: Any T, Any N, M1: The cancer can be any T, any N, but has spread to distant sites such as the liver, lung, peritoneum (the membrane lining the abdominal cavity), or ovary.

The prognosis of early colorectal cancer is generally good although, 30-40% of patients with stage II disease die of recurrent disease. (Liefers et al. 1998, Mori et al. 1998, Rosenberg et al. 2002), and up to 30-50% of all resectable cancers subsequently develop metastatic disease (Deans et al. 1992, Weitz et al. 1998, Weitz et al. 1999). This may be attributable to inadequate primary surgery or disseminated microscopic disease, which cannot be detected by routine histological examination of extirpated tissue (Sanchez-Cespedes et al. 1999). Although adjuvant chemotherapy reduces mortality in up to 22% of poor risk patients (IMPACT investigators 1995), there is no evidence of benefit with adjuvant chemotherapy for patients with node negative disease (IMPACT B2 Investigators 1999). However, chemotherapy might improve prognosis of up 30% patients who are destined to develop recurrence (Tsavellas et al. 2001).

There have been various techniques to detect microscopic or minimal residual disease including immunocytochemical methods. Morphological methods such as Immunocytochemistry against cancer specific antigens (Greenson et al. 1994, Jeffers et al. 1994, Johnon et al. 1995, Broll et al. 1997, Palma et al. 2003, Clarke et al. 2000, Tschmelitsch et al. 2000, van Wyk et al. 2000). Molecular methods (No-morphological) to detect RNA transcripts that express cancer antigens (Futamura et al. 1998, Weitz et al. 1998, Weitz et al. 1999, Rosenberg et al. 2002) and detection of mutation in K-ras (codons 12, 13 and 61) or p53 genes (exons 5-8) (Hayashi et al. 1995). Furthermore, recently, the micrometastatic phenomenon was noted at the hepatic resection margins using both immunocytochemistry (Yokoyama et al. 2002) and genetic assessment for K-ras and p53 mutation (Kokudo et al. 2002).

3.6.1 Micrometastases

Micrometastasis is conventionally defined as single or a group of malignant cells measuring <2-mm in size without any direct continuity with the primary tumour and this definition was based on the morphological identification of micrometastases using immunocytochemical methods (Compton et al. 2000). In an effort to standardise nomenclature, the American College of Pathologists, defined micrometastatic nodal disease as histologically confirmed metastatic tumour that measures less than 2 mm and a tumour detected by non-histological methods was defined as pN0 (Compton et al. 2000). However, despite advances in detection of micrometastatic disease by molecular methods, the original

definition remained (Calaluce et al. 1998). Furthermore, in the morphological method, there has been no distinction between isolated tumour cells and micrometastases, which may have different prognostic implications. This possibility was not considered in any of the studies so far.

The International Union Against Cancer (IUAC) has recommended that the isolated tumour cells be distinguished from micrometastases in order to appreciate their prognostic significance (Hermanek 1999). There is no clear consensus on the significance of the micrometastases in the literature at the present time (Greenson et al. 1994, Jeffers et al. 1994, Adell et al. 1996, Liefers et al. 1998, Oberg et al. 1998, Hermanek et al. 1999). However, the American Joint Committee on Cancer suggests adjuvant chemotherpy trials in N0 PCR positive patients (Yarbro et al. 1999).

Immunological mechanisms, genetic changes and dormancy status of micrometastases are not fully understood. It is possible that some cells are dormant and hence incapable of proliferation (Pantel et al 1993) or do not harbour the genetic changes necessary for progression into overt metastases, as shown by Pantel et al with a reduced expression of proliferation markers such as Ki-67 and p120 in the micrometastases (Pantel et al 1993). This probably explains varied metastatic potential of the individual tumour cells leading to variable reported rates of recurrence. Down regulation of surface molecules responsible for antigen presentation such as major histocompatability complex class I antigens in breast and colorectal carcinoma (Pantel et al 1991) or intercellular adhesion molecule 1

explains the dedifferentiation, facilitating the escape from immunological surveillance (Passlick et al. 1996, Funke et al. 1999) and corresponds to poor survival (Funke et al. 1999, Hosch et al. 2001).

3.6.2 Reasons for research interest on the micrometastases

- In patients with early stage disease with a potential for relapse, detection of micrometastatic disease constitutes an argument for adjuvant therapy. However, larger trails have failed to show improved survival with adjuvant chemotherapy even in stage II and I disease (Buyse et al. 2001).
- Assessment of micrometastases can be a surrogate marker of therapeutic efficacy and permits immediate assessment of therapeutic effects of chemotherapy on the residual malignant cells (Braun et al. 2001).
- Patients may be stratified into various risk groups for the purpose of adjuvant treatment trials.

There is good evidence that the response to chemotherapy may be poor in dormant micrometastases. Further research on micrometastases may lead to the developments in the new therapeutic approaches such as targeted tumour biological therapies. Although there is no evidence of poor response of the micrometastases to chemotherapy in colorectal cancers, there have been reports on breast cancer micrometastases. Circulating tumor cells in breast cancer seem to be non-proliferation cells that persist during chemotherapy (Muller et al. 2005) Slde et al. (2005) have shown that minimal residual disease persists despite adjuvant therapy in a majority of patients with primary breast cancer following

surgery.

- Improved tumour staging as proposed by IUAC with a view to have an internationally accepted classification system for prognostic and therapeutic purposes (See table. 3.1 IUAC classification).

Table 3.1: Proposed classification for isolated tumour cells in the lymph nodes.

Proposed classification for isolated tumour cells	Details of identification method
pN0	No regional lymph node metastasis histologically, no examination for isolated tumour cells (ITC)
pN0 (i-)	No regional lymph node metastasis histologically, negative morphologic findings for ITC
pN0 (i+)	No regional lymph node metastasis histologically, positive morphologic findings for ITC
PN0(mol-)	No regional lymph node metastasis histologically, negative non-morphologic findings for ITC
PN0(mol+)	No regional lymph node metastasis histologically, positive non-morphologic findings for ITC

3.7 Detection of micrometastases

3.7.1 Immunocytochemistry (ICH)

Cytokeratins are an integral part of the cytoskeleton of colorectal epithelial cells that are regularly expressed in the colorectal cancers. Using monoclonal antibodies against Cytokeratins and cancer specific antigens such as CEA, malignant cells may be identified in the lymph nodes, peripheral blood and bone marrow. There are advantages with ICH such as higher rate of detection of micrometastases, higher sensitivity than H&E and easy availability compared to

RT-PCR. However, the results of immunocytochemistry should be interpreted in the light of disadvantages of ICH. ICH is less sensitive than RT-PCR and the destruction of epitope by astringent fixation techniques results in false negativity. Further, lack of standardisation in technique results in limited reproducibility. (See tables 2.4 and 2.5 in the methods section).

3.7.2 Lymph node Immunocytochemistry

The most important prognostic factor in apparently localised colorectal cancer is the presence of metastatic disease in the regional lymph nodes (Dukes 1958, Jass et al. 1986, Jass et al. 1987, Adachi et al. 1994, Lindmark et al. 1994, Broll et al. 1997, Wong et al. 2002). Assessment of metastatic spread to the lymph nodes is crucial in the management of cancer patients in deciding on the adjuvant therapy. Currently this decision is based on the positive lymph node involvement on routine Haematoxylin and Eosin staining of the lymph nodes. Most authors have reported an increase in the detection rate of tumour cells when immunocytochemistry was used on the nodes previously considered being tumour free on routinely stained sections (Pantel et al 1991, Pantel et al 1993, Greenson et al. 1994, Jeffers et al. 1994, Adell et al. 1996, Passlick et al. 1996, Broll et al. 1997, Oberg et al. 1998, Yarbro et al. 1999, Funke et al. 1999, Hosch et al. 2001). Micrometastases in lymph nodes of breast (International LUDWIG Breast cancer study group 1990), oesophageal (Izbicki et al. 1997) and gastric cancer (Yasuda et al. 2002) have been shown to affect the prognosis significantly. Although, the incidence of micrometastases in colorectal cancers in apparently negative nodes

has been reported to be up to 68% (Nakanishi et al. 1999), the clinical significance of micrometastases detected by ICH remains uncertain (Makin et al. 1989, Adell et al. 1996, Passlick et al. 1996, Broll et al. 1997, Oberg et al.1998).

Greenson et al (1994) used anticytokeratin antibodies on lymph nodes of patients with Dukes B carcinoma and reported significant association with poor prognosis. However, using anticytokeratin antibodies other investigators have found no influence on the prognosis or recurrence in other studies (Cutait et al. 1991, Adell et al. 1996, Broll et al. 1997). However, Sasaki et al. (1997) using CAM 5.2 found the significant association with recurrent disease and suggested immunocytochemistry to identify patients with a higher risk of recurrence after primary surgery. A trend towards lower survival in node positive patients was noted in a recent study (Palma et al. 2003). Similarly, Isaka et al. (1999) found significant recurrence rates and positive micrometastases. Yasuda et al. (2001) found the frequency of lymph node micrometastases in 92% of the recurrent group and 70% in the nonrecurrent group.

Differences in choice of antibody, staining procedures and interpretation may explain discrepancy in results in various studies. (Adell et al.1996, Liefers et al. 1998). Varying results are probably because of different selection criteria, and lack of information on statistical details or power of calculation of log rank tests (Broll et al.1997).

Multiple sectioning of lymph nodes increases detection of micrometastases compared to single section staining (Noura et al. 2002). Jeffers et al (1994) found no significant difference in survival and recurrence and therefore suggest that the routine use of ICH not justified. Broll et al (1997) detected metastases in 3 out of 13 patients with rectal carcinoma and 10 out of 36(28%) with colon cancer. Furthermore, these authors have concluded that the presence of micrometastases increases local recurrence or distant metastases, but does not influence survival. The National Surgical Adjuvant Breast and Bowel Project (NSABP) analysis has shown that the immunohistochemical demonstration of nodal mini micrometastases failed to discriminate high- and low-risk groups of patients with colorectal cancer who were designated as being node-negative after routine pathologic examination (Fisher et al. 2003).

3.7.3 Number of lymph node sections

Initial studies concentrated on the single section lymph node ICH, but soon it was clear that subjecting multiple sections of lymph node would detect more cases with micrometastases. (Ratto et al. 1998, Noura et al. 2002). Although, multiple sectioning of lymph nodes has resulted in conflicting results, it is a standard practice to perform ICH on multiple sections (Clarke et al. 1999). Single section sampling will result in small metastases being missed (Cutait et al. 1991, Greenson et al. 1994, Broll et al. 1997, Oberg et al. 1998, Isaka et al. 1999). Therefore, Davidson et al. (Davidson et al 1990) have suggested multiple sectioning, the use

of multiple panels of antibodies and sampling larger number of lymph nodes from the specimen to improve the detection rate.

Furthermore, there is inconsistency in the distribution of micrometastases and a variable presence in number and frequency within the lymph node. Therefore, subjecting a random single section lymph node to ICH is more likely to miss micrometastases (Ratto et al 1998). The General consensus was that multiple sections from a lymph node are required to prevent missing micrometastases. Table 3.2 summarizes various studies of ICH on lymph nodes with brief outcomes.

Table 3.2 Lymph Node immunocytochemistry

Reference	mAb	Node sectioning	Increased Detection of Tumour cells	Prognostic significance
Makin et al. 1989	CAM 5.2	Single	No	NS
Davidson et al. 1990	Anti-CEA Anti-EMA	Single	No	NA
Cutait et al. 1991	Anti-CEA AE1/AE3	Single	Yes	NS
Haboubi et al. 1992	CAM 5.2	Multiple	Yes	NA
Jeffers et al. 1994	AE1/AE3	Single	Yes	NS
Greenson et al. 1994	AE1/AE3	Single	Yes	Adverse
Adell et al. 1996	Anti-CK	Multiple	Yes	NS
Broll et al. 1997	AE1/AE3	Multiple	Yes	NS
Sasaki et al. 1997	CAM 5.2	Multiple	Yes	Adverse
Oberg et al. 1998	CAM 5.2	Single	Yes	NS
Nakanishi et al.1999	AE1/AE3	Multiple	Yes	NS
Isaka et al. 1999	CAM 5.2	Multiple	Yes	Adverse*
Clarke et al. 2000	Anti CK5, 6,8, 17	Single	Yes	Adverse
Yasuda et al. 2001*	CAM 5.2	Multiple	Yes	Adverse*
Palma et al. 2003	AE1/AE3	Multiple	Yes	Adverse^
Noura et al. 2002	AE1/AE3	Multiple	Yes	NS

^ Not statistically significant, * Significant, mAb= Monoclonal antibody, Anti-CEA= Anti- Carcinoembryogenic antigen NA=Not assessed, NS=Not Significant EMA= Epithelial Membrane antigen.

3.7.4 Summary of conclusions on the Lymph node ICH

- ICH increases the detection rate of micrometastases significantly over H&E;
- The detection rate is greater with multiple sectioning of the lymph nodes;
- Several studies have concluded that the lymph nodal micrometastases detected by ICH do not affect prognosis adversely in colorectal cancer;
- ICH is a relatively insensitive method and therefore not recommended routinely;
- Targeted ICH studies on sentinel lymph nodes and RIGS positive lymph nodes may increase the detection of micrometastases;
- Mixed results for ICH are due to the use of different types of antibodies, smaller power of the studies, and lack of standardisation of technique and classification systems. Therefore, these studies cannot be compared to appreciate the role of ICH in general;
- Currently there are no adjuvant therapy trials in the literature based on the presence of MM in the lymph nodes detected by ICH.

3.7.5 Bone marrow immunocytochemistry

ICH detection of colorectal malignant cells in the bone marrow has been shown to be significantly associated with decreased survival (Schlimok et al. 1990). Lindemann et al. (1992) have identified tumour cells in bone marrow in 32% of patients using ICH (Lindemann et al 1992). Lindemann et al conclude that tumour cell detection was associated with early relapse and decreased survival and therefore presence of micrometastases is an independent, prognostic factor for relapse (Lindemann et al. 1992).

Table 3.3 Bone marrow Immunocytochemical studies in colorectal cancer

Reference	Antibody	Positive bone marrow percentage	Prognostic significance
Schlimok et al. 1987	CK2	21	NA
Schlimok et al. 1990	CK2	27	Adverse
Lindemann et al. 1992	CK2	32	Adverse
Juhl et al. 1994	KL-1	29	NA
Litle et al. 1997	Anti CK-20	89	NA
Leinung et al. 2000	A45-B/B3	25	Adverse

CK= Cytokeratin, NA= Not assessed.

3.7.6 Summary conclusions on the bone marrow ICH

- Bone marrow ICH studies have shown significant association with poor prognosis;
- ICH detects cancer cells in bone marrow in up to 30% in colorectal cancer patients;
- ICH assessment of bone marrow cancer cell burden may be used to monitor the effect of chemotherapy;
- ICH of bone marrow may be used in the decision for adjuvant therapy, however, there are no research studies as yet to comment on.

3.8 Identification of micrometastatic disease in the peripheral circulation and peritoneal fluid

Efforts to identify malignant cells in the peripheral circulation and peritoneal fluid using immunocytochemistry have led to the successful identification and to the development of improved methods such as immuno-magnetic beads. However, the application is currently limited to the research studies only. Malignant cells have been identified form peripheral circulation (Leather et al. 1993) and peritoneal cavity washings (Leather et al. 1994, Yamamoto et al. 2003). Conventional staining of peritoneal fluid for colorectal cancer cells in T3/T4 colorectal cancers were found to be a useful diagnostic procedure for predicting recurrence, especially peritoneal recurrence (Yamamoto et al. 2003). To enhance

the detection rate of malignant cells, immuno-magnetic beads have been used (Wong et al. 1996).

3.8.1 Summary conclusions on the peripheral circulation ICH

- Colorectal malignant cells can exist in the circulation and are potentially viable (Taniguchi et at. 2000);
- There is an increase in the number of cancer cells in the peripheral circulation during cancer excision (Yamaguchi et al 2000);
- There is no significant association with the presence of cancer cells in the peripheral circulation and prognosis (Bessa et al 2001, 2003);
- Cancer cells may be monitored in the peripheral circulation during and after chemotherapy (Starintz 2004).

3.9 Molecular basis of detection of genetic abnormalities

Any discussion on the detection methods of micrometastases would be incomplete without a reasonable understanding of the latest methods of micrometastases detection such as RT-PCR. The aim of the discussion on the molecular methods of micrometastases detection is to help to understand its applications and potential limitations due to sensitivity problems.

Specific mutations of the defined DNA such as k-ras, tumour suppressor genes such as p53, or microsatillite instability may be used as markers in DNA or RNA based methods. As the alterations are specific to tumour cells, these techniques permit identification of malignant cells. However, DNA molecules are relatively

stable in the human tissues (Weitz et al 1999) and the fragments that are released from decaying cancer cells can give a false positive test. Therefore, detection of mutant sequences or altered sequences does not imply viable malignant cells. Section 3.9.1 summarises advantages and disadvantages of RT-PCR method.

3.9.1 Advantages and disadvantages of RT-PCR method

Advantages
• More sensitive, therefore can detect one malignant cell among 10 million cells; • Can be used on paraffin-blocked specimens and therefore possible to study retrospectively.
Disadvantages
• Extreme sensitivity tends to cause false positivity; • It can only detect tumours with identified genetic changes • Amplification of pseudogenes can influence the false positivity of the m RNA based techniques; • CEA transcripts can give rise to false positive results in normal lymph nodes; • Lack of tissue specific markers in many solid tumours limits usefulness; • Biologically inactive cells and dormant cells can give rise to false positivity; • Sampling error or intermittent shedding of the tumour cells gives false negative PCR.

3.9.2 RT-PCR Studies on lymph nodes

In the majority of the studies, the sample size was small. However, a recent large study using RT-PCR technique to detect m-RNA transcripts to CK-20 concludes that there was significant association with positive RT-PCR in the lymph nodes, and recurrent disease and survival. This suggests that the decision for adjuvant therapy may be made on the basis of RT-PCR detection of occult metastases (Sanchez-Cespedes et al. 1999, Rosenberg et al. 2002). Table 3.4 summarises RT-PCR studies on lymph nodes.

Table 3.4 RT-PCR Studies on the lymph node negative colorectal cancers

Reference	Marker	Increased detection of tumour cells	Effect on Prognosis
Mori et al 1995	CEA	YES	NA
Gunn et al 1996	CK19 CK20	YES	NA
Wong et al 1997	CD44	YES	NA
Dorudi et al 1998	CK20	YES	NA
Futamura et al 1998	CEA CK20	YES	NA
Waldman et al 1998	GCC	YES	NA
Mori et al 1998	CEA	YES	Adverse
Leifers et al 1998	CEA	YES	Adverse
Weitz et al. 1999	CK20	YES	NA
Sanchez-Cespedes^et al 1999	K-*ras* *P* 53	YES	Adverse
Bernini et al 2000	Mucin 2	YES	NA
Rosenberg et al.2002	CK 20	YES	Adverse

^RT-PCR on perihepatic lymph nodes, GCC= Guanylyl cyclase, NA= Not assessed, CK= Cytokeratins, CEA= Carcinoembryogenic antigen.

Hayashi et al. (1995) used mutant allele specific antigen (MASA) to detect somatic mutations in K-ras or p53 and suggested that this method could be used to select patients for adjuvant chemotherapy.

Using the RT-PCR method the incidence of micrometastases in lymph nodes was found to be 87.5% (Mori et al. 1998) and 100% (Futamura et al 1998) in UICC stage I and II colorectal cancer patients respectively. Furthermore, in the blood samples of colorectal cancer patients, the CEA mRNA level was significantly higher in Dukes D patients than in other clinical stages. In perihepatic lymph nodes, genetic detection for mutations of k-ras and P53 genes have been shown to predict recurrence and survival following hepatic resection (Sanchez-Cespedes et al. 1999). In addition, quantification of CEA mRNA may be a useful marker in the evaluation of recurrence. A CK-20 RT-PCR study on lymph nodes, peripheral blood, and bone marrow showed a higher frequency of lymphatic micrometastases than in blood or bone marrow. CK20 PCR in the apical nodes is also of strong prognostic relevance (Weitz et al. 1999).

Table 3.5 PCR Studies on circulating tumour cells

Reference	Marker	Circulating tumour cells positive	Effect on prognosis
Mori et al. 1996	CEA	35%	NA
Jonas et al. 1996	CEA	84%	NA
Soeth et al. 1997	CK20	17%	Adverse
Wong et al. 1997	CD44	17%	NA
Denis et al. 1997	CK8 CK19 CK20	52%	NA
Funaki et al. 1997	CK20	75%	NA
Mori et al. 1998	CEA	38%	Adverse
Wyld et al. 1998	CK20	25%	NA
Weitz et al. 1998	CK20	46%	NA
Funaki et al. 1998	CK20	64%	Adverse
Castells et al. 1998	CK20	41%	NA
Bustin et al. 1999	CK19 CK20 GCC	26% 100% 74%	NA
Hardingham et al. 2000	CK19 CK20 Mucin 1 and 2	20%	Adverse
Yamaguchi et al. 2000	CEA CK20	38% 36%	Adverse
Khan et al 2000	P53	42%	NA
Taniguchi et al 2000	CEA	34%	Adverse
Funaki et al 2000	CK20	75%	NA

The prognostic significance of circulating tumour cells in colorectal cancers is still unclear (Mori et al 1998). Schlimok et al (1990) have shown that the prognosis was worse in cytokeratin positive micrometastases in the bone marrow.

O' Sullivan et al. (1997) has shown a 23%incidence of micrometastases in the bone marrow. The postoperative presence in the bone marrow is significantly

associated with the subsequent development of overt metastases. Table 3.5 summarises various studies on circulating tumour cells using RT-PCR method. Circulating tumour cells were present before treatment in most patients with colorectal cancer regardless of tumour stage or metastases. Clearance of circulating tumour cells within 24 hours of colorectal cancer excision was greatest in tumours with the best prognosis (Weitz et al. 2000). Table 3.6 summarises some of these studies on bone marrow RT-PCR.

Table 3.6 Bone marrow RT-PCR studies

Reference	Marker	Patients with positive bone marrow	Prognostic effect
Gerhard et al 1994	CEA	66%	NA
Soeth et al 1996	CK20	35%	NA
Gunn et al 1996	CK 19 CK 20	6% BMA	NA
Soeth et al 1997	CK 20	31%	Adverse
Weitz et al 1999	CK 20	21%	NA
Weitz et al 2000	CK 20	27%	NA

CEA, Carcinoembryogenic antigen; BMA, bone marrow aspirate; NA, Not assessed.

3.10 Radio-immunoguided surgery (RIGS)

Radio-immunoguided surgery (RIGS) detects lymph nodes containing metastases at surgery that bind radiolabelled antibody using a hand held gamma- detecting probe. These antibodies can be against CEA or other antigens such as TAG-72 (CC49). Cote et al. have shown that RIGS can detect occult lymph nodal metastases and is more sensitive than histopathological examination in the

detection of micrometastases (Cote et al 1996). Radioimmunoguided surgery for colorectal cancer using I^{125}-labeled anti-carcinoembryogenic antigen monoclonal antibody, enables surgeons to define lymphatic metastasis and successfully guides in performing a radical operation.

3.11 Sentinel node ICH/RT-PCR

Sentinel lymph node is the first lymph node that drains the lymph from a cancer. (Edwards et al.2000). Intraoperative mapping of sentinel lymph node (SLN) in colorectal carcinoma identifies lymph nodes likely to contain metastases. Focused pathologic evaluation of the 1 to 4 SLNs so identified can improve the accuracy of pathologic staging (Wietz et al 2000). Similarly, advanced methods of detection of micrometastases, such as RT-PCR or immunocytochemistry may be applied exclusively to sentinel lymph nodes to improve the yield of micrometastatic detection. Lymphatic mapping using patent blue dye is feasible in colorectal cancer. However, Joosten et al (1999) have shown that this technique is associated with a very high false positive rate of up to 60%, and concluded that the concept of lymphatic mapping and sentinel node identification is not valid for colorectal cancer. Currently sentinel ICH/RT-PCR is not a standard practice in the colorectal cancer management.

Hepatic micrometastases, at the periphery of the tumour indicate widespread hepatic involvement and thus predict an increased risk of intrahepatic recurrence after hepatic resection and a poorer prognosis (Yokoyama et al 2002). Genetic and

histological assessment of surgical margins in resected liver metastases from colorectal carcinoma has shown that micrometastases do exist at the periphery of the tumour (Kokudo et al. 2002).

Genetic assessment for the presence of micrometastases has also been evaluated in head and neck cancers. Brennan et al (1995) have shown that the presence of micrometastatic disease at the resection margins is significantly associated with local recurrence. However, currently there is no information on the incidence of recurrence if micrometastases or isolated tumour cells are present in the mesorectum or circumferential margin using either Immunocytochemistry or RT-PCR technology. This information may be important to decide on the necessity for adjuvant therapy and help predict prognosis.

3.12 Summary of conclusions on the MM research

From the literature review, it is evident that micrometastastic disease is as frequent in colorectal cancers as in other solid cancers. Many studies using ICH in lymph nodes have shown no significance relationship with either relapse or survival. Detection rate is higher with more sensitive techniques suggesting an evolution in the detection methodology. However, micrometastastic disease in the bone marrow has been shown to be significantly associated with relapse of the disease with poor prognosis in several studies (Soeth et al 1997).

While the identification of micrometastastic disease has been suggested to help identify high-risk groups, to prognosticate and decide on adjuvant treatment, there are no studies to confirm these potential benefits. More interestingly, there are no studies of adjuvant therapy based on the presence of micrometastases. Therefore, at present micrometastatic disease does not affect the routine management colorectal cancer patients. The results of any adjuvant therapy trials based on the presence of micrometastastic disease may decide the future role of these advanced investigations in the routine clinical application.

RESULTS

4.0.1 Introduction to results

The results of this study are discussed in three sub-sections. Firstly, the sample on which immunocytochemistry was performed will be compared to a larger sample of patients with rectal cancer to confirm their representativeness. For this purpose, we have chosen to compare the research sample with the Welsh cancer intelligence and surveillance unit's data for the year 2002 on rectal cancers. The main measures of comparison will be age and gender. Furthermore, in this section, limitations and validity of the sample will be discussed.

In section two, clinico-pathological and demographic data (descriptive statistics) of all the rectal cancer patients included in the study from both hospitals are discussed. The clinical data were also correlated with pathological data to determine any relationship to local and systemic recurrence. Factors predictive of recurrent disease will also be evaluated.

In section three, the incidence of micrometastases at the circumferential resection margins and the mesorectum will be assessed. The ICH staining method will be compared to Haematoxylin and Eosin staining method to determine the effectiveness of the new method of staining. Furthermore, the significance of the presence of micrometastases, and isolated tumour cells, at the circumferential margin and in the mesorectum will be studied in relation to

local and systemic recurrence. The presence of micrometastatic disease at the circumferential margin and in the mesorectum will be correlated with clinical and tumour characteristics. Using binary logistic regression, the influence of the MM and ITC in the mesorectum and at the circumferential resection margin on recurrence and survival will be analysed.

4.0.2 Problems encountered in the research method

At the beginning of the project, we aimed to carry out the ICH on rectal cancer specimens at four levels so that the chances of detecting the micrometastases will be maximised. However, from the literature on the immunocytochemistry of the lymph nodes, it is not clear as to how many sections of the lymph nodes would be needed to detect the maximum number of micrometastases. In the majority of the initial studies one section was performed (Cutait 1991, Jeffers 1994, Greeenson1994). However, up to a maximum of ten sections have been reported (Isaka 1999, Yasuda 2001). It would be difficult to apply the results of lymph node immunocytochemistry to our study as the limiting factor for lymph nodes is the size of the lymph node, while the circumferential margin and mesorectum extends all along the rectal cancer specimen. Initially, in the first few cases, the pathology specimen block was enough for four levels of micro-sections. However, there was a case without sufficient rectal cancer tissue in the block to perform four levels of microsectioning. Therefore, this

study carried out with microsectioning of the rectal cancer block at three levels. For immunocytochemical analysis of microsections, only three levels were considered in all cases.

There were technical problems with Vantana® machine at Hospital B, therefore, the majority of the work was carried out in another nearby district general hospital where there were facilities for automated immunocytochemistry using same auto- immunostainer. The staining protocol and other staining formalities were the same as in hospital B, including the automation process, antibodies and their dilution as discussed in the methods section.

RESULTS-SECTION 1

4.1.1 The sample

The research sample consisted of all rectal cancer patients who had total mesorectal excision as a treatment with a curative intention (see inclusion criteria table 2.1). These patients were followed-up for at least 24 months post-operatively so that the data on recurrence of rectal cancer could be collected. Those patients who did not qualify for inclusion, were excluded from the study. Patients who had pre-operative chemotherapy or radiotherapy were also included in the study. If a patient died due to recurrent disease before the 24-month follow-up period, such a patient was included in the study for the purposes of immunocytochemistry and all other data analyses. However, any immediate post-operative death was excluded. This being defined as post-operative death within 30 days from surgery. The full exclusion criteria were given in the table 2.2.

4.1.2 Data collection, validity and reliability

Data on all rectal cancer patients were collected from case records, pathology records and hospital mortality records from both Hospitals A and B. All patients were diagnosed to have rectal cancer between the years 1997 to 2000. The Data (Appendix D) was compared with the MDT co-coordinator's data to ensure completeness and consistency.

Case files of rectal cancer patients form both hospitals were retrieved from medical records departments. Rectal cancers were identified using the local

coding system. Mortality and cancer recurrence information was further confirmed with the pathology department.

There were 150 rectal cancers treated in both hospitals from the year 1997 to 2000. Out of 150 of these, only 80 patients were suitable for inclusion in the study (see inclusion criteria in table 2.1). We followed rigorous exclusion criteria (see table 2.2) so that only patients who had a total mesorectal excision with a curative intention with adequate follow-up were included. Table 4.1.1 presents the information on the number of patients included and excluded from the study.

Table 4.1.1 Rectal cancers inclusion and exclusion form Hospitals A and B

Rectal cancer category	Hospital A	Hospital B	Total number
Rectal cancers included in the study[a]	47	33	80
Rectal cancers excluded[b]	34	36	70
All rectal cancers diagnosed between 1997 to 2000 (4 years)	81	69	150

[a] See inclusion criteria in the methods section.
[b] See exclusion criteria in the methods section.

There was no significant imbalance between the two centres due to inclusion and exclusion criteria p=0.11, and 0.81 respectively.

Table 4.1.2 and 4.1.3 summarises the reasons for exclusions in hospitals A and B respectively. The rationale behind the inclusion and exclusion criteria has been discussed in the methods section.

Table 4.1.2 Reasons for exclusion of rectal cancers in hospital A

Reason for exclusion	No of cases
TME/Palliative colostomy due to the presence of metastases in the liver	8
Post-operative death[a]	5
Suicide (follow-up less than 24 months)	1
Recto-sigmoid junction tumour	5
Death due to haematemesis[b]	1
Lost to follow-up	1
Synchronous cancer	2
Upper rectal cancer	4
Carcinoma in a polyp	2
Death due to pneumonia[b]	1
Death due to congestive Heart failure[b]	1
Death due to Coronary artery disease[b]	1
Palliative Abdominoperineal resection	2
Total	34

[a]Causes of post-operative death include anastomotic leak, myocardial infarction and congestive cardiac failure.

[b]Patients died during the follow-up period due to medical illness not related to cancer (Not immediate postoperative death).

Table 4.1.3 Reasons for exclusion of rectal cancers in hospital B

Reason for exclusion	No of cases
Polypoidal rectal cancer treated by Endo anal excision	1
Advanced rectal cancer with metastases. Inoperable, palliative treatment only	8
Advanced rectal cancer with metastases. Received only CT/RT and loop colostomy	9
Postoperative death[a]	9
Laparoscopic assessment only (disseminated disease).	1
Patient had TME but had liver metastases at the time of surgery	4
Patient died of Pneumoconiosis[b]	1
Laparoscopically performed. Blocks sent for assessment CLASICC Trial	1
Post operatively lost to follow-up	1
Chemotherapy related death[b]	1
Total cases excluded.	36

[a]Causes of post-operative death include anastomotic leak, myocardial infarction and congestive cardiac failure.
[b]Patients died of medical causes during the follow-up period (Not immediate postoperative death).

From the tables 4.1.2 and 4.1.3, differences in the types of cases excluded were noticed between hospital A and hospital B. This was probably due to the fact that in hospital B, laparoscopic surgery was practiced. Furthermore, some cases were excluded because of presence of carcinoma in a polyp specimen. Moreover, it is clear from the tables 4.1.2 and 4.1.3 that a significant proportion

of patients with rectal cancers were quite advanced clinically and therefore, were only suitable for palliative operations.

Recently, due to improvements in the surgical treatment of colorectal metastatic liver disease, a small proportion of fit patients in the current study had total mesorectal excision in spite of liver disease (n=5, total= 150, 3.3%). However, these patients were subsequently referred to tertiary centres for consideration of liver resection. Patients who were deemed unsuitable for liver resection were considered for adjuvant treatment. Another important finding from tables 4.1.2 and 4.1.3 is that a significant proportion (n=27, total= 150, 18%) of patients in the study had received only palliative surgical intervention such as colostomy, or laparoscopic assessment of the intra-abdominal dissemination.

Having considered the sample and the inclusion and exclusion criteria, the following discussion in this chapter will be devoted to the comparison between the sample and a wider population of patients with rectal cancer to assess their representativeness.

4.1.3 Sample Representativeness

Representativeness is the extent to which a sample compares with a wider population, so that we can reasonably be assertive on the quality of the sample and the generalisability of the findings established. After a detailed literature review, it became clear that mean ages and gender incidences described in the

scientific papers could not be used for comparison with our sample. The reasons for this are multifactorial. Many studies have combined the rectal and colon cancers (Greenson et al. 1994, Noura et al. 2002) and some scientific studies have chosen not to mention the mean age of the studies sample (Isaka et al. 1999, Noura et al. 2002). Furthermore, the scientific data were from various geographical regions of the world. This makes the comparison difficult and less reliable. Therefore, data on all rectal cancers collated by the Welsh Cancer Intelligence and Surveillance Unit for the year 2002 were chosen for the purposes of comparison. Firstly, there is a rationale in comparing our sample with a larger data set from Wales as they belong to the same geographic area. This would, logically, make the sample more robust and reliable. We have therefore, compared the data of all rectal cancers diagnosed in Wales in the year 2002 with our sample derived form these two Welsh hospitals.

The main measures of comparison between the sample and the Welsh data were age and gender distribution to confirm representativeness. The mean age, with their standard deviations of the sample were compared with the Welsh data. The degree to which the sample compares with the Welsh data will be discussed. The reasons behind any discrepancy in the expected values will be rationally explained. The data on all rectal cancers that were included

in the Welsh Cancer Intelligence and Surveillance data are included in the Appendix I and J.

A detailed calculation of the mean age and standard deviation for the rectal cancer patients in Welsh population data are presented in Appendix K.

The mean age of the Welsh data for males was 69.134 (10.85 SEM) years, while the mean age for the females was 70.350(12.98 SEM) years.

Table 4.1.4 Gender comparison between the sample and the Welsh data*

Gender	Sample	Welsh Data[a]	Significance
Males	55 (68.75%)	438 (60.09%)	
Females	25 (31.25%)	291(39.91%)	0.15[b]
Male: Female	55:25 (2.2:10)	438:291 (1.5:1)	
Total	n= 80 (100%)	n=729 (100%)	

[a]Welsh Cancer Intelligence and Surveillance unit's data for the year 2002(Appendix I and J)
[b]Chi Square goodness of fit X^2=2.000, df=1.

Table 4.1.4 presents the number of rectal cancer patients affected in Wales in the year 2002 with male and female distribution. The gender comparison in the sample revealed a ratio of 2.2: 1 male to female affliction of rectal cancers compared to 1.5: 1 in the Welsh data. Our targeted sample of rectal cancers had a total mesorectal excision as a surgical procedure, with a curative intention as a main inclusion criterion. However, while the Welsh data were on all rectal cancers that were diagnosed in Wales, in the year 2002, without

any regard to the type of treatment, whether curative or palliative. The mean ages of the sample and the Welsh data were 65.46 and 69.74 years respectively as shown in the table 4.1.5.

Table 4.1.5 Mean age comparison between the sample and Welsh population[a]

Variable	Number considered for comparison	Mean age
Sample data	80	65.46
Welsh Population data[b]	729	69.742[b]
X^2 goodness of fit test(X^2= 2.000, df=1)		p= 0.15

[a]Data based on the Welsh Cancer Intelligence and Surveillance units' information 2002 (Appendix I and J).
[b]Mean age calculated from table in appendix K.

Although, there is a difference between the two mean ages, this was not statistically significant (p=0.5). Note that the test was performed on mean ages for simplicity, otherwise a test would have been appropriate. Any differences in mean age could be due to a selection bias of patients who were fit enough to undergo a major surgical intervention. Naturally, the sample age would be younger than the Welsh data, which has included all patients with rectal cancer. In the next table 4.1.6 differences in the mean number of males and females will be considered.

Table 4.1.6 Comparison between mean ages of males and females in the sample with Welsh Data[a]

Variable	Number of males	Mean age	Number of females	Mean age
Sample data	55	66.13	25	64.0
Welsh Population data[b]	438	69.13[b]	291	70.35[b]
X2 goodness of fit test	X^2=2.000 df=1	p-value =0.15	X^2=2.000,df=1	p-value =0.79

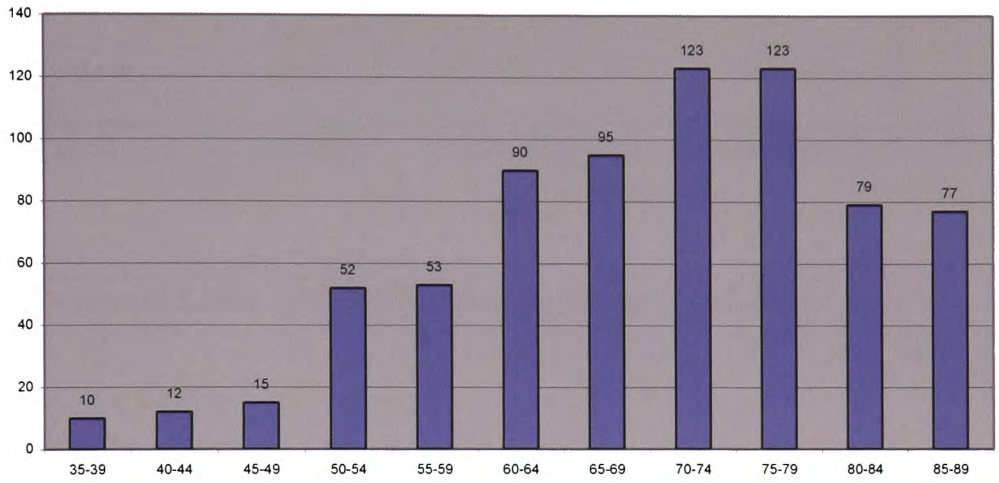
[a]Data based on the Welsh Cancer Intelligence and Surveillance units' information 2002. (Appendix I and J)
[b]Mean age calculated form table in the appendix K.

From the table 4.1.6 it is clear that there was no statistically significant difference in the mean ages of males and females in the sample compared to the Welsh rectal cancer data. However, the sample appears to be younger possibly due to selection bias in the sample who had a definitive surgical intervention with a curative intention.

In figure 4.1.1, the age distribution of rectal cancers in larger Welsh data for the year 2002 is presented. It is clear from 4.1.1 that the majority of the patients

were in the sixth and seventh decade the Welsh population data.

Figure 4.1.1: Age distribution of rectal cancers in the Welsh population for the year 2002



The proportion of patients in their 6th and 7th decade between the sample and Welsh data is demonstrated in the following table. There was no statistically significant difference between the sample and the Welsh population in 6th and 7th decade age groups.

Table 4.1.7 Rectal Cancer comparison between the sample and the Welsh data in the 6th and 7th decade group

Type of data	Total number of patients	No of patients in the 6th decade	No of patients in the 7th decade
Sample data	n=80	20/80 (25.00%)	31/81 (38.75%)
Welsh data	n=738	185/738 (25.06%)	256/738 (34.68%)

X^2 goodness of fit test: p=0.78(6th decade), p=0.78(7th decade)

From the above data on both the sample and from the Welsh population, it is apparent that there is no statistically significant difference in the mean age for the whole sample and the Welsh Population, and that there is no statistically significant difference between the males and females in their mean ages. From the above discussion, we can confidently express that the sample we have chosen in our study does represent the Welsh rectal cancer data for the year 2002.

4.1.4 Validity and robustness of the comparison between the sample and the Welsh data

The Welsh cancer data is the closest data that could be compared with the sample due to the problems with the literature information as stated before. Further, the true values of the sample data are likely to lie within the Welsh data as the sample was derived from the same geographical region. From the previous data on rectal cancers from 1992 to 2001, the distribution of rectal cancers was similar with similar male to female ratios. (See Appendix L for the Welsh data from the year 1992 to 2001). Therefore, it is inappropriate to compare with literature reports outside Wales. However, there were limitations with the nature of the research undertaken that make the direct comparison more difficult with the literature.

4.1.5 Limitations and problems with the sample and the data

- Sample was a highly selective group of rectal cancers;
- Data from rectal cancer patients were drawn from two hospitals were included in the study;
- Two or more surgeons were involved in the operative treatment of rectal cancer;
- All the data including local recurrence was collected retrospectively;
- There were several exclusion criteria;
- No similar studies were found in the literature for direct comparison.

4.1.6 Summary and conclusions

In this section we have defined the sample, explained the inclusion and exclusion criteria with their rationale, data quality was highlighted and various sources of data were explained. The sample was tested against the Welsh data to examine representativeness, which confirms a good representation in terms of age and gender. There was no significant difference in the mean ages of the sample and the Welsh data; not only for the whole sample but also between sample males and females and the Welsh population of males and females. Having investigated the sample, data and their sources, and confirmed the representativeness, the next chapter will consider the sample demographics and clinico-pathological information. The clinico-pathological correlations for local and systemic recurrence and survival will be highlighted.

RESULTS-SECTION 2

4.2.0 Introduction

In this section, only the clinical and pathological information pertinent to the sample will be discussed. This data will then be correlated to examine the relationship to the local and systemic recurrence. Mortality and survival will be analysed in relation with clinico-pathological data.

4.2.1 Sample demographics

Table 4.2.1 The distribution of the sample by gender

Gender	n=80 (100%)
Males	55 (68.75)
Females	25 (31.25)

Male to female ratio of the sample: Males: Females= 55:25=2.2: 1.0

The proportion of males to females in the sample is not statistically significant compared with the Welsh data for the year 2002. In figure no 4.1, the distribution of age in relation to gender is presented.

Figure 4.2.1 Bar chart demonstrating the gender distribution frequency

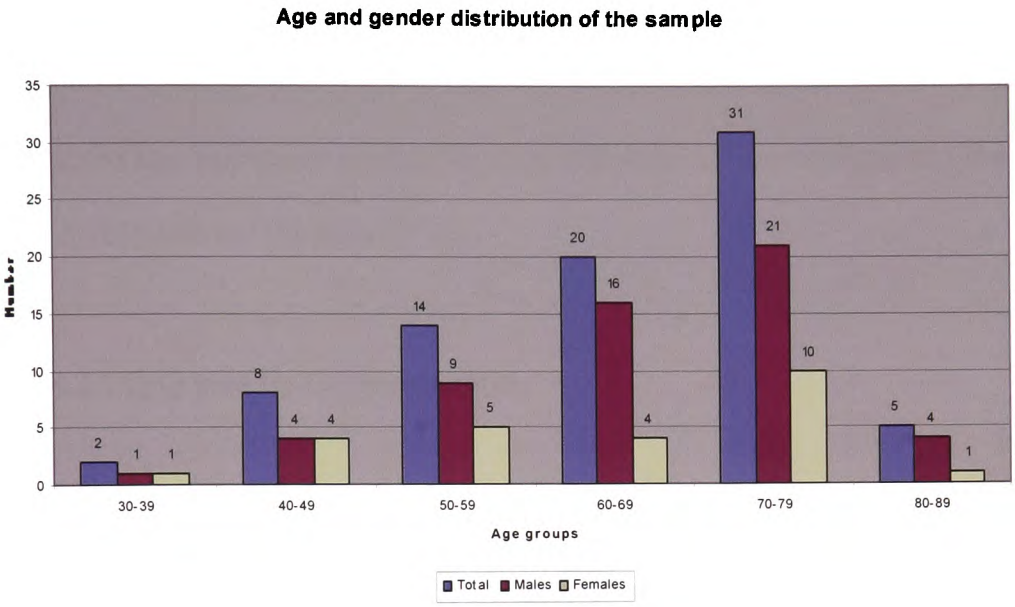


Table 4.2.2 Sample distribution by age group

Age range	n=80
35-39	2 (2.50)
40-44	3 (3.75)
45-49	5 (6.25)
50-54	5 (6.25)
55-59	9 (11.25)
60-64	8 (10.00)
65-69	12 (15.00)
70-74	16 (20.00)
75-79	15 (18.75)
80-84	3 (3.75)
85+	2 (2.50)
Total	80 (100)
Test of normality	p=0.19 [a], 0.20 [b]

[a] Significance for males, [b] Significance for females
Figures in parenthesis are percentages.

the rectal cancer patients in our research included all age groups on the basis of proportional representation and did not concentrate on a particular age group. The Quantile-Quantile plot (figures 4.2.2 and 4.2.3) for both genders show that the observed age values are around the expected normal curve suggesting the normal distribution of the data for age.

Figure 4.2.2 Q-Q plot for the male sample

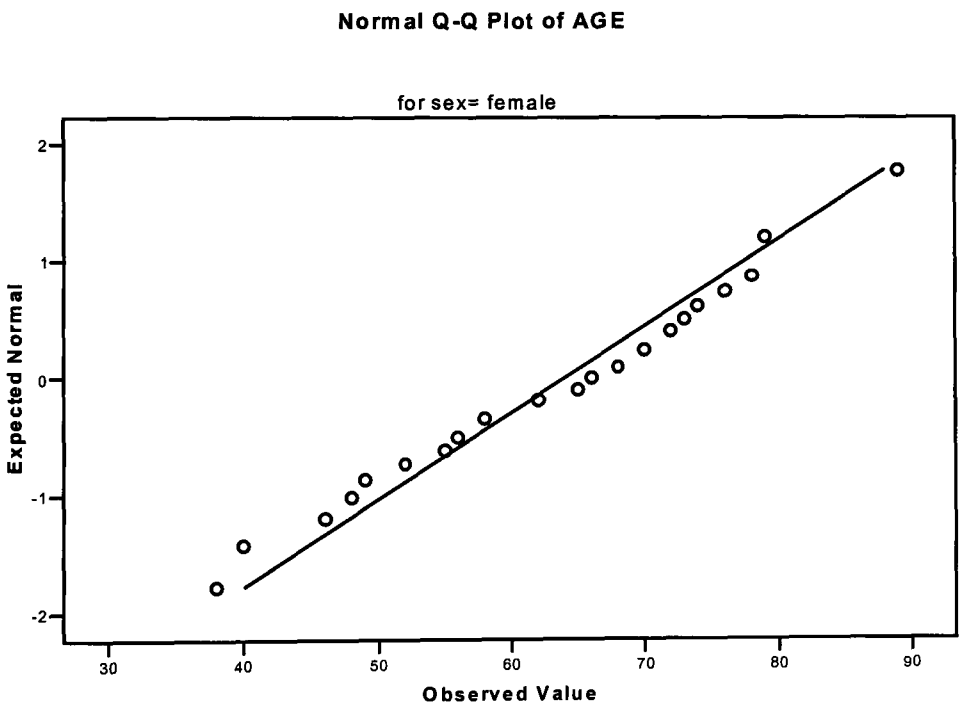


Figure 4.2.3 Q-Q plot for female gender in the sample

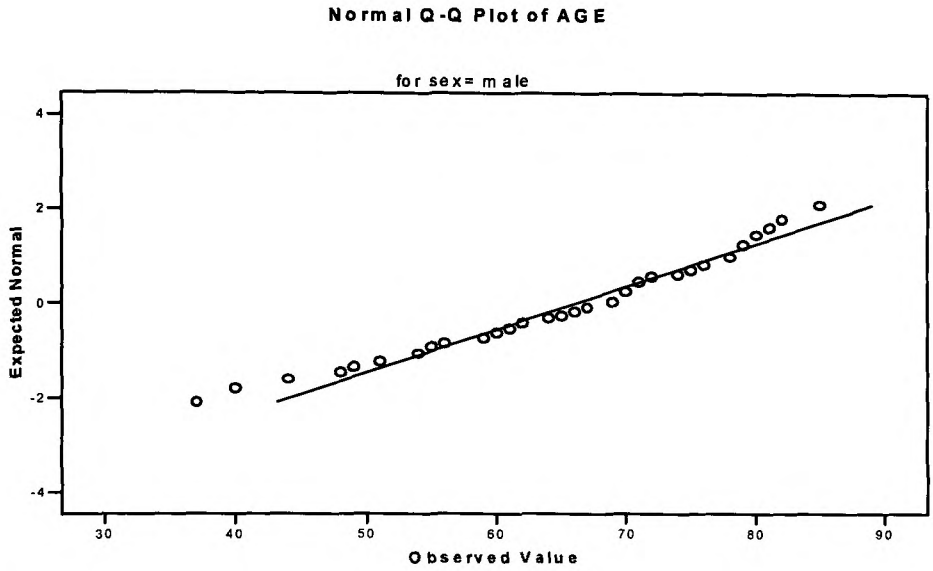
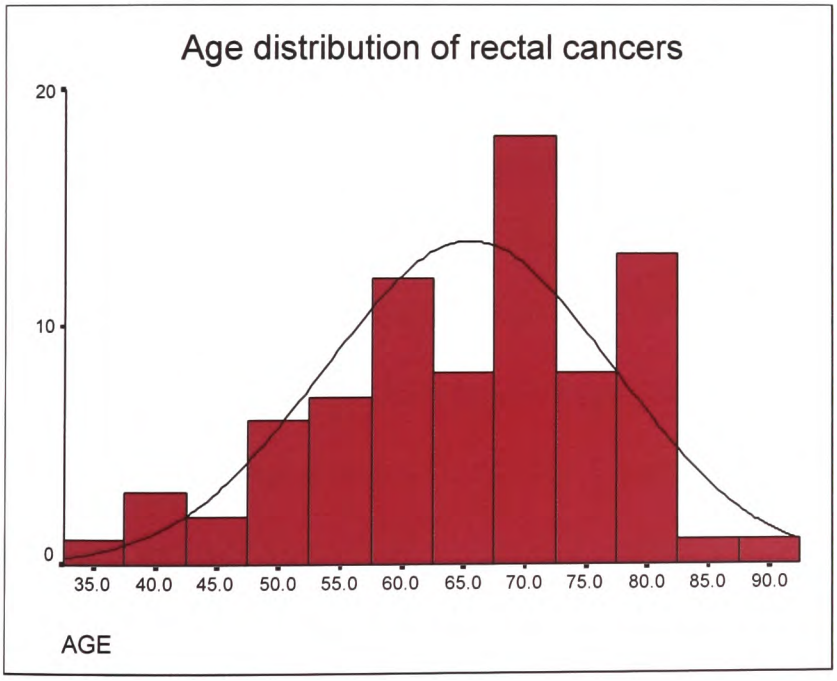


Figure 4.2.4 Age distribution shown as a histogram with a standard distribution curve superimposed



The above histogram demonstrates the age distribution of the patients with rectal cancers who were considered for further analysis, and immunocytochemical examination. The graph shows apparent skewed distribution suggesting that age is not normally distributed. However, the distribution appears to be comparable to the Welsh data. Furthermore, the Q-Q plot of age distribution is around the straight line suggesting normality (fig 4.2.2 and 4.2.3). This is unsurprising as it could be explained by the fact that cancers naturally are more common in older population therefore, the same reasoning applies to rectal cancers. Epidemiological studies have shown that colorectal cancers are more common in older populations; consequently, one would expect more cancers in the older population resulting in skewed distribution.

4.2.2 Type of surgical procedure

All the patients in our sample underwent total mesorectal excision (TME) as the surgical intervention. This involved either an anterior resection where the rectum bearing the tumour was resected (anterior resection) or removal of entire rectum and the anal canal (Abdominoperineal resection) dependent on the position of the rectal tumour in relation to the anal canal and also on the functional status of a patient in general and in specific, anal canal functions. Out of 80 patients, 52(65.0%) underwent anterior resection while 28(35.0%) underwent abdominoperineal resection.

Table 4.2.3 Gender and type of operation performed for rectal cancers

Gender	AP Resection	Anterior Resection	Significance Chi-Squared goodness of fit
Males	21(26.25)	34(42.5)	P=0.07
Females	7 (8.75)	18 (22.5)	P=0.02
Total	28 (35)	52 (65)	

NB: Figures in parenthesis are percentages

The hypothesis in the above table was to assess the equality of distribution of gender between two different types of operation abdominoperineal resection and anterior resection. Table 4.2.3 shows that there was significant difference in the type of operations performed in female gender p= 0.02, but not in male gender p=0.07, Chi square goodness of fit test. This is probably a chance observation.

The decision to perform abdominoperineal resection depends on several factors including tumour location, functional status and invasion of anal sphincter. The literature shows that the lowest rates of permanent stoma formation following rectal cancer surgery using staples was 9% (Karanjia 1994), although other specialist units have reported low stoma rates of 10% (Williams et al. 1985), and 19% (Matheson et al. 1985). However, in the Wales/Trent audit on bowel cancer, (ACPGBI guidelines 2001) a permanent stoma rate of 47% was reported. In this study, a stoma rate of 35% was noted. Thus, there seems to be a variation in the permanent stoma formation, largely

due to case mix and increasing elderly population. Failure to recognise the fact that only 1 cm distal clearance margin is required may also result in an unacceptably high rate of permanent stoma formation (ACPGBI guidelines, 2001). As highlighted in figure 4.2.1, the majority (63.75%) of our patients were in the sixth and seventh decade. This would probably explain the high incidence of permanent stoma formation. However, the permanent stoma formation rate in our study complies with the recommendations of the ACPGBI.

Table 4.2.4 Age distribution by operation type

Age range	Abdominoperineal resection	Anterior resection
35-39	1 (1.25%)	1 (1.25%)
40-44	2 (2.50%)	1 (1.25%)
45-49	0 (0%)	5 (6.25%)
50-54	2 (2.50%)	3 (3.75%)
55-59	2 (2.50%)	7 (8.75%)
60-64	3 (3.75%)	4 (5.0%)
65-69	3 (3.75%)	11 (13.75%)
70-74	6 (7.50%)	9 (11.25%)
75-79	7 (8.75%)	8 (10%)
80-84	1 (1.25%)	2 (2.50%)
85+	1 (1.25%)	1 (1.25%)
Total	28 (35%)	52 (65%)
X2 goodness of fit test of uniformity	p=0.650	

Although there was statistically significant differences between the gender and the type of operation, there was no statistically significant difference (p=0.650) in the type of operation for various age ranges.

Table 4.2.5 Type of operation performed in hospital A and B

Centre	No of patients	Type of operation APR	Type of operation AR
Hospital A	47	18	29
Hospital B	33	10	23
Total	80	28	52
X2 test of association	p=0.46(X^2=0.545, df=1)		

APR= Abdominoperineal resection, AR= Anterior resection.

In table 4.2.5, differences in the types of operations between hospital A and B are presented. There were no statistically significant differences in the type of operation carried out between the hospitals A and B.

Table 4.2.6 Mean age and type of operation performed in hospitals

Centre	Mean age of patients undergoing AR	Mean age of patients undergoing APR
Hospital A	68.41	68.41
Hospital B	60.56	60.57
Independent t-test	P=0.009, t=2.70, df=50	

AR= Anterior resection, APR= Abdominoperineal resection.

After removing outliers from the records, the mean ages were 71.0 (hospital A) and 60.57 (hospital B) years. Boxplots revealed normality in both cases and an independent t test was carried out showed significant differences in the means (p=0.000, t=4.14, df=24). In the case of APR, no outliers were observed and normality could be established for age groups within hospitals (Boxplots). Independent t test revealed significant age difference between the two hospitals (P=0.009, t=2.70, df=50).

Table 4.2.7 Gender differences between hospital A and B

Center	Gender Males	Gender Females	TOTAL
Hospital A	34	13	47
Hospital B	21	12	33
Total	55	25	80
X2 goodness of fit test of uniformity	p = 0.21	p =0.98	0.29

No significant gender differences were found between the two hospitals A and B.

4.2.3 Tumour differentiation

The majority of cases demonstrated moderate differentiation 58 (72.5%) while only 14 (17.5%) tumours were well differentiated, with fewer than 8(10%) cases demonstrated poor differentiation.

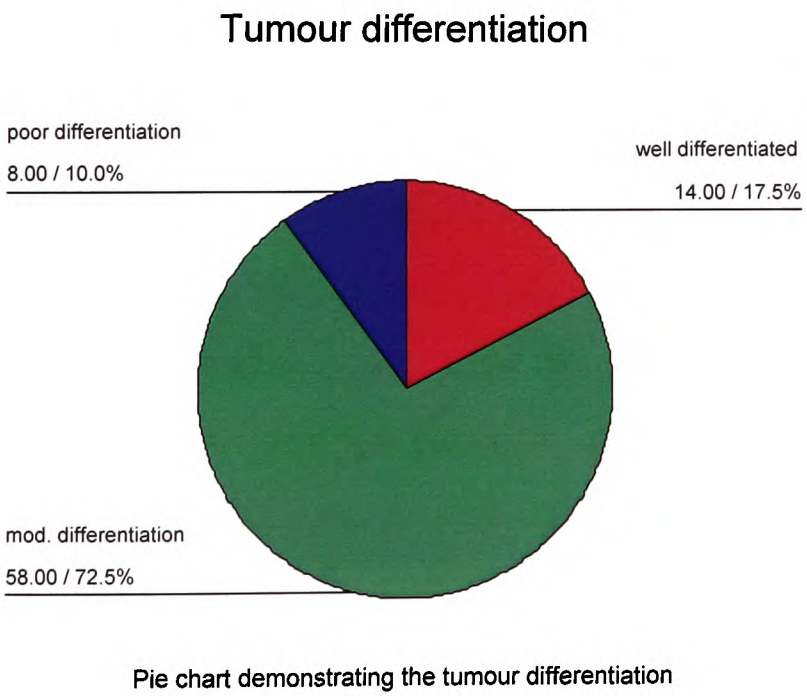


Figure 4.2.5 Distribution of tumour differentiation

4.2.4 Perineural invasion

Perineural invasion is considered to be one of the poor prognostic factors in the colorectal cancer (Bognel et al. 1995). Moreira et al. (1999) found neural invasion in 27% of the rectal cancer cases. Five-year survival rates (Kaplan-Meier method) were less in patients with neural invasion ($p < 0.01$). Neural invasion was not noted in Dukes stage A tumours in their series. However, neural invasion increased with tumour stage. Both Dukes B and C cases with local recurrence had a higher incidence of neural invasion as compared with the disease-free group. These results suggest that postoperative assessment of neural invasion may provide valuable information to determine which patients with low rectal cancers would benefit from adjuvant treatment. The incidence of neural invasion increased with the frequency of venous invasion and the degree of lymph node metastasis, but not significantly (Kenmotsu et al. 1994). Matsushima et al. (1998), found neural invasion in 38 (29.7%) of 128 of rectal cancers in their study. The rectal cancers with neural invasion were characterized by infiltrative growth pattern, frequent lymphatic and venous permeation, frequent lymph node metastasis and advanced stage of disease. The cases with neural invasion also showed a higher rate of recurrence and worse prognosis in this study. In the current study, the incidence of perineural invasion was 13.8%.

Furthermore, perineural invasion was significantly associated with local and systemic recurrence of rectal cancer (p=0.005 for local and systemic recurrence respectively, see table 4.2.8).

Table 4.2.8 Cross tabulation between Perineural invasion and local and systemic recurrence

Perineural invasion	Recurrence[a]		Total (n=80)
	No	Yes	
No invasion	49	20	69
Invasion present	3	8	11
Total	52	28	Significance p=0.005(X^2=7.979, df=1)

[a]Includes both systemic and local recurrence.

It is important to emphasize that the findings from the above test and a number of other tests carried out, need to be taken with caution due to sample size limitation. Note that Yates correction has been adopted in these situations.

4.2.5 Extra-mural vascular invasion

Extra-mural vascular invasion is another important prognostic factor that influences the local and systemic recurrence. Bayar et al. (2002) found the presence of venous invasion in 60% of early rectal cancers with lymph node metastases, and suggested that venous invasion may provide valuable information to determine which patients would benefit from radical surgery, or adjuvant radiation therapy after sphincter-sparing surgery. Furthermore, there is a close relationship between venous invasion and the development of liver metastases in patients with colorectal carcinoma. In patients without venous invasion, the incidence of liver metastases was less (Ouchi et al. 1996). When venous invasion was observed, liver metastases developed over three times as frequently in these patients as when metastases were not demonstrated (Knudsen et al. 1983). Various pathologic features have been shown to have stage-independent prognostic significance in colorectal cancer and may help to define risk of adverse outcome. Such features include tumour grade; histologic type; extent of extramural penetration by tumour; neural, venous, and lymphatic invasion tumour border configuration; tumour budding; and host lymphoid response (Compton et al. 2002). In the current study, the extra-mural vascular invasion was noted in 15% (n= 12) of patients. This was shown to be significantly associated with cancer recurrence (p=0.013, see table 4.2.9).

Table 4.2.9 Cross tabulation between vascular invasion and recurrence

Extramural vascular invasion	Recurrence[a]		Total (n=80)
	No	Yes	
No invasion	48	20	68
Invasion present	4	8	12
Total	52	28	p=0.013 (X^2=6.233, df=1)

[a]Includes both systemic and local recurrence.

4.2.6 Lymph node involvement

Lymphatic involvement is the most important prognostic factor in colorectal disease (Muto et al. 2003, Dukes et al. 1958). The majority (40-50%) of cases treated in the UK are Dukes C tumours, which alters the prognosis significantly. In our study the incidence of lymph node involvement was 43.8% (35/80). In the literature the reported rate of incidence of lymph node involvement has been quoted variably. This principally is dependent on the tumour penetration. In series with early rectal cancers such as T1 lesions, the incidence of lymph node involvement has been reported to be up to 10%. Haggit et al. (1985) reported that level of invasion is the major factor for prognosis in early cancers. Among T1 rectal lesions, the incidence of lymph node involvement was dependent on the lympho-vascular invasion and depth of submucosal invasion. The incidence of lymph node involvement was higher

in tumours with lymphovascular invasion (11%) and submucosal invasion level 2 and 3 (8.4%) (Matsuda et al. 1999). Furthermore, when all these factors were added, the incidence of lymphatic involvement was highest 20% (Muto et al. 2003).

In this study the overall incidence of lymph node involvement was 43.8% (35/80). Table 4.2.10 shows the incidence of node metastases and recurrence. In addition, the node involvement was significantly associated with cancer recurrence (p=0.076) and poor survival (P=0.001) as shown in table 4.2.11 respectively.

Table 4.2.10 Cross tabulation between lymph node involvement and recurrence of the rectal cancer

Lymph node involvement	**Recurrence[a]**		**Total (n=80)**
	YES	**NO**	
No Lymph node involvement	12	33	45
Nodes involved	19	16	35
Total	31	49	Significance[b] p= 0.042 (X^2=8.227, df=1)

[a]Includes both systemic and local recurrence.
[b]Chi-Squared test of association.

Table 4.2.11 Cross tabulation between lymph node involvement and survival

Lymph node involvement	Patient alive or dead at analysis		Total (n=80)
	Alive	Dead	
No Lymph node involvement	38	7	45
Nodes involved	18	17	35
Total	56	24	Significance[a] p= 0.001(X^2=10.219, df=1)

[a] Chi-Squared test of association.

Prabhudesai et al (2003) have recently reported mesorectal tumour deposits, discontinuous with the main tumour margin in 52.7% of Dukes B and C patients with rectal cancer. Furthermore, they found a significant association between the number of extranodal deposits and intramural vascular invasion (P = 0.017), extramural vascular invasion (P = 0.039), perineural invasion (P = 0.039), and lymph node involvement (P = 0.008). Furthermore, Prabhudesai et al. have concluded that extranodal deposits are a distinct form of metastatic disease in patients with rectal cancer. The association with vascular invasion and an earlier development of metastases suggests that a significant proportion of extranodal deposits may occur from blood-borne spread. These tumour foci should be considered as indicators of poor prognosis (Prabhudesai et al. 2003). In our study, the mesorectal tumour deposits were found in 12.5%(10/80) of patients. However, there was no statistically significant association with recurrence rate and the mesorectal deposits (p=0.288) and survival (p=0.140) (4.2.12 and 4.2.12a).

Table 4.2.12 Cross tabulation between mesorectal deposits and recurrence

Mesorectal deposits	Recurrence		Total (n=80)
	Yes	No	
No deposits	47	23	70
Deposits present	5	5	10
Total	56	24	Significance[a] p= 0.288 (X^2= 1.13, df=1)

[a] Chi-Squared test of association.

Table 4.2.12a Cross tabulation between mesorectal deposits and survival

Mesorectal deposits	Patient alive or dead at analysis		Total (n=80)
	Alive	Dead	
No deposits	51	19	70
Deposits present	5	5	10
Total	56	24	Significance[a] p= 0.140 (X^2= 2.177, df=1)

[a] Chi-Squared test of association.

The removal and identification of lymph nodes containing tumour is crucial both to reduce the risk of recurrence to decide on the adjuvant therapy. The currently there seems to be a consensus on the optimum of lymph nodes that should be examined (Caplin et al. 1998, Cserni et al. 1999, Maurel et al. 1999, Wong et al. 1999, Chen et al. 2000, Cianchi et al. 2002, Goldstein et al. 2002, Kuru et al. 2002, Prandi et al. 2002,). However, there is also evidence that removing 10 or more nodes improves staging (Kuru et al. 2002, Pocard et al. 1998, Esser et al. 2001). Patients classed as node negative, based on the examination of fewer nodes demonstrate a higher recurrence rate and poorer survival than those patients classed as node negative on examination of more

nodes (Goldstein et al. 2002, Esser et al. 2001, Pocard et al. 1998, Tepper et al. 2001). In a recent publication Sarli et al. (Sarli et al. 2005) have concluded that five-year survival rates for patients with stage III tumours with only 1-3 positive lymph nodes (52.6%) was similar to that of patients with stage II tumour who had nine or fewer lymph nodes examined (51.3%). These results demonstrate that the prognosis of TNM stage II colorectal cancer is dependent on the number of lymph nodes examined. A study carried out based on the cancer registry data from 1988- 1991 reported that in the UK only 14% patients with colorectal cancer had 12 or more lymph nodes examined (Gutta et al. 2000).

Thorn et al (2004), have suggested that the number of lymph nodes identified within the excised specimen, in patients undergoing resection of a rectal cancer positively correlates with the size of the tumour and is also dependent on the examining histopathologist. In addition, in node-positive patients the number of involved nodes increases with increasing lymph node yield (Thorn et al. 2004). In our study in 16/80(20%) of pathology specimens, more than 13 nodes were retrieved. However, there were 10/80 (12.5%) patients in whom the actual numbers of nodes retrieved were not indicated in the pathology reports (Tables 4.2.13 and 4.2.14). The reasons for this were not clear from pathology reports. However, it is widely believed that pathologists' workload has increased significantly, due to the standardisation of comprehensive

The number of nodes positive for malignancy ranged from one to eighteen (Table 4.2.14). The more the number of nodes involved, the more aggressively the carcinoma behaves biologically (Sarli 2005).

Table 4.2.14 Positive lymph nodes

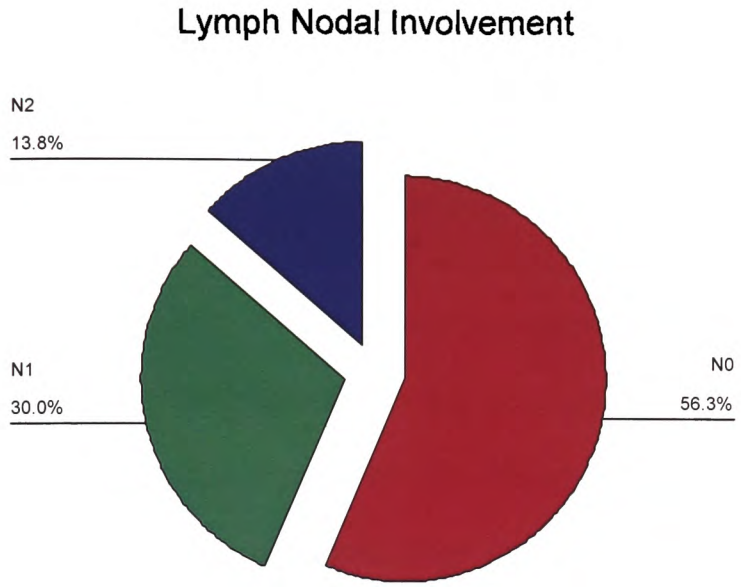
Positive lymph nodes	Number of cases n=80
0	42 (52.5)
1-5	28(35.0)
6-10	5 (6.25)
11-15	2 (2.5)
15+	1 (1.25)
Missing[a]	2 (2.5)
Total (n=80)	80 (100.0)

[a]Missing values, figures in parenthesis are percentages.

Furthermore, the prognosis of node positive cancer also depends on the involvement of apical nodes, situated at the apex of the vascular ligation. In the latest TNM classification (6th edition) the distinction between the N1 and N2 node involvement was based on the number of nodes involved, N1 being less than 4 nodes while N2 being more than 4 nodes involved by the cancer, irrespective of whether the apical nodes are involved or not. In this study the node positive patients were further separated for those with N1 disease and those with N2 disease, based on whether the apical node is involved or not. Of all the node positive patients, there were 25/80(30%) patients with N1 disease category, while there was 11/80(13.75%) with N2 disease. The following Pie

chart demonstrates the node involvement into N1 and N2 categories as percentages.

Figure 4.2.6 Pie chart demonstrating the proportion of patients with N1 and N2 lymph node disease



In the resection of rectal cancer, an adequate margin free from tumour is essential for an R0 resection to achieve microscopic clearance. In rectal cancers, where an anterior resection is appropriate, the distal resection margins are as important as circumferential margins. Studies have shown that a distal clearance of 2 cm is adequate for a cancer clearance (Williams et al. 1983). In this study there was one patient with obvious involvement of distal margin. However, there were 4 (5%) patients with distal clearance less than 1 centimetre (Table 4.2.15).

Table 4.2.15 Involvement of distal resection margin with malignancy

Distal resection margin	N=80
Clear, AR	47 (58.8%)
Clear, APR	28 (35.0%)
Involved	1 (1.25%)
Less than 1 cm	4 (5.0%)
Total (n=80)	80 (100.0)

The importance of clear circumferential margins cannot be over emphasised. Nagtegaal et al (2002) have shown that the positive CRM margin with carcinoma was a strong predictor of local recurrence after TME surgery. A margin of less than 2 mm is associated with local recurrence risk of 16% compared with 5.8% in patients with more mesorectal tissue surrounding the tumour. Further, patients with margins less than 1mm have an increased risk of distant metastases (37.6% vs. 12.7%, p=<0.0001) as well as shorter survival (Nagtegaal 2002). Currently, the circumferential margin is considered to be involved if the tumour is obviously involving the circumferential margin and if the tumour advancing margin is less than 1mm (ACPGBI, 2001) from the circumferential margin. In this study there were 14 (17.5%) patients with positive circumferential margins (see table 4.2.16). (This includes patients with direct involvement and those with less than 1 mm).

However, in this study the involvement of circumferential margin was not commented in the pathology reports in ten (12.5%) patients. The reasons for this were not very clear from pathology reports, except in one case where the technical assessment of the circumferential margin was reported as difficult.

Table 4.2.16 Circumferential margin involvement in rectal cancers

Circumferential margin involvement	**Total n=80**
Involved or less than 1 mm	14 (17.5)
Not involved*	56 (70.0)
Not Commented	10 (12.5)
Total (n=80)	80 (100.0)

*More than 1 mm circumferential margin is considered not involved.

Sphincter invasion is relevant in cases where the surgical treatment of rectal cancer is carried out as abdominoperineal resection. In this study there were 3(3.75%) patients where the sphincter was involved (Table 4.2.17).

Table 4.2.17 Sphincter invasion by the tumour in patients who had abdominoperineal resection

Sphincter invasion	n=80
Not Relevant, AR	52 (65.0)
APR Sphincter not involved	25 (31.25)
APR Sphincter involved	3 (3.75)
Total (n=80)	80 (100.0)

AR= Anterior resection, APR= Abdominoperineal resection

Table 4.2.18 Tumour staging

Tumour staging	n=80
T1	6 (7.5)
T2	16 (20.0)
T3	53 (66.3)
T4	5 (6.3)
Total (n=80)	80 (100.0)

The above table clearly demonstrates that the majority of rectal cancers were T3 stage suggesting advanced nature of the tumour at presentation. Furthermore, only a small proportion of patients 22/80 (27.5%) had T1 and T2 cancers, treated by total mesorectal excision.

Table 4.2.19 Dukes staging

Dukes staging	n=80
A	17 (21.3)
B	28 (35.0)
C1	24 (30.0)
C2	11 (13.8)
Total (n=80)	80 (100.0)

Dukes staging of rectal cancer (Dukes et al. 1958) has been traditionally used in the UK. However, TNM classification is the current standard for disease staging. In this study 35 (43.8%) patients were of Dukes stage C, again suggesting advanced nature of the disease at presentation.

Location of the tumour not only influences the type of treatment for rectal cancers but also affects prognosis (Heald et al. 1986). In this study, due to the inherent bias involved in the selection of mid and low rectal cases, the majority of the rectal cancers were below the peritoneal reflection 69 (86.3), while there were 11 (13.8) patients with rectal cancers at the peritoneal reflection.

4.2.7 Adjuvant pre or post-operative chemo-radiotherapy

As a part of the treatment for rectal cancer, chemo-radiotherapy is currently an option in order to optimize the cure and improve the survival. In this study 15 (18.8) patients underwent post-operative chemotherapy, belonging to Dukes C

category. Some patients were not suitable for chemotherapy and some declined this treatment option post-operatively.

There was no significant difference in the incidence of ITCs in patients who had post adjuvant chemotherapy ($p=0.352$, $X2= 0.867$, $df=1$), compared with patients who did not have chemotherapy. Furthermore, there was no statistically significant difference in the incidence of ITC in patients who had pre-operative radiotherapy ($p=0.658$).

There is consistent evidence that local recurrence can be reduced by up to 50% by pre-operative radiotherapy. In the trials reported to date, the local recurrence rates in the surgery only group were in the region of 10 -20%. With improvement in surgical techniques such as total mesorectal excision (TME), the local recurrence rates are falling to less than 10% in most specialist units. Results from a Dutch trial show that the local recurrence rates were reduced from 8.2% with TME alone to 2.4% with the addition of a pre-operative short course radiotherapy (Kapiteijn et al. 2001). Pre-operative radiotherapy as a short course regimen for 5 days at a rate of 5 Gray (Gy) daily, followed immediately by surgical treatment was considered in 7 patients (7/80, 8.8%). While post-operative chemotherapy was given in only 2 patients for positive circumferential margins in this study.

4.2.8 Tumour recurrence

Rectal cancer recurrence is one of the standard variables considered in the quality of rectal cancer treatment, offered to patients in addition to survival. In this study, 28 patients (35%) had recurred within 24 months following surgical treatment. These recurrences include local and systemic recurrences. Local recurrence was noted in 9 patients (11.25%) out of whom there were only 3 pure local recurrences while the remaining 6 patients had both local and systemic recurrences. In earlier studies some authorities have mentioned that recurrence following rectal cancer surgery occur in up to 50% in the pelvis (Silen et al. 1983). With the introduction of total mesorectal excision in majority of the centres, the local recurrence is becoming less.

Table 4.2.20 Nature of recurrence within 24 months

Site of recurrence	**n=80**
No recurrence	52(65.0)
Local recurrence	3 (3.8)
Systemic recurrence	19 (23.8)
Both local and systemic recurrence	6 (7.5)
	Total= 100%

The majority of recurrences were systemic in nature with predominant metastases to liver (16, 64%) followed by lung (5, 20%)and intra-abdominal disease. Other rare sites of recurrences include bone and brain metastases (4% each).

Table 4.2.21 Site of systemic recurrence

Site of recurrence	**(n=25)***
Liver	14 (56)
Lungs	4 (16)
Liver and Lungs	1 (4)
Brain	1 (4)
Intra-Abdominal Disease	4 (16)
Liver and Bone	1 (4)
Total (n=80)	25 (100.0)

*Only 25 (31.25%) patients developed systemic recurrence (n=80) within the study period.

Various investigative tools were applied to detect recurrences. Most commonly, a computerized tomogram of the abdomen and pelvis was used followed by Ultrasound examination. MRI of the abdomen and pelvis, examination under anesthetic, and laparotomy were other means of detection methods for recurrence in this study (table 4.2.22). Positron Emission Tomogram (PET) is currently being assessed as an investigative tool, with

promising results (Veit et al. 2005). However, PET scan was not used on the study patients to detect recurrence, probably due to relative rarity of the availability of this new investigative tool.

Table 4.2.22 Investigations used to assess recurrence

Investigation	n=80
Clinical	2 (2.5)
CT Scan	11 (13.8)
CT Scan and Laparotomy	1 (1.3)
EUA And Biopsy	1 (1.3)
EUA, CT	1 (1.3)
Laparotomy	1 (1.3)
MRI	2 (2.5)
Not Relevant	52 (65.0)
US And FNAC	1 (1.3)
US Scan	8 (10.0)
Total (n=80)	80 (100.0)

CT= Computerised Tomography, US= Ultrasound Scan, EUA= Examination under Anesthetic, FNAC= Fine Needle Aspiration Cytology.

Local recurrence following rectal cancer surgery depends on several factors such as surgical technique, application of pre-operative radiotherapy, and stage of the disease. However, the majority of recurrences will present within 24 months after surgery. In this study, 6 (66%) local recurrences were noted within 24 months and the rest of the recurrences were noted between 25 months to 48 months (table 4.2.20).

Table 4.2.23 Time to local recurrence in months

Local recurrence	**n=80**
Manifested between 0- 24 months	6
Manifested between 25-48 months	3
Total	9

Survival following rectal cancer surgery depends on several factors such as stage of the disease at presentation, tumour factors, type and quality of surgical procedure performed, location of the rectal tumour, with lower third rectal cancers having a bad prognosis (Heald et al. 1986). Treatment related factors such as the use of pre and post-operative chemo-radiotherapy are

relevant. In this study, there were 24 (30%) cancer related deaths due to metastases, resulting in a mortality rate of 30% at 2 years (table 4.2.24). The overall 5-year survival rates for rectal cancer are 80%, 55% and 32% for Dukes A, B and C respectively (Slaney et al. 1991).

Table 4.2.24 Patient survival at analysis

Alive or deceased at analysis	**n=80**
Deceased	24 (30.0)
Patient Alive	56 (70.0)
Total (n=80)	80 (100.0)

From the table 4.2.25, there were no significant clinico-pathological association in the patients between hospital A and B, except for a significant association with the tumour size p= 0.047 and tumour differentiation p=0.001. It is important to note that pathologist's assessment of tumour differentiation is exposed to subjectivity. Furthermore, in Hospital B, there were several pathologists were involved in the assessment. Therefore, it is likely that there would be differences in the assessment and ensuing results.

Table 4.2.25 Clinicopathological associations between Hospital A and B

Clinicopathological variable		Hospital A	Hospital B	Significance
Tumour size	Less than 4 cms More than 4cms	31 15	15 13	0.047 (X^2=6.11, df=1)
Dukes Stage	A B C1 C2	11 15 15 6	6 13 9 5	0.855 (X^2=0.778, df=1)
Nodal stage	N0 N1 N2	26 15 6	19 9 5	0.888 (X^2=0.237, df=1)
Perineural invasion	Absent Present	40 7	29 4	0.723 (X^2=0.345, df=1)
Vascular invasion	Absent Present	39 8	29 4	0.546 (X^2=0.365, df=1)
Tumour differentiation	Well Moderate Poor	2 41 4	12 17 4	0.001 (X^2=5086, df=1)

4.2.9 Summary and conclusions

The rectal cancer patients included in the study for the purpose of immunocytochemistry have exhibited the following clino-pathological attributes.

- A large proportion of the tumours (72.5%) were advanced at presentation included T3 and T4 cancers;
- Only 6.5% cancers were of T1 stage;
- The majority of the cancers were moderately differentiated 72.5%;
- There was no significant difference in the type of operation between the two centres, nor between age and type of operation, sex and the type of operation. This suggests uniform case mix;
- Lymph node invasion was significantly associated with decreased survival p= 0.001 and increased recurrence of cancer (p= 0.07);
- Perineural invasion was significantly associated with decreased survival and increased tumour recurrence (p=0.005);
- Vascular invasion was significantly associated with decreased survival and increased tumour recurrence (p=0.013);

- Mesorectal deposits were not significantly associated with increased recurrence (p= 0.288) or decreased survival (p= 0.140);

- Overall survival at 2 years was 70%;
- Overall recurrence at 2 years was 35%;
- Systemic recurrence was 31.25% and local recurrence was 11.25%.

Having considered the representativeness of the sample compared to the Welsh rectal cancer data in Section one and its clinico-pathological attributes in section two, section three of the results chapter concentrates on the two different detection methods of micrometastases and isolated tumour cells. These two techniques will be compared to assess the effectiveness of the new method. Furthermore, the relationship of micrometastases and isolated tumour cells to the clinico-pathological parameters including the crucial question of their relevance to recurrent disease and survival will be analysed.

RESULTS-SECTION 3

4.3.1 Introduction

In this section, the two different staining methods for the detection of micrometastatic disease will be compared to examine their efficacy. Furthermore, micrometastatic disease detected by the Immunocytochemical method will be correlated with patient and tumour factors to assess their association. In particular, the association between disease recurrence and survival and the presence of micrometastatic disease at the circumferential margin and the mesorectum will be considered and analysed.

This section concentrates on the impact of the new staining method on the detection rate of micrometastases and isolated tumour cells at the CRM and in the mesorectum. It examines the likely effects of the MM and ITC detected through the Immunocytochemistry on local and systemic recurrence as well as survival.

4.3.2 Comparison between Immunocytochemistry and Haematoxylin and Eosin

Immunocytochemistry seems to be significantly more efficient in identifying isolated tumour cells in the mesorectum than standard Haemotoxylin and Eosin staining 36.25% vs. 26.25%, p=0.05. However, the improvement in the detection of micrometastases at the CRM with Immunocytochemistry was only marginal and insignificant 7.5% vs. 3.75 (p=0.16) (Table 4.3.1).

Table 4.3.1 Number of cases (out of 80) with isolated tumour cells and micrometastases found at the circumferential margin and in the mesorectum on haematoxylin and Eosin staining and Immunocytochemistry

Entity	Site	Haemotoxylin and Eosin staining method	Immunocyto-chemistry method[a]	Significance Chi-Squared goodness of fit test
ITC	Mesorectum	16(20)	29(36.25)	0.05*
	CRM	2(2.5)	6(7.5)	0.16
MM	Mesorectum	5(6.25)	6(7.5)	0.76
	CRM	3(3.75)	6(7.5)	0.31

[a]Includes both the types of the antibodies·
Figures in parenthesis are percentages.

In the assessment of micrometastatic disease by Immunocytochemistry and by the Haematoxylin and Eosin method, slides from all three levels of microsections were considered for analysis. In the case of immunocytochemistry both antibodies were considered for the purpose of analysis. On the similar principle, sections from all levels were included in the assessment of MM and ITC detected by Haemotoxylin and Eosin method.

It has been suggested that the Immunocytochemistry is superior to Haemotoxylin and Eosin staining in the detection of micrometastatic disease in the lymph nodes (Isaka et al. 1999). In this study the significance of its superiority was evident only in the detection of isolated tumour cells in the

mesorectum (p=0.05). A non-significant improvement in the detection of micrometastases and isolated tumour cells was also noted at the CRM. Micrometastases and ITC were demonstrated on Immunocytochemistry both at the CRM and in the mesorectum as shown in the figures 4.3.1 to 4.3.5.
If all the ITC were considered together in relation to the detection method in question, there was significant improvement in the detection with ICH than H&E (p= 0.0003).

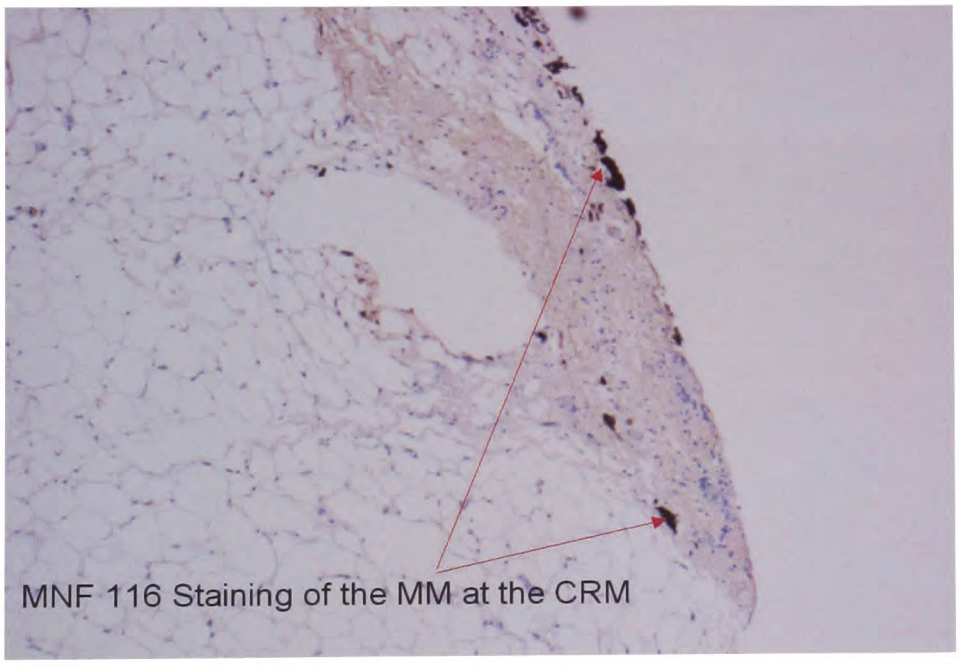


Figure 4.3.1 MNF 116 Immunocytochemistry of the rectal cancer circumferential margin demonstrating MM.

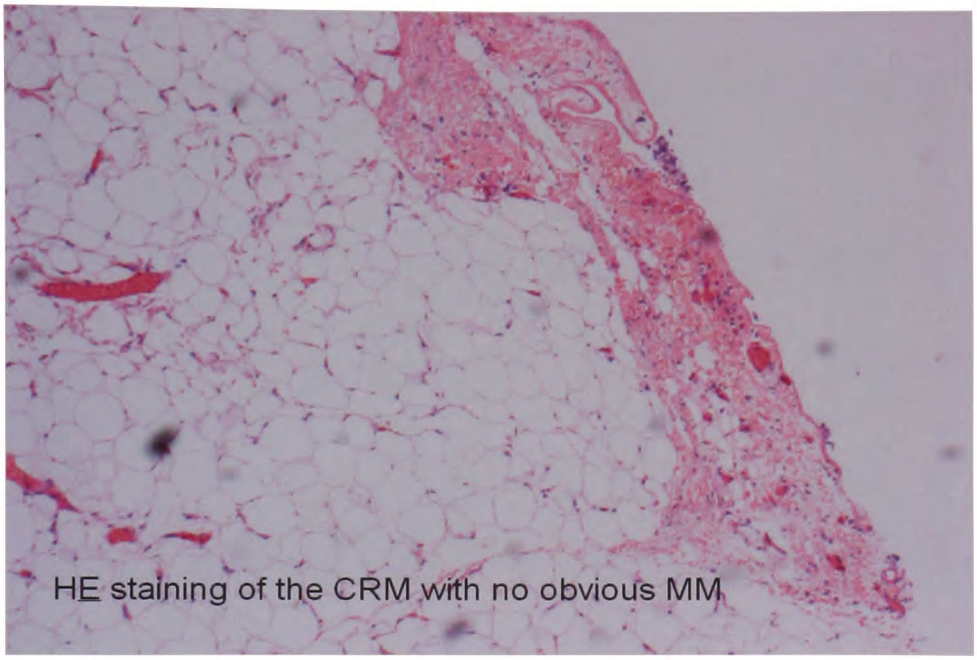


Figure 4.3.2 H&E staining of the CRM with no obvious evidence of micrometastases. However, ICH with MNF 116 demonstrated micrometastases as shown in the figure 4.3.1.

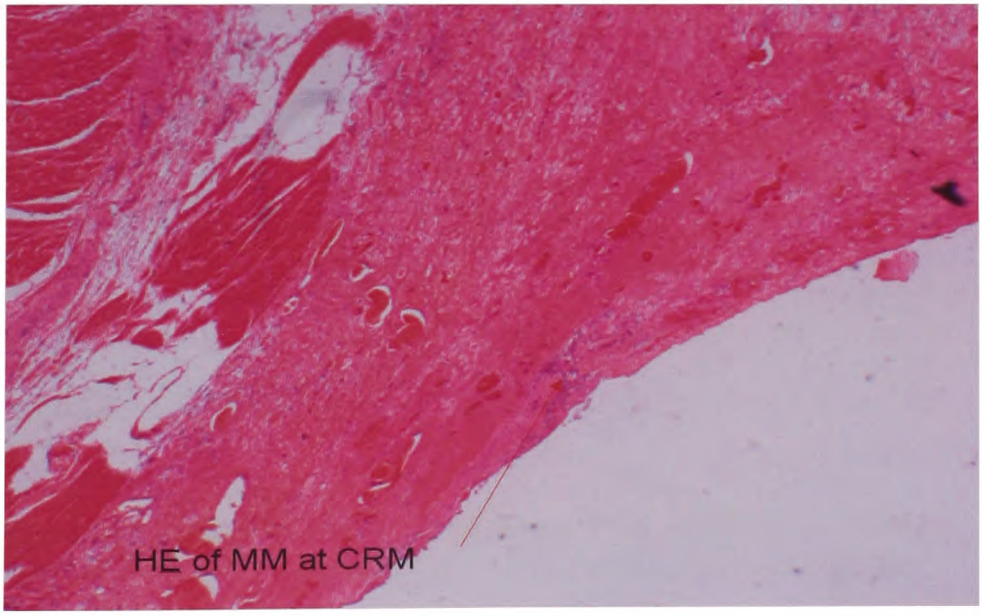


Figure 4.3.3 H&E staining of the circumferential margin showing (arrowed) suspected area of malignant cells.

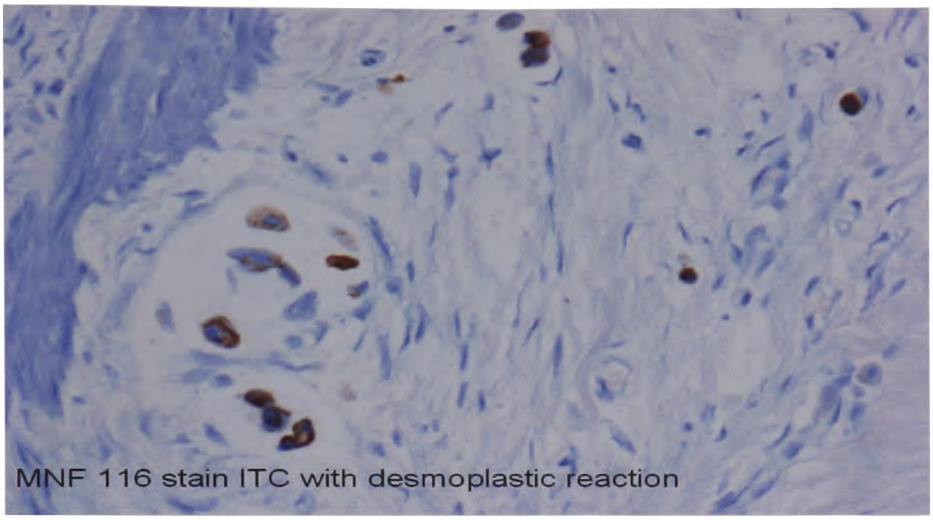


Figure 4.3.4 MNF 116 Immunocytochemistry demonstrating ITC with desmoplastic reaction.

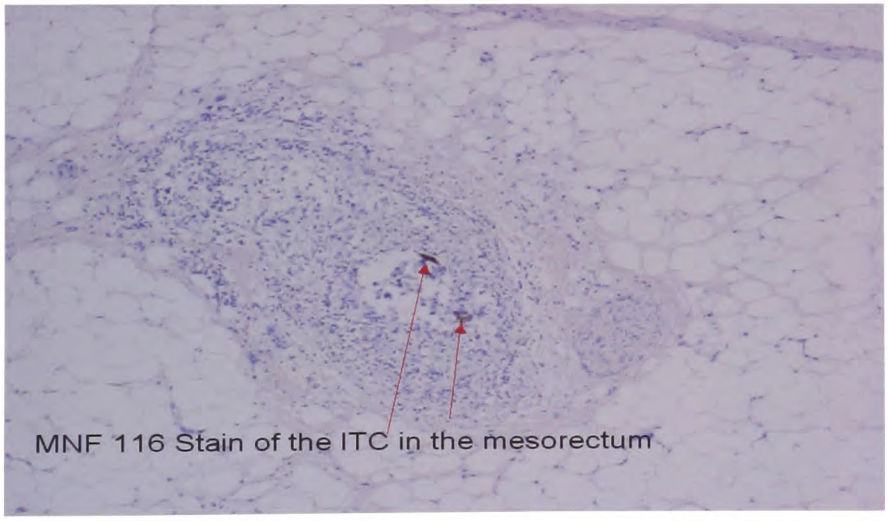


Figure 4.3.5 MNF Immunocytochemistry demonstrating ITC in the mesorectum with no evidence of tumour stain in the near vicinity.

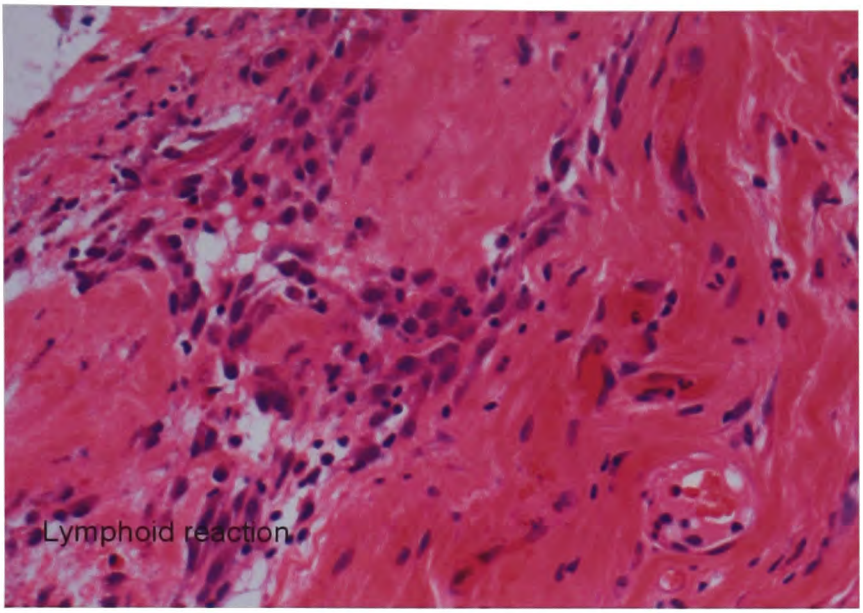


Figure 4.3.6 H&E staining showing an area of lymphoid reaction, which was shown to be an area with ITC on ICH.

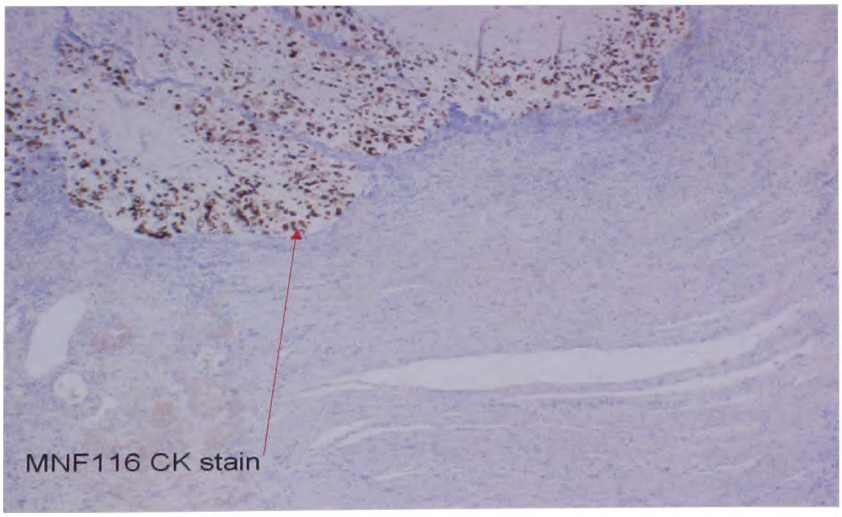


Figure 4.3.7 MNF Immunocytochemistry demonstrating positive cytokeratin of the tumour-advancing margin.

Having demonstrated the superiority of the immunocytochemistry in the detection of both MM and ITC at the CRM and in the mesorectum, it would be appropriate to further appreciate the micrometastatic disease. The following data is pertinent to the further understanding of the relationship between the ITC and MM in the mesorectum and patient related factors. The data in the following tables 4.3.2 to 4.3.4 feature the association of the isolated tumour cells with patient related factors. Most of the tests of association carried out in this section have sample size limitations, therefore, the findings need to be taken with caution.

4.3.2 Association between isolated tumour cells in the mesorectum and age

Age	Haematoxylin and Eosin staining	Immunocytochemistry
<60 Years (n=26)	6	8
≥60 Years (n=54)	10	21
Significance (Chi-Square goodness of fit test)		p=0.722 (X^2=0.126, df=1)

Table 4.3.3 Association between isolated tumour cells in the mesorectum and gender

Gender	Haematoxylin and Eosin staining	Immunocytochemistry
Male (n=55)	13	22
Females (n=25)	3	7
Significance (Chi-Square goodness of fit test)		P= 0.301 X^2=1.071, df=1

Table 4.3.4 Association between isolated tumour cells in the mesorectum and hospital

Hospital	Haematoxylin and Eosin staining	Immunocytochemistry
Hospital A (n=47)	6	10
Hospital B (n=33)	10	19
Significance (Chi-Square goodness of fit test)		P=0.001 X^2=11.054, df=1

Table 4.3.5 Association between isolated tumour cells at the circumferential margin and patient related factors

Patient factor	Feature	Cases with ITC found at the CRM	Significance
Age	< 60years=26	1	
	> 60years=54	5	0.38
Gender	Males=55	4	
	Females=25	2	0.90
Hospital	Hospital A=47	3	
	Hospital B=33	3	0.65

Table 4.3.6 Association between micrometastases at the circumferential margin and patient related factors

Patient factor	Feature	Number	Cases with Micrometastases found at the CRM	Significance
Age	< 60years	26	1	0.38
	> 60years	54	5	
Gender	Males	55	4	0.90
	Females	25	2	
Hospital	Hospital A	47	4	0.65
	Hospital B	33	2	

From table 4.3.6, it appears that there was no statistically significant association between patient related factors such as age and gender and micrometastatic

disease at the circumferential resection margin. However, isolated tumour cells in the mesorectum appear to differ significantly between hospitals A and B (Table 4.3.4a), although the cases selected between hospitals were similar in terms of clinicopathological features (Table 4.3.4b), the significant differences in tumour differentiation and tumour sizes may explain the significant variation in the ITCs found in the mesorectum of cases between two hospitals. Having considered the micrometastatic disease at the circumferential margin, in the following tables (tables 4.3.7 and 4.3.8), the association of micrometastatic disease in the mesorectum will be considered.

Table 4.3.7 Association between micrometastases in the mesorectum and patient related factors

Patient factor	Feature	Micrometastases at the mesorectum	Significance
Age	< 60years=26	1	
	> 60years=54	5	0.38
Gender	Males=55	3	
	Females=25	3	0.30
Hospital	Hospital A=47	3	
	Hospital B=33	3	0.65

Table 4.3.8 Number of cases with isolated tumour cells found in relation to the various pathological factors

Pathological factor	Pathological feature	ITC at the CRM	Significance
Tumour size	< 4cms=46	1	
	>4 cms=28	5	
	Missing values [a] =6		0.035 [a]
Vascular invasion	Present=12	1	
	Absent=68	5	0.90
Perineural invasion	Present	1	
	Absent	5	0.82
Tumour Differentiation	Well differentiation=14	1	
	Moderate differentiation=58	3	
	Poor differentiation=8	2	0.13
Lymph node Involvement	Present=35	3	
	Absent=45	3	0.74
Mesorectal Deposits	Present=10	1	
	Absent=70	5	074
Tumour Stage	T1=6	0	
	T2=16	0	
	T3=53	6	
	T4=5	0	0.34
Node stage	N0=45	3	
	N1=24	1	
	N2=11	2	0.32
Dukes Stage	A=17	0	
	B=28	3	
	C1=24	1	
	C2=11	2	0.26

[a] Chi squared test of association

Chi-Squared test of association between tumour factors and the presence of isolated tumour cells at the circumferential margin revealed significant association between tumour size and presence of ITC at the CRM when the

tumour is more than 4 centimetres in size p=0.035. However, there was no significant association with any of the pathological tumour factors considered in the table 4.3.8. One of the most striking finding was that there was no association of ITC with mesorectal deposits, which one would expect to be associated. This may be due to several reasons including incomplete reporting on the mesorectal deposits along with small sample size. However, due to the smaller sample size the test of association may be unreliable.

From table 4.3.8, it appears that the presence of micrometastases at the circumferential margin is independent of several tumour pathological factors including Dukes staging. However, micrometastases at the CRM were significantly associated with moderately differentiated tumours p=0.09 compared with tumours with well or poor differentiation. One would expect significant association of micrometastastic disease with poorly differentiated tumours compared with well or moderately differentiated tumours. This may be due to the biologically aggressive behavior of poorly differentiated tumours. This may be explained by the larger number of cases with moderately differentiated tumours compared with poorly differentiated tumours. Again the sample size of the study is small (type II error).

Table 4.3.9 Number of cases with micrometastases at the CRM found in relation to the various pathological factors

Pathological factor	Pathological feature	Micrometastases at the Circumferential margin	Significance
Tumour size	< 4cms=46	2	
	>4 cms=28	4	
	Missing=6	0	0.22
Vascular invasion	Present=12	1	
	Absent=68	5	0.90
Perineural invasion	Present=11	2	
	Absent=69	4	0.14
Tumour Differentiation	Well differentiation=14	0	
	Moderate differentiation=58	4	
	Poor differentiation=8	2	0.09[a]
Lymph node Involvement	Present=35	3	
	Absent=45	3	0.74
Mesorectal Deposits	Present=10	1	0.74
	Absent=70	5	
Tumour Staging	T1=6	0	
	T2=16	0	
	T3=53	6	
	T4=5	0	0.34
Node staging	N0=45	3	
	N1=24	2	
	N2=11	1	0.94
Dukes Stage	A=17	0	
	B=28	3	
	C1=24	2	
	C2=11	1	0.60

[a]Missing values.

It is important to consider that pathological reporting of the tumour differentiation, statistical misinterpretation is possible.

Table 4.3.10 Number of cases with isolated tumour cells in the mesorectum and tumour related factors

Pathological factor	Pathological feature	Isolated tumour cells detected in the mesorectum with		Significance
		H&E	ICH	
Tumour size	< 4cms=46 >4 cms=28 Missing=6	9 7 0	13 12 4	0.12
Vascular invasion	Present=12 Absent=68	3 13	3 26	0.37
Perineural invasion	Present=11 Absent=69	3 13	4 25	0.99
Tumour Differentiation	Well differentiation=14 Moderate differentiation=58 Poor differentiation=8	4 8 4	7 18 4	0.28
Lymph node Involvement	Present=35 Absent=45	6 10	12 17	0.74
Mesorectal Deposits	Present=10 Absent=70	3 16	3 26	0.66
Tumour Staging	T1=6 T2=16 T3=53 T4=5	1 5 9 1	3 7 18 1	0.66
Nodal staging	N0=45 N1=24 N2=11	10 4 2	17 8 4	0.93
Dukes Stage	A=17 B=28 C1=24 C2=11	4 6 4 2	7 10 8 4	0.96

Chi X^2 test of association between tumour related factors and isolated tumour cells in the mesorectum revealed that there was no association between these

factors. It is therefore, likely that the presence of isolated tumour cells was independent of any of the tumour factors considered in the table 4.3.10.

It is clear that immunocytochemistry consistently was able to detect more cases with isolated tumour cells than conventional Haematoxylin and Eosin staining.

Table 4.3.11 Number of cases with micrometastases in the mesorectum and tumour related factors

Pathological factor	Pathological feature	Micrometastases in the mesorectum	Significance
Tumour size	< 4cms=46	4	
	>4 cms=28	2	
	Missing =6	0	0.74
Vascular invasion	Present=12	4	
	Absent=68	2	0.000[a]
Perineural invasion	Present=11	2	
	Absent=69	4	0.14
Tumour Differentiation	Well differentiation=14	2	
	Moderate differentiation=58	3	
	Poor differentiation=8	1	0.43
Lymph node Involvement	Present=35	6	
	Absent=45	0	0.004[a]
Mesorectal Deposits	Present=10	4	
	Absent=70	2	0.10
Tumour Stage	T1=6	0	
	T2=16	0	
	T3=53	4	
	T4=5	2	0.02[a]
Nodal stage	N0=45	0	
	N1=24	2	
	N2=11	4	0.000[a]
Dukes Stage	A=17	0	
	B=28	0	
	C1=24	2	
	C2=11	4	0.001[a]

[a]Chi-Squared test

There was a significant association between micrometastases in the mesorectum and each of tumour stage ($p=0.02$), node stage ($p<0.001$), Dukes stage ($p=0.001$), node involvement ($p=0.004$) and vascular invasion ($p<0.001$). It must be emphasised that these tests of association may be unreliable due to small sample size.

Table 4.3.12 Systemic recurrence and micrometastases at the circumferential resection margin

Systemic recurrence[a]	MM at the CRM		Total
	Not present	Present	
Recurrence present	20	5	25
No recurrence	54	1	55
Total	74	6	80
Significance: p=0.001			

[a]Recurrence at all systemic sites included

Table 4.3.13 Systemic recurrence and isolated tumour cells at the circumferential resection margin

Systemic recurrence[a]	ITC at the CRM		Total
	Not present	Present	
Recurrence present	20	5	25
No recurrence	54	1	55
Total	74	6	80
Significance: p=0.001			

[a]Recurrence at all sites included

From the above tables (4.3.12 and 4.3.13), it appears that there was a significant association between the systemic recurrence of the disease and the presence of both micrometastases and isolated tumour cells at the circumferential resection margin. Whether this was related due to micrometastatic disease or due to combination of other known prognostic factors such as lymph node involvement, extra mural vascular invasion and/or tumour stage will be considered by using binary logistic regression. Similarly, systemic recurrence was significantly associated with micrometastases (p= 0.005), but not isolated tumour cells in the mesorectum (small sample size limits the significance).

Table 4.3.14 Systemic recurrence and micrometastases in the mesorectum

Systemic recurrence[a]	**MM in the mesorectum**		**Total**
	Not present	**Present**	
Systemic recurrence Present	21	4	25
No systemic recurrence	53	2	55
Total	74	6	80
Significance: $X^2_{(1)}=3.787$, p=0.05.			

[a]Recurrence at all sites included

Table 4.3.15 Systemic recurrence and isolated tumour cells in the mesorectum

Systemic recurrence[a]	No ITC in the mesorectum	ITC in the mesorectum	Total
Systemic recurrence present	16	9	25
No systemic recurrence	35	20	55
Total	51	29	80
Significance: $X^2_{(1)}=0.001$, p=0.975.			

[a]Recurrence at all sites included

When systemic recurrence in the liver was considered in relation to the micrometastatic disease at the CRM, there was statistically significant association p= 0.004, and p=0.004 with both MM and ITC respectively at the circumferential margin (table 4.3.16 and 4.3.17).

Table 4.3.16 Hepatic recurrence and Micrometastases in the mesorectum

Hepatic recurrence	No MM at the CRM	MM at the CRM	Total
Hepatic recurrence present	12	4	16
No hepatic recurrence	62	2	64
Total	74	6	80
Significance: $X^2_{(1)}=9.776$; p= 0.002			

Table 4.3.17 Hepatic recurrence and isolated tumour cells in the mesorectum

Hepatic recurrence	No ITC at the CRM	ITC at the CRM	Total
Hepatic recurrence present	13	3	16
No hepatic recurrence	62	2	64
Total	75	5	80
Significance: $X^2_{(1)}$=4.158; p=0.041.			

Local recurrence following rectal cancer surgery is a serious complication leading to intractable pain leading to poor quality of life. Following implementation of total mesorectal excision (TME) in most centres the local recurrence problem is generally on the decline (Wibe et al. 2002). There are several factors that lead on to the development of local recurrence such as locally advanced tumour, poor quality of the TME surgery and tumour rupture during surgery. However, inspite of good quality TME surgery and the use of selective pre-operative radiotherapy, the problem of local recurrence still exist. Whether this is related to the biological behavior of the rectal cancer or due to the presence of micrometastatic disease is yet to be resolved.

Table 4.3.18 Local recurrence and isolated tumour cells in the mesorectum

Local recurrence	No ITC in the mesorectum	ITC in the mesorectum	Total
Developed local recurrence	6	3	9
No local recurrence	45	26	71
Total	51	29	80
Significance: $X^2_{(1)}=0.037$; p= 0.847.			

Table 4.3.19 Local recurrence and micrometastases in the mesorectum

Local recurrence	No MM in the mesorectum	MM in the mesorectum present	Total
Developed local recurrence	8	1	9
No local recurrence	66	5	71
Total	74	6	80
Significance: $X^2_{(1)}=0.191$; p= 0.662.			

Table 4.3.19, considers the local recurrence and the presence of micrometastases in the mesorectum to assess their association. There was no statistically significant association between the micrometastatic disease in the mesorectum and the local recurrence. Furthermore, a similar observation was

noted from the table 4.3.20, that there was no statistically significant association between local recurrence and the presence of isolated tumour cells at the CRM. The numbers are small for comparison and hence, the results of the X^2 test of association may be unreliable.

Table 4.3.20 Local recurrence and isolated tumour cells at the circumferential margin

Local recurrence	No ITC at the CRM	ITC at the CRM present	Total
Developed recurrence	8	1	9
No local recurrence	66	5	71
Total	74	6	80
Significance: $X^2_{(1)}=0.191$; p=0.662.			

Table 4.3.21 Local recurrence and circumferential margin micrometastatic disease

Local recurrence	No MM at the CRM	MM present at the CRM present	Total
Developed recurrence	8	1	9
No local recurrence	66	5	71
Total	74	6	80
Significance: $X^2_{(1)}=0.191$; p=0.662.			

4.3.3 Patient survival

From tables 4.3.22 to 4.3.25, it appears that there was a significant association between the patient survival and micrometastatic disease both at the circumferential margin and in the mesorectum. The association between the presence of micrometastatic disease and survival only will be considered. A detailed survival curves analysis is beyond the scope of the current thesis and will be performed separately in the future.

Table 4.3.22 Number of patients survived at analysis and mesorectal isolated tumour cells

Patients survived at analysis	**No mesorectal ITC**	**Mesorectal ITC**	**Total**
Survived	36	20	56
Death from disease[a]	15	9	24
Total	51	29	80
Significance= $X^2_{(1)}$=0.435; p= 0.50			

[a]Deaths due to cancer recurrence

Deaths due to disease recurrence only were included. All other deaths due to some other cause were excluded at the outset.

Table 4.3.23 Number of patients with mesorectal micrometastases surviving at the time of analysis

Patients survived at analysis	No mesorectal MM	Mesorectal MM	Total
Survived	55	1	56
Death from disease[a]	19	5	24
Total	74	6	80
Significance= $X^2_{(1)}$=8.78, p=0.003*			

[a]Deaths due to cancer recurrence

Table 4.3.24 Number of patients surviving at the time of analysis having circumferential margin micrometastases

Patients survived at analysis	Circumferential MM absent	Circumferential MM present	Total
Survived	54	2	56
Death from disease[a]	20	4	24
Total	74	6	80
Significance= $X^2_{(1)}$=4.153, p=0.042*			

[a]Deaths due to cancer recurrence

Table 4.3.25 Number of patients surviving at the time of analysis and circumferential margin isolated tumour cells

Patients survived at analysis	Circumferential ITC absent	Circumferential ITC present	Total
Survived	55	1	56
Death from disease[a]	19	5	24
Total	74	6	80
Significance= $X^2_{(1)}=8.78$, p=0.003*			

[a]Deaths due to cancer recurrence

The MM at the CRM and in the mesorectum were found be significantly related to the recurrence calculated based on Binary Logistic regression p=0.032;

p= 0.071 respectively (overall recurrence). The calculated odds ratio of recurrence with MM at the CRM was 12.89 (CI= 1.413 to 117.7). Where as the odds ratio of recurrence with MM in the mesorectum were 5.158 (CI= 0.871 to 30.528 appendix M). One unit change in the MM at the CRM is predicted to correspond to 12.89 points change in recurrence.

The ITC at the CRM and MM in the mesorectum were found be significantly affecting the survival calculated using Binary logistic regression p=0.032; p= 0.071 respectively. The calculated odds ratio of death with ITC at the CRM

were 19.286 (CI=2.082 to 178.647). Where as the odds of death with MM in the mesorectum were 2.959 (CI= 2.082 to 178.647, appendix N).

4.3.4 Summary and conclusions of section three

- The Immunocytochemistry of the rectal cancers resulted in improved detection of both micrometastases and isolated tumour cells in the mesorectum and at the circumferential margin compared with Haematoxylin and Eosin staining;
- The improvement in the detection of isolated tumour cells in the mesorectum was statistically significant (p=0.05) compared with routine staining with Haematoxylin and Eosin staining. However, improvement in the detection of micrometastases in the mesorectum and at the CRM was not statistically significant (p= 0.22 and 0.31 respectively);
- Where micrometastases or ITC were detected using H&E, the immunocytochemistry did not miss the micrometastatic disease. This was noted at all levels of staining and at both CRM and in the mesorectum;
- Isolated tumour cells at the circumferential resection margin were independent of any of tumour related factors such as vascular and neural invasion, tumour differentiation and lymph node involvement;

- Isolated tumour cells were associated significantly with tumours greater than 4 centimeters in size;
- Micrometastases at the CRM were associated significantly with moderately differentiated tumours than tumours with other differentiation;
- Micrometastases at the CRM were significantly associated with Dukes stage C2;
- No significant association was found between micrometastases or isolated tumour cells at the circumferential resection margin with patient related factors such as age and gender;
- There was no significant association of micrometastases or isolated tumour cells in the mesorectum with patient related factors such as age and gender;
- Isolated tumour cells in the mesorectum have no significant association with any of the tumour related factors;
- Micrometastases in the mesorectum were significantly associated with vascular invasion, Tumour stage T3 and T4 disease, N1and N2 disease, and Dukes stages C1 and C2 stage;
- Micrometastases and isolated tumour cells at the circumferential resection margin were significantly associated with systemic recurrence;

- Mesorectal micrometastases were significantly associated with poor survival;
- Circumferential margin micrometastases and isolated tumour cells were significantly associated with poor survival;
- There was no significant association noted between circumferential margin isolated tumour cells and micrometastases and local recurrence;
- There was no significant association noted between isolated tumour cells and micrometastases in the mesorectum and local recurrence.

In the next chapter, the findings of the research are discussed in relation to the literature and implications are presented. Furthermore, the strengths and limitations of this study are explained.

DISCUSSION

5.1 Introduction

Colorectal cancer is one of the common gastrointestinal cancers in the UK with 34, 000 new cases diagnosed in the year 2003 (Cancer Research UK), and is the second commonest cause of cancer deaths in the UK. Although the incidence of colorectal cancers appears to be stable, there has been slow progress in improving treatment. Furthermore, the majority of colorectal cancers are advanced at the time of presentation resulting in only moderate overall resectability rates in the UK contributing to the poor survival (Mella et al. 1997).

5.2 Measures to improve outcome

In an effort to improve the survival in the UK, there have been improvements in several areas such as introduction of fast track referral criteria so that patients with high risk symptoms are seen within 2 weeks from the time of referral (ACPGBI guidelines 2002), standardisation of surgical techniques such as the routine practice of total mesorectal excision, the introduction of new generation of chemotherapeutic agents such Oxaliplatin and Irinotecan and resectional surgery for the metastatic disease.

In the UK, the National Institute of Clinical Excellence has recommended measures to improve early diagnosis and the establishment of efficient systems to refer patients to fast track clinics; and stressed the urgent need for

expansion of endoscopy services to improve access (NICE 2004). All these efforts may improve to detect cases at an early stage so that prognosis might be improved. As discussed earlier, ten-year local recurrence and survival rates for early rectal cancers were 17% and 74% for T1 tumours and 26% and 72% for T2 tumours respectively (Paty et al. 2002), suggesting that even in early stage cancers, the recurrence is a problem with associated cancer related mortality. Cancer recurrence in these early cases with complete surgical excision may be explained by the micrometastatic phenomenon, which is not routinely detected by standard staining methods. It is in these cases that any further research should be focused on to improve survival and prevent recurrence.

5.3 Research progress to understand recurrence

In the field of colorectal cancer in general, several other improvements have taken place in the laboratory investigations to improve the diagnosis and metastasis detection. These methods include immunocytochemistry, RT-PCR methods, molecular diagnostics to detect p53 identification and mutation identification in colorectal cancers helped to improve the identification of and characterization of micrometastases.

5.4 Research on micrometastases

There has been considerable research into the micrometastases in colorectal cancer to establish their relation to local recurrence and survival.

Micrometastases were shown to be present in the draining regional lymph nodes (Davidson et al. 1990, Cutait et al. 1991, Adell et al. 1996 Broll et al. 1997), bone marrow (Schlimock et al. 1990, Lindemann et al. 1992, Leinung et al. 2000) and peripheral circulation (Leather et al. 1993, Wong et al. 1997). Several methods of detection have been employed to optimize the detection rate of the metastases (Hayashi et al. 1995, Sasaki et al. 1997, Leifers et al. 1998, Sanchez-Cespedes et al. 1999, Funaki et al. 2000, Rosenberg et al. 2002).

There have been problems with the technical methods and interpretation of the published literature leading to considerable ambiguity on the relative significance of micrometastases in both local recurrence and decreased survival. It has been recommended that the isolated tumour cells and micrometastases should be considered as two separate entities (Hermanek et al. 1999). Surprisingly the published literature on micrometastases has not followed a uniform terminology, rendering the analysis of published literature difficult and less meaningful. To understand the significance of micrometastases and isolated tumour cells, IUAC recommends a systematic approach to the terminology and to consider isolated metastases and micrometastases as distinct entities. (Hermanek et al. 1999). In this study, we followed the IUAC recommendations as highlighted in the table 2.3.

5.5.1 Local recurrence

The importance of positive circumferential margin involvement in rectal cancer has been well established with adverse effects on local recurrence rates and poor survival (Adam et al. 1994, Nagtegaal et al. 2002). In a study (Quirke et al. 1998), local recurrence rate was found to be 78% in patients with microscopic positive margin, while those with negative margin were 10%. Even in the margin negative patients, the local recurrence is a problem. This may be explained by the presence of micrometastatic disease not detected by routine staining methods. Involvement of circumferential margins independently influences both local recurrence and survival. Microscopic involvement of circumferential resection margin is a good predictor of local recurrence, especially when multiple sections are examined (Quirke et al. 1986).

5.5.2 Importance of circumferential margins

Positive circumferential margins are very well documented to be a strong predictor of not only local recurrence of rectal cancer, but also of systemic failure (Nagtegaal et al. 2002). The local recurrence rates of less than 10% are considered to be excellent in rectal cancer surgical outcomes (Abulafi et al. 1994). The local recurrence rate depends on several factors such as tumour related and surgeon related factors. Improperly performed total mesorectal excision was shown to be associated with higher local recurrence than a

specialist performed total mesorectal excision. Locally advanced rectal tumour into the mesorectum with or without lymph node involvement, thus has more chance of local recurrence than an early rectal cancer. Even in specialist centres, there is a degree of local recurrence in pathologically early stage cancers such as Dukes A and B (Heald et al. 1998). In larger trials such as the Dutch rectal cancer trial (Kapiteijn et al. 2001), local recurrences have been reported in early stage tumours. From these studies, it can be understood that local recurrence is a challenging issue even in early rectal cancers significantly affecting the quality of life after rectal cancer. Furthermore, studies have shown that the development of local recurrence predicts the systemic failure significantly leading to decreased survival (Nagtegaal et al. 2002).

It would be interesting to know the reasons for local recurrence in early cancers. Considering the evidence presented in the above discussion, it can be hypothesized that even in early rectal cancers, micrometastatic disease process exist to cause subsequent local failure. This may be a plausible explanation for the local recurrence in early rectal cancers providing the surgeon and tumour related factors do not differ significantly. The central theme of this study was to investigate this hypothesis.

Immunocytochemistry was investigated as a detection method to evaluate the MM and ITC at the circumferential margins and in the mesorectum. Further, we have systematically employed the IUAC classification to categorise the microscopic disease as MM and ITC.

Improved staining and detection methods such as immunocytochemistry and RT-PCR methods have not been applied in the detection of micrometastases in rectal cancers at the circumferential resection margins. However, in head neck cancers, the resection margins were assessed for micrometastases using RT-PCR method and it has been shown that a positive margin with cancer detected with more sensitive technique such as RT-PCR was significantly associated with subsequent local recurrence (Brennan et al. 1995).

5.6 The main findings of this study

The main aim of this thesis was to apply a more sensitive method of cancer cell detection such as immunocytochemistry to detect micrometastases at the circumferential margins and in the mesorectum of rectal cancers and to examine if the micrometastases had any impact on local recurrence and survival. This chapter will highlight and discuss the main findings that are observed and achieved through the data examination and analysis of the qualitative and quantitative information.

The objectives were achieved by ensuring that the sample representativeness of the selected rectal cancers compared with the wider population. In the present study, the sample was compared with rectal cancer data from the Welsh cancer intelligence and surveillance unit for the year 2002. As highlighted in section one of the results chapter, the sample was comparable to the larger population data such as Welsh rectal cancer data for the year 2002. There was no statistically significant difference in the mean ages between the sample and Welsh data between the males and females and among males and female proportions (X^2 goodness of fit p= 0.71). There was non-significant difference in the mean age between the sample and the Welsh data, this may be explained by the fact that the research project involved a selection process with robust inclusion and exclusion criteria (see table 2.1 and 2.2). This lead to the selection of younger patients who had total mesorectal excision as a form of therapeutic intervention. Consequently, the sample mean appears to be different from the Welsh data. The sample in the research process was therefore appropriate and does represent the larger population of Welsh rectal cancer data. Furthermore, the sample was derived from same geographic region.

The hypothesis was that rectal cancers have malignant cells at the circumferential resection margins and in the mesorectum that are not detected by routine histological staining; and that the Immunocytochemical method

can successfully detect these cells at these sites. The second hypothesis was that the malignant cells detected by immunocytochemistry at the circumferential margins or in the mesorectum influence the local recurrence rate and overall survival.

With the above aims and hypothesis, the thesis was set out to investigate basic clinico pathological information on rectal cancers that are subsequently investigated with Haemotoxylin and Eosin and immunocytochemistry with a view to identify micrometastases. Any differences in detection rate of micrometastases between the routine Haematoxylin and Eosin staining and Immunocytochemistry were investigated.

The main clinico-pathological findings of this study were presented in the summary and conclusions of the section two of the results chapter.

5.7 Incidence of micrometastases and isolated tumour cells

There was a definite presence of both micrometastases and isolated tumour cells in the mesorectum and at the circumferential margin detected both on Haematoxylin and Eosin staining and Immunocytochemistry (4.3.1 to 4.3.7).

Haematoxylin and Eosin staining

With Haematoxylin and Eosin staining, micrometastases were detected in 3 (3.75%) cases at the CRM and in 5 cases (6.25%) in the mesorectum. Although, isolated tumour cells were identified in only 2 (2.5%) cases at the CRM, with

routine staining, isolated tumour cells were detected in 16 (20%) cases in the mesorectum.

Immunocytochemistry

However, when Immunocytochemical method was applied, the micrometastases were detected in 7.5% of cases at both the CRM and in the mesorectum. Isolated tumour cells were found in 7.5% of cases at the CRM and more significantly so in 36.25% of cases in the mesorectum. Thus we were able to detect Isolated tumour cells significantly better than H&E only in mesorectum p=0.05. Furthermore, both ITC's and MM were not significantly associated with any specific age groups or gender. However, Isolated tumour cells in the Hospital B, were significantly found more frequently. This may be explained by the fact that the Hospital B had more cases with tumours larger than 4 cms.

Isolated tumour cells at CRM were significantly more frequent in tumours greater than 4 centimetres (p=0.035), however, these were not significantly associated with other pathological factors such as tumour stage, nodal stage, vascular and perineural invasion, tumour differentiation, lymph nodal involvement, and mesorectal nodes (table 4.3.8). In contrast to ITC, micrometastases in mesorectum were significantly associated with vascular invasion (p=0.00), lymph nodal involvement (p=0.004), nodal stage (p=0.000) and Dukes stage (p=0.001).

Specificity and sensitivity of ICH in the detection of ITC and MM

Immunocytochemistry is more sensitive than H&E in the detection of micrometastases. As there were no studies in literature on the ICH of the CRM of the rectal cancers to compare with this study results. Furthermore, the exact incidence of MM and ITC is dependent on the method of detection. Compared with H&E, ICH detected more cases with MM and ITC. Where the ICH was negative for both ITC and MM, the H&E method could not add any further information. In terms of specificity, although false positivity is possible with ICH, higher magnification has resolved this issue. Furthermore, The problem of non-specific staining due to Mesenchymal cells is not relevant in case of assessment involving mesorectum and CRM as Mesenchymal cells do not exist normally in these areas. The fact that desmoplastic reaction surrounding cancer cells was one of the criteria increases the specificity of the ICH.

5.8 Local and systemic recurrence and micrometastatic disease

Local recurrence of rectal cancers has been consistently shown to be positively associated with the involvement of circumferential margin. The closer the advancing tumour margin to the circumferential margin, the local recurrence increases proportionately. Currently the circumferential margins are considered positive if the tumour advancing margin or a tumour deposit lies within two mm of the margin (Nagtegaal et al. 2002).

In this study, we attempted to establish if any micrometastases or isolated tumour cells existed in the circumferential margin and in the mesorectum. We have not made attempts to assess the distance to the circumferential margin from the position of the micrometastases in the mesorectum. Although, isolated tumour cells and micrometastases were detected in an equal number of cases at the CRM, there was no significant association with local recurrence ($X^2_{(1)} = 0.191$; p=0.662, Isolated tumour cells, $X^2_{(1)} = 0.191$; p=0.662, micrometastases). Furthermore, there was no significant association between both ITC and MM in the mesorectum and local recurrence.

Reasons for no significant association of micrometastases with local recurrence

This observation suggests that not all patients who are positive for micrometastatic disease at the circumferential margins are likely to recur locally. Similar observations were noted even when circumferential margins are frankly involved on routine staining (Nagtegaal et al. 2002). This may be explained by the immunological factors, tumour growth factors for the survival of malignant cells and the genetic alterations that occur in the micrometastases and isolated tumour cells. Furthermore, the survival and further establishment of malignant cells in to significant clinical metastases depends on various factors such as tumour dormancy and expression of proliferating genes such as Ki-67.

Systemic recurrence

Systemic recurrence is a function of several tumour prognostic factors such as poor differentiation, vascular invasion, lymphatic invasion and the existence of lymph nodal involvement. Furthermore, patients with positive circumferential margins subsequently develop significant systemic relapse (Nagtegaal et al. 2002, Luna-Perez et al. 2005). Systemic recurrence is commonly seen in the liver and lungs, although other sites such as bones and adrenal glands have been reported.

In this study, systemic recurrence was associated with presence of MM and ITC at the circumferential margin. However, only MM in the mesorectum was associated with significant systemic recurrence but not the ITC in the mesorectum (tables 4.3.12 to 4.3.14). Whether this is related to the significantly enhanced metastatic growth potential of the micrometastases than isolated tumour cells or other variables influencing the systemic recurrence together with isolated tumour cells remains inexplicable.

All other main findings related to patient demographics, local recurrence and systemic recurrence were presented in the results section of the thesis.

5.9 Implications of this study

5.9.1 Adjuvant therapy

Current treatment options following total mesorectal excision depend on the high-risk histological features such as lympho-vascular invasion, the involvement of surgical resection margins and incomplete excision. Post-operative radiotherapy has the advantage that its use may be guided by the histological findings, but it is generally reported to have greater toxicity. In the Uppsala study (Frykholm et al. 1993), more patients had symptoms or signs of morbidity at 5 years after post-operative radiotherapy than pre-operative radiotherapy. Further, a policy of postoperative radiotherapy application based on the histological findings is more cost effective. Chemotherapy may be offered to patients with positive circumferential surgical resection margins. Currently as with colon cancer, adjuvant therapy for rectal cancer is based on the host of high-risk histological factors such as lympho-vascular invasion, poor differentiation and lymph node involvement. Although there have been many studies elucidating the presence of micrometastases, there are currently no adjuvant therapy trials based on the micrometastases data.

Interestingly, in a recent study on in stage II distal rectal cancers, there were significantly less micrometastases in the lymph nodes in the radiotherapy group compared to the group without any radiotherapy. Similarly, patients with rectal cancer treated by preoperative chemoradiation showed a

surprisingly low rate of micrometastasis detection (7%), even in high-risk patients such as T3 and T4 tumors (Perez et al. 2005).

Specifically, in Dukes A and B cancers with a possible local recurrence who are positive for MM/ITC could potentially be offered adjuvant therapy to prevent recurrence. As with other high risk factors, presence of micrometastases may be considered as one of the indications for adjuvant therapy. However, to date there is no evidence of the application of adjuvant therapy based on micrometastatic detection. Current adjuvant therapeutic regimes are based on robust information obtained from randomized trials. Until a randomized trial is carried out, it is unlikely that patients would be offered adjuvant therapy based on the findings of any micrometastases research. Furthermore, recently there have been developments in the understanding of biological behavior of micrometastases. This may help to develop targeted tumour biological therapy, as conventional treatment is known to be ineffective on dormant micrometastases.

5.9.2 Staging implications

Current TNM staging method does not account micrometastases in the clinical staging of rectal cancers. However, micrometastases may help to stratify patients into various risk groups with varying recurrence and the adjuvant therapy needs. Furthermore, this improves the staging of the rectal cancers (see table 3.1).

5.9.3 Therapeutic response monitoring

Monitoring of micrometastases burden during and after chemotherapy may be an effective method of evaluating the therapeutic efficiency of the adjuvant treatment. This is potentially possible to apply in clinical situations to monitor the tumour burden in the bone marrow and peripheral circulation. However, this may not be applicable to monitor micrometastases in rectal cancer, as their detection is only possible after resectional surgery.

5.10 Strengths of this study

This is a unique study, which was attempted to detect micrometastases using ICH as a detection method. Although, detection of metastases using RT-PCR method was employed in head and neck cancer resection margins, there is no literature on the assessment of micrometastatic disease in rectal cancers. Furthermore, it would be inappropriate to compare with the results of ICH detection methods in other areas of the rectal cancer spread such as lymph nodes as limiting factor in lymph nodal MM or ITC detection is its size while the circumferential margin extends along the entire length of the rectal cancer

specimen. Examination of the pathology slides by an experienced pathologist avoided any bias in our study. In fact, this is one of the main strengths of this study. More importantly, the rigorous selection of cases employing a strict inclusion and exclusion criteria increases the robustness of this study.

5.11 Problems and limitations of this study

This study was conducted on a small sample of rectal cancers. Although the sample did represent the wider Welsh population in terms of age and gender, there are wider issues that need to be considered so that the findings of this study could be generalized to a wider population.

5.11.1 Sample size

The current sample size was restricted mainly because of the available resources and availability of the suitable cases. Emphasis was laid on the strict inclusion and exclusion criteria. Consequently, with such rigorous criteria, the cases suitable for evaluating micrometastatic disease will be limited. Perhaps a similar study needs to be repeated on a larger sample size in order to evaluate the true incidence of the micrometastastic disease.

5.11.2 Technique based issues

Although the Immunocytochemistry has a higher sensitivity than Haemotoxylin and Eosin, studies based on the comparison between ICH and RT-PCR suggest that ICH method is less sensitive and less specific than RT-PCR and genetic based methods such as in mutation detection in oncogenes.

However, in this study ICH was chosen as a reasonable compromise between the standard method and RT-PCR due to the limited availability.

5.11.3 Rectal cancer specimen

This study was performed on rectal cancer paraffin blocked specimens. Thus, only a part of the tumour specimen was involved in the examination process looking for micrometastastic disease. There was a study to suggest the entire rectal cancer specimen may have to be subjected to properly assess the circumferential margin. This approach increases the true incidence rate of CRM involvement (Quirke et al. 1989).

5.11.4 Retrospective study

Immunocytochemistry was performed on formalin fixed rectal cancer specimens. It is well known that the formalin fixation has an astringent effect, which can destroy cytokeratin epitopes. Thus in retrospectively performed studies, as is the case in many of the reported studies in the literature, immunocytochemistry may underestimate the exact incidence of micrometastases. All other limitations of the ICH staining method may impair the detection rate.

Furthermore, the data on recurrence was collected retrospectively. Only symptomatic patients were investigated for local and systemic recurrence. It is well established that both local and systemic recurrences can be asymptomatic. Furthermore, application of sensitive investigations such as

endorectal ultrasound scan can detect asymptomatic recurrences early on (Beynon et al. 1990). Similarly, the systemic recurrences could be detected at a much earlier stage if investigations such as CT scan and FDG-PET scan were applied even in the asymptomatic stage.

5.11.5 Follow-up limitation

In this study, patients who were followed up to at least 24 months following total mesorectal excision were included. Those patients who were followed for longer periods were assessed further to examine the survival. The majority of the recurrences were reported within 2 years from the surgical intervention (Kapiteijn et al. 2001). More than 90 % of the recurrences have been reported within 5 years (Nagtegaal et al. 2002). Thus ideally, a follow-up of at least 5 years would be appropriate.

The recurrences found in this study were based on investigations when the patients became symptomatic during the follow-up period. However, there is evidence to suggest that the rectal cancer recurrences can be asymptomatic. Further, by routine use of endorectal ultrasound scan, the local recurrence may be detected at a higher rate and at an earlier stage because of the improved sensitivity of the ultrasound examination images (Beynon 1990, Lohnert et al. 2000, Manfredi et al. 2001, de Anda et al. 2004). Similarly, the systemic recurrence was investigated only when patients became symptomatic. With recent advances in the detection of recurrent cancer using Fluoro-Deoxy- Glucose based Positron emission scan, the recurrent cancer can

be detected at an early stage when the patients are asymptomatic. By applying conventional investigations such as ultrasound scan in symptomatic patients it is likely that the true incidence of systemic recurrence may be underestimated. Thus, there is measurement bias in the detection of local and systemic recurrence in this study. Measurement bias needs to be kept in mind in the interpretation of results. However, the methods of detection of recurrence applied in our study are currently acceptable clinical standards.

5.11.6 Follow-up variation

Between hospital A and B, there was a wide variation in the follow-up practices. In hospital B, the patients were followed up intensively especially younger patients who were more likely to be suitable for liver resection should they develop metastases. On the other hand, in hospital A, where three different surgeons were involved in the management of rectal cancers, there was no standard follow-up protocol among surgeons. This may have implication in the metastases detection and detection of local recurrence.

5.11.7 Highly selected group of patients

The research was carried out on selected cases of rectal cancer patients who had total mesorectal excision as the main treatment. Further, we have excluded (table 2.2) patients with recto-sigmoid and upper rectal cancers in view of the fact that there is inadequate mesorectum at that level of the

rectum. Therefore, this study was performed mainly on mid rectal and low rectal cancers. How the results of this study can be applicable to upper rectal tumours remains debatable. Furthermore, a selection bias must be considered in the interpretation of results.

5.11.8 Validation of pathological data

In this study, an experienced pathologist affirmed the pathological data. The pathological data is robust as the pathologist reviewed slides twice to reconfirm the positive slides. Review of pathology slides by two pathologists and came to an agreement on the presence of micrometastases further strengthens the study. However, in view of lack of resources, in this study, only one pathologist reviewed the pathology slides.

5.11.9 Limitations from the lack of data

Inadequate pathological data on the involvement of circumferential margins, the number of lymph nodes retrieved and the number of positive nodes compromises the analysis of data. In this study, the pathological data was available in the majority of the cases. However, in Hospital B, the circumferential margin information was not available in 10 patients (12.5%). In addition, more than two surgeons carried out the total mesorectal excision.

5.12 Suggestions

Considering the limitations under which this study was carried out, different results in the incidence of micrometastases and isolated tumour cells may emerge if this study was repeated keeping the limitations of this to a

minimum. Here are some of the suggestions that may have to be considered prior to re-starting any research project concerning micrometastases. Subjecting the entire fresh rectal cancer specimen for ICH prospectively on more cases avoids the problem of epitope destruction and sample size limitations. Further, the pathology slides may have to be examined by two pathologists to validate the results. Micro-dissection techniques as utilized in a study to assess the micrometastatic disease in head and neck tumours using RT-PCR may be considered to avoid the confounding factor of tumour cell contamination and improved sensitivity.

The methods to detect recurrence have improved recently. This may involve utilisation of sensitive recurrence detection methods such as FDG-PET scan. Furthermore, routine endo-rectal ultrasound has been shown to be superior in the detection of local recurrence than routine clinical examination, which is quite insensitive (Lohnert et al. 2000, Manfredi et al. 2001, de Anda et al. 2004). Perhaps these sensitive methods should be incorporated to detect the local recurrence early. This hopefully avoids measurement bias on "asymptomatic" local and systemic recurrences.

CONCLUSIONS

Conclusions

Rectal cancer is one of the commonest gastrointestinal cancers affecting the population of the Western countries. The current standard treatment for an operable carcinoma of the rectum is total mesorectal excision (TME). There has been continuing research on colorectal caners leading to innovation in diagnosis and treatment leading to overall improvement of cancer outcomes such as reduced recurrence and improved survival. There is a definite improvement in the results over the years due to improvements in the early diagnosis, treatment and standardisation of surgical procedures. However, there is no doubt that continuing research on early diagnosis and treatment is likely to result in the detection of cancers at an early stage with further improvement in survival rates.

In the review of literature, it was clear that the research on MM and ITC has been topical and there is a continued pursuit for research on the micrometastases to understand the ways of improving the prognostic factors that affect the recurrence of rectal cancers. More crucially, the compelling reason for continuing research on MM is to ultimately improve survival. Despite many studies showing existence of MM and ITC, it can be concluded that on the basis of the literature, at present the interpretation of data on micrometastasis research is complex, and more crucially, does not influence the decision making for adjuvant treatment at this stage. The basis for this conclusion is that the interpretation of the results is difficult due to a lack of standardisation of

terminology until recently. Even after the IUAC's publication (Harmanek et al. 1999) subsequent publications continue to follow random terminology and there has been no uniformity in the classification of the micrometastatic disease. This is further compounded by the fact that the definition of micrometastatic disease is morphological. The results of more sensitive methods of detection of micrometastases such as RT-PCR are not based on the morphological identification of the malignant cells. However, a different classification for micrometastatic disease based on the molecular methods has been proposed (Table 3.1). Therefore, the extensive research carried out so far on micrometastatic disease has not been translated into the clinical treatment of cancer patients at this stage.

In this study, we have made an attempt to assess if MM and ITC exist at the CRM and in the mesorectum using a simple, yet sensitive method of micrometastasis detection method i.e the immunocytochemical method, which is available in most district general hospitals without much pressure on the National Health Service (NHS) resources.

However, there are other complex and expensive methods of micrometastasis detection such as RT-PCR, P53, and K-RAS for example, which are available with a higher detection rate. These sensitive methods may not be available in most centres of the NHS and suffer from a very high false positivity. It would be reasonable therefore to examine the relevant problem of micrometastases with a

simpler method of investigation such as Immunocytochemistry against tumour expressed proteins or the epithelial derived proteins such as cytokeratins.

In this study, we chose to employ monoclonal antibodies against cytokeratins. The authority of the current study is underpinned by the uniqueness of this study, as there have been no other studies in the literature on the assessment of micrometastases or isolated tumour cells at the circumferential resection margin.

The application of a simple staining method to detect the micrometastases more effectively than standard haematoxylin and eosin staining method seems to be more appropriate and reasonable in the context of National Health Service (NHS) setting. The staining process we utilised was automated; consequently it has eliminated human error. A single qualified pathologist examined the pathology slides.

Before the conclusions are made, it is worthwhile to revisit the main the research questions highlighted in the methods chapter and to see if the research process we undertook answered these questions.

The first objective was to investigate various clinico-pathological factors of the patients with rectal cancers in the two district hospitals in South Wales.

The analysis revealed a large proportion of the tumours at advanced stage of presentation with 72.5% being T3 and T4 cancers with only 6.5% T1 cancers.

Tumour differentiation was typical with the majority being moderately differentiated (72.5%). There was no significant difference in the type of operation between the two centres, age and type of operation, sex and the type of operation suggesting uniform mix of case mix. Lymph nodal invasion was significantly associated with decreased survival (p= 0.001) and increased recurrence of cancer (p= 0.07). Perineural invasion was significantly associated with decreased survival and increased tumour recurrence (p=0.005). Vascular invasion was significantly associated with decreased survival and increased tumour recurrence (p=0.013). Mesorectal deposits were not significantly associated with increased recurrence (p= 0.288) or decreased survival (p= 0.140). Overall survival at 2 years was 70% with overall recurrence of 35%at 2 years. The systemic recurrence was 31.25% while local recurrence was 11.25%.

The second objective was to stain rectal cancers using immunocytochemical techniques and to assess the presence of malignant cells at the circumferential resection margins and in the mesorectum.

We have successfully applied Immunocytochemistry on the rectal cancer paraffin microsections in an attempt to detect micrometastases and isolated tumour cells in the mesorectum and circumferential margin. We have shown an improvement in the detection of both micrometastases and isolated tumour cells overall in the mesorectum and at the circumferential margin. The improvement in the detection of isolated tumour cells in the mesorectum was statistically

significant (p=0.05) compared with routine staining with Haematoxylin and Eosin staining. However, improvement in the detection of micrometastases in the mesorectum and at the CRM was not statistically significant (p= 0.22 and 0.31). Furthermore, where MM or ITC were detected using H&E, the immunocytochemistry did not miss the micrometastatic disease. This was consistently noted at all levels of staining and at both CRM and in the mesorectum.

The third objective was to examine the association between the presence of malignant cells in the mesorectum and at the circumferential resection margins with patient and tumour related factors. Furthermore, an association of the micrometastatic disease with either local recurrence or systemic recurrence of rectal cancer assess any.

Isolated tumour cells at the circumferential resection margin were independent of any of the tumour related factors (table 4.3.8) and were significantly associated with tumours more than 4 centimeters in size (p=0.035). Micrometastases at the CRM were significantly (p=0.09) associated with moderately differentiated tumours than tumours with other differentiation. Micrometastases in the mesorectum were significantly associated with Dukes stage C2 than with other stages (p=0.001). No significant association of micrometastases or isolated tumour cells at the circumferential resection margin and in the mesorectum with patient related factors such as age and gender was noted (tables 4.3.8 and 4.3.9). Isolated tumour cells in the mesorectum had no significant association with any

of the tumour related factors. However, micrometastases in the mesorectum were significantly associated with vascular invasion, tumour stage T3 and T4 disease, N1 and N2 disease, and Dukes stages C1 and C2 stage (table 4.3.11). Micrometastases and isolated tumour cells at the circumferential resection margin were significantly (p=0.001) associated with systemic recurrence. No significant association was noted between circumferential margins isolated tumour cells and micrometastases and local recurrence. No significant association was noted between isolated tumour cells and micrometastases in the mesorectum and local recurrence.

The final objective was to examine the impact of the presence of malignant cells in the mesorectum or the malignant cells at the circumferential resection margins on the survival outcomes.

Circumferential margin micrometastases and isolated tumour cells were significantly associated with poor survival. Furthermore, mesorectal micrometastases were also significantly associated with poor survival.

From the findings of this study, the following conclusions are reasonable.

- There was definite presence of micrometastases and isolated tumour cells at both CRM and the mesorectum;
- Immunocytochemistry was superior in the detection of MM and ITC compared to Haematoxylin and eosin;
- Circumferential margin and mesorectal MM and ITC were significantly associated with systemic recurrence but did not affect local recurrence;
- Mesorectal MM and circumferential margin MM and ITC were significantly associated with poor survival.

RECOMMENDATIONS

Recommendations for future researchers and research projects:

The following recommendations may be made based on our study.

- Considering the small sample size of our study and that this is the first ever study to assess the circumferential margins and the mesorectum using immunocytochemistry, further studies with a larger sample size may be needed to validate the results of our study;
- The immunocytochemical methods may be used to assess the presence of micrometastatic disease both in the mesorectum and circumferential margins of rectal cancer;
- The precise number of levels of sectioning of rectal cancer specimens for Immunocytochemistry is yet to be established;
- If a consistently positive correlation is observed in other observational studies between micrometastatic disease and local recurrence, then, a randomised controlled study of adjuvant therapy in the presence of micrometastatic disease should be proposed;

  The assessment of distal resection margins of rectal cancers using more sensitive investigations such as RT-PCR or ICH is a potential area for research.

REFERENCES

Adachi Y, Mori M, Maysushima T et al. Distribution of lymph node metastasesin right-sided colon cancer. J Clin Gastroenterol. 1994; 19: 210-213.

Adam IJ, Mohmadee MO, Martin IG et al. Role of circumferential margin involvement in the local recurrence of rectal cancer. Lancet 1994; 344: 707-11.

Adell G, Boeryd B, Franlund B et al. Occurrence and prognostic importance of micrometastases in regional lymph nodes in Dukes' B colorectal carcinoma: an immunohistochemical study. Eur J Surg. 1996 Aug; 162(8): 637-42.

Ambiru S, Miyazaki M, Isono T et al. Hepatic resection for colorectal metastases. Analysis of prognostic factors. Dis Colon Rectum 1999; 42: 632-39.

American Joint Committee on Cancer. Colon and Rectum. In: *AJCC Cancer Staging Manual*. 6th ed. New York: Springer. 113-124.

Angus B, Purvis J, Stock D, Westley et al. NCL-5D3: a new monoclonal antibody recognizing low molecular weight cytokeratins effective for mmunohistochemistry using fixed paraffin-embedded tissue. J Pathol. 1987 Dec; 153(4): 377-84.

Arbman G, Nilsson E, Hallbook O, Sjodahl R. Local recurrence following total mesorectal excision for rectal cancer. Br J Surg. 1996 Mar; 83(3): 375-9.

Association of Coloproctology of the Great Britain and the Ireland (ACPGBI). Guidelines for the management of colorectal cancer. 2001; 52.

Bayar S, Saxena R, Emir B et al. Venous invasion may predict lymph node metastasis in early rectal cancer. Eur J Surg Oncol. 2002 Jun; 28(4): 413-7.

Bernini A, Spencer M, Frizelle S, Madoff RD. Evidence for colorectal cancer micrometastases using reverse transcriptase-polymerase chain reaction analysis of MUC2 in lymph nodes. Cancer Detect Prev. 2000; 24(1): 72-9.

Bessa X, Lack of prognostic influence of circulating tumor cells in peripheral blood of patients with colorectal cancer. Gastroenterology. 2001 Apr;120(5):1084-92.

Bessa X et al. Prognostic value of postoperative detection of blood circulating tumor cells in patients with colorectal cancer operated on for cure. Ann Surg. 2003 Mar;237(3):368-75.

Bokey EL, Ojerskog B, Chapuis PH et al. Local recurrence after curative excision of the rectum for cancer without adjuvant therapy: role of total anatomical dissection. Br J Surg. 1999 Sep; 86(9): 1164-70.

Bognel C, Rekacewicz C, Mankarios H et al. Prognostic value of neural invasion in rectal carcinoma: a multivariate analysis on 339 patients with curative resection. Eur J Cancer. 1995 Jun; 31A(6): 894-8.

Bolognese A, Cardi M, Muttillo IA et al. Total mesorectal excision for surgical treatment of rectal cancer. J Surg Oncol. 2000 May; 74(1): 21-3.

Braun S, Rosenberg R, Thorban S et al. Implications of occult metastatic cells for systemic cancer treatment in patients with breast and gastrointestinal cancer. Surg Oncol 2001; 20: 334-346.

Broll R, Schauer V, Schimmelpenning H et al. Prognostic relevance of occult tumour cells in lymph nodes of colorectal carcinomas: an immunohistochemical study. Dis Colon Rectum. 1997 Dec; 40(12): 1465-71.

Brennan J, Mao L, Huban R H at al. Molecular assessment of histopathological staging in squamous cell carcinoma of the head and neck. N Engl J Med 1995; 332: 429-35.

Bustin SA, Gyselman VG, Williams NS et al. Detection of cytokeratins 19/20 and guanylyl cyclase C in peripheral blood of colorectal cancer patients. Br J Cancer. 1999 Apr; 79(11-12): 1813-20.

Buyse M, Piedbois P. Should Dukes B patients receive adjuvant therapy? A statistical perspective. Semin Oncol 2001; 28: 20-24.

Cancer Research UK Statistics report April 2003.

Calaluce R, Miedema BW, Yesus YW. Micrometastasis in colorectal carcinoma: a review. J Surg Oncol. 1998 Mar; 67(3): 194-202.

Caplin S, Cerottini JP, Bosman FT et al. For patients with Dukes' B (TNM Stage II) colorectal carcinoma, examination of 6 or fewer lymph nodes is related to poor prognosis. Cancer 1998; 83: 666-72.

Castells A, Boix L, Bessa X et al. Detection of colonic cells in peripheral blood of colorectal cancer patients by means of reverse transcriptase and polymerase chain reaction. Br J Cancer. 1998 Nov; 78(10): 1368-72.

Cianchi F, Palomba A, Boddi V et al. Lymph node recovery from colorectal tumour specimens: recommendation for a minimum number of lymph nodes to be examined. World J Surg 2002; 26: 384-9.

Clarke G, Ryan E, O'Keane JC et al. The detection of cytokeratins in lymph nodes of Duke's B colorectal cancer subjects predicts a poor outcome.
Eur J Gastroenterol Hepatol. 2000 May; 12(5): 549-52.

Compton CC. Pathologic prognostic factors in the recurrence of rectal cancer.
Clin Colorectal Cancer. 2002 Nov; 2(3): 149-60.

Compton CC, Fielding LP, Burgart LJ et al. Prognostic factors in colorectal cancer. College of American Pathologists Consensus Statement 1999. Arch Pathol Lab Med. 2000 Jul; 124(7): 979-94.

Cote R, Houchens DP, Hitchcock C L et al. Intraoperative detection of occult colon cancer micrometastases using 125 I -radiolabelled monoclonal antibody CC49. Cancer 1996; 77: 613-20.

Cserni G, Vajda K Tarjan M et al. Nodal staging of colorectal carcinomas from quantitative and qualitative aspects: can lymph node mapping help staging? Pathol Oncol Res 1999; 5: 291-6.

Cutait R, Alves VA, Lopes LC et al. Restaging of colorectal cancer based on the identification of lymph node micrometastases through immunoperoxidase staining of CEA and cytokeratins. Dis Colon Rectum. 1991 Oct; 34(10): 917-20.

Dahlberg M, Glimelius B, Pahlman L. Changing strategy for rectal cancer is associated with improved outcome. Br J Surg. 1999 Mar; 86(3): 379-84.

Davidson BR, Sams VR, Styles J et al. Detection of occult nodal metastases in patients with colorectal carcinoma. Cancer 1990; 65: 967-70.

Deans GT, Parks TG, Rowlands BJ et al. Prognostic factors in colorectal cancer Br J Surg. 1992 Jul; 79(7): 608-13.

de Anda EH, Lee SH, Finne CO, Rothenberger DA, Endorectal ultrasound in the follow-up of rectal cancer patients treated by local excision or radical surgery. Dis Colon Rectum. 2004 Jun; 47(6): 818-24.

Delaney CP, Lavery IC, Brenner A et al. Preoperative radiotherapy improves survival for patients undergoing total mesorectal excision for stage T3 low rectal cancers. Ann Surg. 2002 Aug; 236(2): 203-7.

Denis MG, Lipart C, Leborgne J et al. Detection of disseminated tumour cells in peripheral blood of colorectal cancer patients. Int J Cancer. 1997 Oct 21; 74(5): 540-4.

Dorudi S, Kinrade E, Marshall NC et al. Genetic detection of lymph node micrometastases in patients with colorectal cancer. Br J Surg. 1998 Jan; 85(1): 98-100.

Dukes CE, Bussy HJR. The spread of rectal cancer and its effect on prognosis. Br J cancer. 1958; **12**: 309-320.

Edwards, MJ; Whitworth, P; Tafra, L; McMasters, KM; "The Details of Successful Sentinel Lymph Node Staging for Breast Cancer." Am J Surg 2000; 180(4):257-261.

Elias JM et al. Special report: Quality control in immunocytochemistry. Am J Clin Pathol 1989; 92(6): 836-43.

Eriksen MT, Wibe A, Syse A, Haffner J Inadvertent perforation during rectal cancer resection in Norway. Br J Surg. 2004 Feb;91(2):210-6

Esser S, Reilly WT, Riley LB et al. The role of sentinel lymph node mapping in staging of colon and rectal cancer. Dis Colon Rectum. 2001 Jun; 44(6): 850-4

Fisher ER, Colangelo L, Wieand S et al. Lack of influence of cytokeratin-positive mini micrometastases in "Negative Node" patients with colorectal cancer: findings from the national surgical adjuvant breast and bowel projects protocols R-01 and C-01. Dis Colon Rectum. 2003 Aug; 46(8): 1021-5.

Frykholm G, Glemelius B, Pahlman L. Pre-operative or post-operative irradiation in adenocarcinoma of rectum: final treatment results if a randomised trial and evaluation of late secondary effects. Dis Colon Rectum 1993; 36: 564-72.

Funaki NO, Tanaka J, Itami A et al. Detection of colorectal carcinoma cells in circulating peripheral blood by reverse transcription-polymerase chain reaction targeting cytokeratin-20 mRNA. Life Sci. 1997; 60(9): 643-52.

Funaki NO, Tanaka J, Ohshio G et al. Cytokeratin 20 mRNA in peripheral venous blood of colorectal carcinoma patients. Br J Cancer. 1998 Apr;77(8):1327-32.

Funaki NO, Tanaka J, Sugiyama T. Perioperative quantitative analysis of cytokeratin 20 mRNA in peripheral venous blood of patients with colorectal adenocarcinoma. Oncol Rep. 2000 Mar-Apr; 7(2): 271-6.

Funke I, Zia A, Gabka C et al. Loss of HLA class I antigen expression on disseminated tumour cells in bone marrow of breast cancer patients relates to poor prognosis. Langenbecks Arch Surg 1999; 1: 707-710.

Futamura M, Takagi Y, Koumura H et al. Spread of colorectal cancer micrometastases in regional lymph nodes by reverse transcriptase-polymerase chain reactions for carcinoembryonic antigen and cytokeratin 20. J Surg Oncol. 1998 May; 68(1): 34-40.

Gatta G, Capocaccia R, Sant M et al. Understanding variations in survival for colorectal cancer in Europe: a EUROCARE high resolution study. Gut. 2000 Oct;47(4):533-8.

Gatter KC. Diagnostic immunocytochemistry: Achievements and challenges. J Pathol 1989; 159: 183-190.

Gerhard M, Juhl H, Kalthoff H. Specific detection of carcinoembryonic antigen-expressing tumour cells in bone marrow aspirates by polymerase chain reaction. J Clin Oncol. 1994 Apr; 12(4): 725-9.

Goddard MJ, Wilson B, Grant JW. Comparison of commercially available cytokeratin antibodies in normal and neoplastic adult epithelial and non-epithelial tissues. J Clin Pathol 1991; 44: 660-3.

Goldstein NS. Lymph node recoveries from 2427 pT3 colorectal resection specimens spanning 45 years: recommendations for a minimum number of recovered lymph nodes based on predictive probabilities. Am J Surg Pathol 2002; 26: 179-89.

Greenson JK, Isenhart CE, Rice R et al. Identification of occult micrometastases in pericolic lymph nodes of Duke's B colorectal cancer patients using monoclonal

antibodies against cytokeratin and CC49. Correlation with long-term survival. Cancer. 1994 Feb 1; 73(3): 563-9.

Gunn J, McCall JL, Yun K, Wright PA. Detection of micrometastases in colorectal cancer patients by K19 and K20 reverse-transcription polymerase chain reaction. Lab Invest. 1996 Oct;75(4):611-6.

Haboubi NY, Clark P, Kaftan SM et al. The importance of combining xylene clearance and immunohistochemistry in the accurate staging of colorectal carcinoma. J R Soc Med. 1992 Jul; 85(7): 386-8.

Haggit RC, Glotzbach RE, Soggar EE et al. Prognostic factors in colorectal carcinomas arising in adenomas: Implications for lesions removed by endoscopy. Gasrtoenterology 1985; 89: 328-36.

Hall NR, Finan PJ, al-Jaberi T et al. Circumferential margin involvement after mesorectal excision of rectal cancer with curative intent. Predictor of survival but not local recurrence? Dis Colon Rectum. 1998 Aug; 41(8): 979-83.

Hardingham JE, Hewett PJ, Sage RE et al. Molecular detection of blood-borne epithelial cells in colorectal cancer patients and in patients with benign bowel disease. Int J Cancer. 2000 Jan 20;89(1):8-13.

Hayashi N, Ito I, Yanagisawa A et al. Genetic diagnosis of lymph-node metastasis in colorectal cancer. Lancet. 1995 May 20; 345(8960): 1257-9.

Heald R J, Ryall RDH. Recurrence and survival after total mesorectal excision for rectal cancer. THE LANCET 1986; June28th: 1479-1482.

Hermanek P, Hutter RV, Sobin LH et al. International Union Against Cancer. Classification of isolated tumor cells and micrometastasis. Cancer. 1999 Dec 15; 86(12): 2668-73.

Hosch S, Braun S, Pantel K. Characterisation of disseminated tumour cells. Semin. Surg. Oncol. 2001; 20: 265-271.

International (LUDWIG) Breast cancer study group. Prognostic importance of occult axillary lymph node micrometastases from breast cancers. Lancet 1990; 335: 1565-8.

Isaka N, Nozue M, Doy M et al. Prognostic significance of perirectal lymph node micrometastases in Dukes' B rectal carcinoma: an immunohistochemical study by CAM5.2. Clin Cancer Res. 1999 Aug; 5(8): 2065-8.

Izbicki JR, Hosch SB, Pichlmeier U et al. .Prognostic value of immunohistochemically identifiable tumor cells in lymph nodes of patients with completely resected esophageal cancer. N Engl J Med. 1997 Oct 23;337(17):1188-94

Jass JR, Atkin WS, Cuzick J et al. The grading of rectal cancer: historical perspectives and a multivariate analysis of 447 cases. Histopathology. 1986 May; 10(5): 437-59.

Jass J.R, Love SB, Northover JM. New prognostic classification of rectal cancer 1987; 1: 1303-6.

Jeffers MD, O'Dowd GM, Mulcahy H et al. The prognostic significance of immunohistochemically detected lymph node micrometastases in colorectal carcinoma. J Pathol. 1994 Feb; 172(2): 183-7.

Johnon P.W.M, Burchill SA, Selby PJ. Molecular detection of circulating tumour cells. Br J Cancer 1995; 72: 268-76.

Jonas S, Windeatt S, O-Boateng A et al. Identification of carcinoembryonic antigen-producing cells circulating in the blood of patients with colorectal carcinoma by reverse transcriptase polymerase chain reaction. Gut. 1996 Nov;39(5):717-21.

Joosten JJ, Strobbe LJ, Wauters CA et al. Intraoperative lymphatic mapping and the sentinel node concept in colorectal carcinoma. Br J Surg. 1999 Apr; 86(4): 482-6.

Juhl H, Stritzel M, Wroblewski A et al. Immunocytological detection of micrometastatic cells: comparative evaluation of findings in the peritoneal cavity and the bone marrow of gastric, colorectal and pancreatic cancer patients. Int J Cancer. 1994 May 1; 57(3): 330-5.

Kapiteijn E, Marijnen CA, Nagtegaal ID, Putter H et al. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. N Engl J Med. 2001 Aug 30;345(9):638-46.

Karanjia ND, Corder AP, Bearn P et al. Leakage from stapled low anastomosis after total mesorectal excision for carcinoma of the rectum. Br J Surg. 1994 Aug; 81(8): 1224-6.

Kenmotsu M, Gouchi A, Maruo Y et al. The expression of neural cell adhesion molecule (NCAM), neural invasion and recurrence patterns in rectal cancer--a study using anti-NACM (neural cell adhesion molecule) antibody] Nippon Geka Gakkai Zasshi. 1994 Feb; 95(2): 66-70.

Khan ZA, Jonas SK, Le-Marer N et al. P53 mutations in primary and metastatic tumors and circulating tumor cells from colorectal carcinoma patients. Clin Cancer Res. 2000 Sep; 6(9): 3499-504.

Kokudo N, Miki Y, Sugai S et al. Genetic and histological assessment of surgical margins in resected liver metastases from colorectal carcinoma: minimum surgical margins for successful resection. Arch Surg. 2002 Jul;

137(7): 833-40.

Kuru B, Camlibel M, Dinc S et al. Prognostic factors affecting local recurrence and survival for operable rectal cancers. J Exp Clin Cancer Res. 2002 Sep; 21(3): 329-35.

Leather AJ, Kocjan G, Savage F et al. Detection of free malignant cells in the peritoneal cavity before and after resection of colorectal cancer. Dis Colon Rectum. 1994 Aug; 37(8): 814-9.

Leather AJ, Gallegos NC, Kocjan G et al. Detection and enumeration of circulating tumour cells in colorectal cancer. Br J Surg. 1993 Jun; 80(6): 777-80.

Leinung S, Wurl P, Weiss CL et al. Cytokeratin-positive cells in bone marrow in comparison with other prognostic factors in colon carcinoma. Langenbecks Arch Surg. 2000 Aug; 385(5): 337-43.

Liefers GJ, Cleton-Jansen AM, van de Velde CJ et al. Micrometastases and survival in stage II colorectal cancer. N Engl J Med. 1998 Jul 23; 339(4): 223-8.

Lindemann F, Schlimok G, Dirschedl P et al. Prognostic significance of micrometastatic tumour cells in bone marrow of colorectal cancer patients. Lancet 1992; 340: 685-89.

Litle VR, Warren RS, Moore D et al. Molecular cytogenetic analysis of cytokeratin 20-labeled cells in primary tumors and bone marrow aspirates from colorectal carcinoma patients. Cancer. 1997 May 1; 79(9): 1664-70.

Lohnert MS, Doniec JM, Henne-Bruns D.Effectiveness of endoluminal sonography in the identification of occult local rectal cancer recurrences. Dis Colon Rectum. 2000 Apr;43(4):483-91.

Luna-Perez P, Bustos-Cholico E, Alvarado I et al. Prognostic significance of circumferential margin involvement in rectal adenocarcinoma treated with preoperative chemo radiotherapy and low anterior resection. J Surg Oncol. 2005 Apr 1; 90(1): 20-5

Makin CA, Bobrow LG, Nicholls RJ. Can immunohistology improve detection of lymph-node metastases in large-bowel cancer? Dis Colon Rectum. 1989 Feb; 32(2): 99-102.

Matsushima T, Mori M, Kido A et al. Preoperative estimation of neural invasion in rectal carcinoma. Oncol Rep. 1998 Jan-Feb; 5(1): 73-6.

Matsuda K. Metastatic rate of colorectal invasive carcinoma. Inquiry study (In Japanese) In Muto T, Tada M, Eds. Colorectal sm cancer. Endoscopic diagnosis and treatment. Tokyo: Japan Medical Centre, 1999: 99-116.

Manfredi S, Benhamiche AM, Meny B, Cheynel N, Rat P, Faivre J. Population-based study of factors influencing occurrence and prognosis of local recurrence after surgery for rectal cancer. Br J Surg. 2001 Sep;88(9):1221-7

Maurel J, Launoy G, Grosclaude P et al. Lymph node harvest reporting in patients with carcinoma of the large bowel: a French population-based study. Cancer. 1998 Apr 15; 82(8): 1482-6.

Maxwell-Armstrong CA, Scholefield JH. Colorectal cancer. In: Clinical evidence. BMJ Publishing group, 2003; 10: 509-17.

Mella J, Biffin A, Radcliffe AG et al. Population-based audit of colorectal cancer management in two UK health regions. Colorectal Cancer Working Group, Royal College of Surgeons of England Clinical Epidemiology and Audit Unit. Br J Surg. 1997 Dec; 84(12): 1731-6.

Michie SA et al. A panel approach to the evaluation of sensitivity and specificity of antibodies for diagnosis of routinely processed histologically undifferentiated human neoplasms. Am J Clin Pathol 1987; 88(4): 457-62.

Minimum dataset for colorectal cancer histopathology reports: The Royal College of Pathologists. July 1998.

Moll R. Cytokeratins in the histological diagnosis of malignant tumours. Int J Biol Markers. 1994 Apr-Jun; 9(2): 63-9. 9.

Moreira LF, Kenmotsu M, Gochi A et al. Lymphovascular and neural invasion in low-lying rectal carcinoma. Cancer Detect Prev. 1999; 23(2): 123-8.

Mori M, Mimori K, Inoue H et al. Detection of cancer micrometastases in lymph nodes by reverse transcriptase-polymerase chain reaction. Cancer Res. 1995 Aug 1; 55(15): 3417-20.

Mori M, Mimori K, Ueo H et al. Molecular detection of circulating solid carcinoma cells in the peripheral blood: the concept of early systemic disease. Int J Cancer. 1996 Dec 11; 68(6): 739-43.

Mori M, Mimori K, Ueo H et al. Clinical significance of molecular detection of carcinoma cells in lymph nodes and peripheral blood by reverse transcription-polymerase chain reaction in patients with gastrointestinal or breast carcinomas. J Clin Oncol. 1998 Jan; 16(1): 128-32.

Muller V, Stahmann N, Riethdorf S et al. Circulating tumor cells in breast cancer: correlation to bone marrow micrometastases, heterogeneous response to systemic therapy and low proliferative activity. Clinical Can Res 2005; 15;11(10):3678-85.

Muto T, Oya M. Recent advances in diagnosis and treatment of colorectal T_1 carcinoma. Dis Colon Rectum 2003; 46(suppl): s89-s93.

Nagtegaal ID, Marijnen CA, Kranenbarg EKCircumferential margin involvement is still an important predictor of local recurrence in rectal carcinoma: not one millimeter but two millimeters is the limit. Am J Surg Pathol. 2002 Mar;26(3):350-7.

Nakanishi Y, Ochiai A, Yamauchi Y et al. Clinical implications of lymph node micrometastases in patients with colorectal cancers. A case control study. Oncology. 1999 Nov; 57(4): 276-80.

National Institute of Clinical excellence. Guidance on cancer services. Improving outcomes in colorectal cancers. 2004; 6-7.

No authors listed. Improved survival with preoperative radiotherapy in resectable rectal cancer. Swedish Rectal Cancer Trial. N Engl J Med. 1997 Apr 3; 336(14): 980-7.

No authors listed. Efficacy of adjuvant fluorouracil and folinic acid in colon cancer. International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators. Lancet. 1995 Apr 15; 345(8955): 939-44.

No authors listed. Efficacy of adjuvant fluorouracil and folinic acid in B2 colon cancer. International Multicentre Pooled Analysis of B2 Colon Cancer Trials (IMPACT B2) Investigators J Clin Oncol. 1999 May; 17(5): 1356-63.

Noura S, Yamamoto H, Miyake Y et al. Immunohistochemical assessment of localisation and frequency of micrometastases in lymph nodes of colorectal cancer. Clin Cancer Res 2002; 8: 759-67.

NHS Direct. Making changes. NHS direct 2003.

Oberg A, Stenling R, Tavelin B et al. Are lymph node micrometastases of any clinical significance in Dukes Stages A and B colorectal cancer? Dis Colon Rectum. 1998 Oct; 41(10): 1244-9.

Okuyama T, Oya M, Ishikawa H. Budding as a risk factor for lymph node metastases in pT1 or pT2 well differentiated colorectal adenocarcinoma. Dis Colon Rectum 2002; 45: 628-34.

O'Sullivan GC, Collins JK, Kelly J et al. Micrometastases: marker of metastatic potential or evidence of residual disease? Gut. 1997 Apr; 40(4): 512-5.

Ouchi K, Sugawara T, Ono H et al. Histologic features and clinical significance of venous invasion in colorectal carcinoma with hepatic metastasis. Cancer. 1996 Dec 1; 78(11): 2313-7.

Okuyama T, Oya M, Ishikawa H. Budding as a useful prognostic marker in pT3 well- or moderately-differentiated rectal adenocarcinoma. J Surg Oncol. 2003 May; 83(1): 42-7.

Palma RT, Waisberg J, Bromberg SH et al. Micrometastasis in regional lymph nodes of extirpated colorectal carcinoma: immunohistochemical study using anti-cytokeratin antibodies AE1/AE3. Colorectal Dis. 2003 Mar; 5(2): 164-8.

Pantel K, Schlimok G, Kutter D et al. Frequent down regulation of major histocompatability class I antigen expression on individual micrometastatic carcinoma cells. Cancer Res 1991; 51:4712-15.

Pantel K, Schlimok G, Braun S et al. Differential expression of proliferation-associated molecules in individual micrometastatic carcinoma cells. J Natl Cancer Inst. 1993 Sep 1; 85(17): 1419-24.

Passlick B, Pantel K, Kubuschok B. Expression of MHC molecules and ICAM-1 on non-small cell lung carcinomas: Association with early lymphatic spread of the tumour cells. Eur J Cancer1996; 32A: 141-145.

Paty PB, Nash GM, Baron PLong-term results of local excision for rectal cancer.Ann Surg. 2002 Oct;236(4):522-29.

Patel H, Le Marer N, Wharton RQ et al. Clearance of circulating tumour cells after excision of primary colorectal cancer. Ann Surg. 2002 Feb; 235(2): 226-31.

Perez RO, Habr-Gama A, Arazawa ST Lymph node micrometastasis in stage II distal rectal cancer following neoadjuvant chemoradiation therapy. Int J Colorectal Dis. 2005 Sep; 20(5): 434-9.

Pocard M, Panis Y, Malassagne B et al. Assessing the effectiveness of mesorectal excision in rectal cancer: prognostic value of the number of lymph nodes found in resected specimens. Dis Colon Rectum. 1998 Jul; 41(7): 839-45.

Poller DN. Method of specimen fixation and pathological dissection of colorectal cancer influences retrieval of lymph nodes and tumour nodal stage. Eur J Surg Oncol. 2000 Dec;26(8):758-62.

Prabhudesai A, Arif S, Finlayson CJ et al. Impact of microscopic extranodal tumour deposits on the outcome of patients with rectal cancer. Dis Colon Rectum. 2003 Nov; 46(11): 1531-7.

Prandi M, Lionetto R, Bini A et al. Prognostic evaluation of stage B colon cancer patients is improved by an adequate lymphadenectomy: results of a secondary analysis of a large scale adjuvant trial. Ann Surg. 2002 Apr; 235(4): 458-63.

Prieto VG, Lugo J, Mc Nutt NS. Intermediate and low- molecular weight keratin detection with monoclonal antibody MNF 116. An immunohistochemical study on 232 paraffin-embedded cutaneous lesions. J Cutan Pathol 1996; 23: 234-41.

Porter GA, O'Keefe GE, Yakimets WW. Inadvertent perforation of the rectum during abdominoperineal resection. Am J Surg. 1996 Oct; 172(4): 324-7.

Quirke P, Durdey P, Dixon ME et al. Local recurrence of rectal adenocarcinoma due to inadequate surgical resection. Histological study of lateral tumour spread and surgical excision. Lancet 1986; I: 996-9.

Ratto C, Sofo L, Ippoliti M et al. Prognostic factors in colorectal cancer. Literature review for clinical application. Dis Colon Rectum. 1998 Aug; 41(8): 1033-49.

Rosenberg R, Hoos A, Mueller J et al. Impact of cytokeratin-20 and carcinoembryonic antigen mRNA detection by RT-PCR in regional lymph nodes of patients with colorectal cancer. Br J Cancer. 2000 Nov; 83(10): 1323-9.

Rosenberg R, Hoos A, Mueller J et al. Prognostic significance of cytokeratin-20 reverse transcriptase polymerase chain reaction in lymph nodes of node-negative colorectal cancer patients. J Clin Oncol. 2002 Feb 15; 20(4): 1049-55.

Sanchez-Cespedes M, Esteller M, Hibi K et al. Molecular detection of neoplastic cells in lymph nodes of metastatic colorectal cancer patients predicts recurrence Clin Cancer Res. 1999 Sep; 5(9): 2450-4.

Sarli L, Bader G, Iusco D et al. Number of lymph nodes examined and prognosis of TNM stage II colorectal cancer. Eur J Cancer. 2005 Jan; 41(2): 272-9.

Sasaki M, Watanabe H, Jass JR et al. Occult lymph node metastases detected by cytokeratin immunohistochemistry predict recurrence in "node-negative" colorectal cancer. J Gastroenterol. 1997 Dec; 32(6): 758-64.

Schlimok G, Funke I, Holzmann B et al. Micrometastatic cancer cells in bone marrow: in vitro detection with anti-cytokeratin and in vivo labelling with anti-17-1A monoclonal antibodies. Proc Natl Acad Sci U S A. 1987 Dec; 84(23): 8672-6.

Schlimok G, Funke I, Bock B et al. Epithelial tumour cells in bone marrow of patients with colorectal cancer: Immunocytochemical detection, phenotypic characterization, and prognostic significance. J Clin Oncol. 1990 May;8(5): 831-7.

Scott KW, Grace RH. Detection of lymph node metastases in colorectal carcinoma before and after fat clearance. Br J Surg. 1989 Nov;76(11):1165-7.

Silen W. Colon and Rectum in Schwartz E, Ed. The year book of surgery. Chicago: Yearbook 1983: 368-70.

Slaney G et al. (eds) Cancer of the large bowel. Clinical cancer monograph Vol 4. MacMillan Press 1991.

Slade MJ, Singh A, Smith BM Persistence of bone marrow micrometastases in patients receiving adjuvant therapy for breast cancer: results at 4 years. Int J Cancer. 2005; 114(1): 94-100.

Slanetz CA JrThe effect of inadvertent intraoperative perforation on survival and recurrence in colorectal cancer. Dis Colon Rectum. 1984 Dec;27(12):792-7.

Soeth E, Vogel I, Roder C et al. Comparative analysis of bone marrow and venous blood isolates from gastrointestinal cancer patients for the detection of disseminated tumour cells using reverse transcription PCR. Cancer Res. 1997 Aug 1; 57(15): 3106-10.

Soeth E, Roder C, Juhl H et al. The detection of disseminated tumour cells in bone marrow from colorectal-cancer patients by a cytokeratin-20-specific nested reverse-transcriptase-polymerase-chain reaction is related to the stage of disease. Int J Cancer. 1996 Aug 22; 69(4): 278-82.

Starintz P et al. Detection of disseminated tumour cells as a potential surrogate-marker for monitoring palliative chemotherapy in colorectal cancer patients. J Exp Clin Cancer Res. 2004 Dec;23(4):633-9.

Taniguchi T, Makino M, Suzuki K et al. Prognostic significance of reverse transcriptase-polymerase chain reaction measurement of carcinoembryonic antigen mRNA levels in tumour drainage blood and peripheral blood of patients with colorectal carcinoma. Cancer. 2000 Sep 1; 89(5): 970-6.

Tepper JE, O'Connell MJ, Niedzwiecki D et al. Impact of number of nodes retrieved on outcome in patients with rectal cancer. J Clin Oncol. 2001 Jan 1; 19(1): 157-63.

Thorn CC, Woodcock NP, Scott N et al. What factors affect lymph node yield in surgery for rectal cancer? Colorectal Dis. 2004 Sep; 6(5): 356-61.

Tsavellas G, Patel H, Allen-Mersh TG. Detection and clinical significance of occult tumour cells in colorectal cancer. Br J Surg. 2001 Oct; 88(10): 1307-20.

Tschmelitsch J, Klimstra DS, Cohen AM. Lymph node micrometastases do not predict relapse in stage II colon cancer. Ann Surg Oncol. 2000 Sep; 7(8): 601-8.

van Wyk Q, Hosie KB, Balsitis M. Histopathological detection of lymph node metastases from colorectal carcinoma. J Clin Pathol. 2000 Sep; 53(9): 685-7.

Veit P, Kuehle C, Beyer T,Whole-body PET/CT tumour staging with integrated PET/CT- colonography: technical feasibility and first experiences in patients with colorectal cancer. Gut. 2005 Jun 14; [Epub ahead of print].

Waldman SA, Cagir B, Rakinic J et al. Use of guanylyl cyclase C for detecting micrometastases in lymph nodes of patients with colon cancer. Dis Colon Rectum. 1998 Mar; 41(3): 310-5.

Wiese DA, Saha S, Badin J et al. Pathologic evaluation of sentinel lymph nodes in colorectal carcinoma. Arch Pathol Lab Med. 2000 Dec; 124(12): 1759-63.

Weitz J, Kienle P, Magener A et al. Detection of disseminated colorectal cancer cells in lymph nodes, blood and bone marrow. Clin Cancer Res. 1999 Jul; 5(7): 1830-6.

Weitz J, Koch M, Kienle P et al. Detection of hematogenic tumour cell dissemination in patients undergoing resection of liver metastases of colorectal cancer. Ann Surg. 2000 Jul; 232(1): 66-72.

Weitz J, Kienle P, Lacroix J et al. Dissemination of tumour cells in patients undergoing surgery for colorectal cancer. Clin Cancer Res. 1998 Feb; 4(2): 343-8.

Weitz J, Kienle P, Magener A et al. Detection of disseminated colorectal cancer cells in lymph nodes, blood and bone marrow. Clin Cancer Res. 1999 Jul; 5(7): 1830-6.

Weitz J, Kienle P, Lacroix J et al. Dissemination of tumour cells in patients undergoing surgery for colorectal cancer. Clin Cancer Res. 1998 Feb; 4(2): 343-8.

Wibe A, Rendedal PR, Svensson E et al. Prognostic significance of the circumferential resection margin following total mesorectal excision for rectal cancer. Br J Surg. 2002 Mar; 89(3): 327-34.

Williams NS, Dixon MF, Johnston D. Reappraisal of the 5cm rule of distal excision for the carcinoma of the rectum: a study of distal intramural spread and of patient survival. Br J Surg 1983; 70: 150-154.

Wold LE et al. Interlaboratory variability of immunohistochemical stains. Arch of Pathol Lab Med 1989; 113: 680-83.

Wong LS, Morris AG, Fraser IA. The exfoliation of free malignant cells in the peritoneal cavity during resection of colorectal cancer. Surg Oncol. 1996 Jun; 5(3): 115-21.

Wong H, Steinmann S, Tom P et al. Volume of lymphatic metastases does not independently influence prognosis in colorectal cancer. J Clin Oncol 2002; 20: 1506-11.

Wong JH, Severino R, Honnebier MB et al. Number of nodes examined and staging accuracy in colon and rectal cancer. Dis Colon Rectum 2001; 44: 850-4.

Wong LS, Cantrill JE, Odogwu S et al. Detection of circulating tumour cells and nodal metastasis by reverse transcriptase-polymerase chain reaction technique. Br J Surg. 1997 Jun; 84(6): 834-9.

www.cancerscreening.nhs.uk/bowel/index.html#how

Wyld DK, Selby P, Perren TJ et al. Detection of colorectal cancer cells in peripheral blood by reverse-transcriptase polymerase chain reaction for cytokeratin 20. Int J Cancer. 1998 Jun 19; 79(3): 288-93.

Yamaguchi K, Takagi Y, Aoki S et al. Significant detection of circulating cancer cells in the blood by reverse transcriptase-polymerase chain reaction during

colorectal cancer resection. Ann Surg. 2000 Jul; 232(1): 58-65.

Yamamoto S, Akasu T, Fujita S et al. Long-term prognostic value of conventional peritoneal cytology after curative resection for colorectal carcinoma. Jpn J Clin Oncol. 2003 Jan; 33(1): 33-7.

Yarbro JW, Page DL, Fielding LP et al. American Joint Committee on Cancer prognostic factors consensus conference. Cancer. 1999 Dec 1; 86(11): 2436-46.

Yasuda K, Adachi Y, Shiraishi N et al. Pattern of lymph node micrometastasis and prognosis of patients with colorectal cancer. Ann Surg Oncol. 2001 May;8(4): 300-4.

Yasuda K, Adachi Y, Shiraishi N et al. Prognostic effect of lymph node micrometastasis in patients with histologically node-negative gastric cancer. Ann Surg Oncol. 2002 Oct; 9(8): 771-4.

Yokoyama N, Shirai Y, Ajioka Y et al. Immunohistochemically detected hepatic micrometastases predict a high risk of intrahepatic recurrence after resection of colorectal carcinoma liver metastases. Cancer. 2002 Mar 15; 94(6): 1642-7.

APPENDICES

AWDURDOD IECHYD
BRO TAF
HEALTH AUTHORITY

Appendix A

26 February 2003

JS/SR

Mr M E Foster
Consultant Surgeon
Royal Glamorgan Hospital
Ynysmaerdy
Llantrisant
CF72 8XR

Dear Mr Foster

03/4939 - Local recurrence of rectal cancer : Role of immunocytochemistry in the evaluation of circumferential resection margins in rectal cancer

Thank you for your letter dated 14 February 2003.

The Chairman of the Bro Taf Local Research Ethics Committee (Panel D), Dr D E B Powell has asked me to inform you that your response satisfactorily addressed the Panel's concerns.

I can therefore confirm that full ethical approval has been granted to the above study.

I trust that this is satisfactory.

Yours sincerely

Mrs Jagjit Sidhu
Deputy Executive Officer
Local Research Ethics Committee

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Appendix B

YMDDIRIEDOLAETH GIG
PONTYPRIDD & RHONDDA
NHS TRUST

Eich Cyf Your Ref	Ein Cyf Our Ref	Est. Tel Tel. Ext.	Dyddiad Date
	01443 44/3421		**3 April 2003**

E-mail: Trish.Southway@Pr-Tr.wales.nhs.uk

Research & Development Office

Dear Mr Harinath

Research & Development Project P&R 98:
Local Recurrence of Rectal Cancers: Role of immunocytochemistry in the assessment of circumferential margins of rectal cancer

With regard to the above research project, the Trust has now received notification of Local Research Ethics Committee (LREC) approval, and after studying the relevant paperwork I am pleased to confirm that this project may go ahead.

You are required to provide the Trust with regular updates in order that we may track the progress of the project. Notification of Start and Interim Progress Report forms are enclosed and should be completed and returned to Mrs Trish Southway by the date shown. Random checks will be carried out to ensure that projects comply with clinical guidelines for research.

All researchers should be aware of and adhere to Data Protection and Caldicott guidelines.

Yours sincerely

Dr M Hasan
Director Research & Development

Enc.
cc Mrs J Murray
Mrs C Bright/Mr G Walker

Mr G Harinath
17 Clos dol Heulog
Pontprennua
Cardiff
CF23 8AT

North Glamorgan
NHS Trust
Ymddiriedolaeth GIG
Gogledd Morgannwg

Chair/Cadeirydd:
Mrs. J. Penn

Acting Chief Executive/Prif Weithredwr Dros Dro:
Mrs. J.M. Lloyd
MA, FIPD

Appendix C

Your Ref/Eich Cyf: DC/sjt
E-mail address:

Our Ref/Ein Cyf:

Direct Line:

Date/Dyddiad: 26 March 2003

Mr Harinath
C/O Mr Haray's Secretary
Prince Charles Hospital

Dear Mr Harinath

Local recurrence of rectal cancer: role of immunocytochemistry in the evaluation of circumferential resection margins in rectal cancer

I am pleased to confirm that having received satisfactory answers to the issues as outlined in my letter to you of 14 March 2003 I have taken Chairman's action to fully approve your project which may now commence with immediate effect.

As stated in a previous correspondence approval lapses if the project does not commence within 24 months of approval. The R&D office reserve the right to information on the progress of the project at any time and should receive a progress report six monthly and a written report on completion. Random audits will be carried out to ensure that projects comply with the clinical guidelines for research. Any serious adverse incidents relating to the project should be reported to the R&D office and a Clinical Incident Form filled in.

On completion of the project please inform the Trust R&D office who will arrange for you to attend a Research Risk Review Group meeting at which to present your project.

I would like to take this opportunity to wish you well with your research and look forward to the presentation of your findings.

If you require any further assistance please contact either Alison Stroud or Sarah Townsend in the Trust R&D Office which is situated in Block 10, at Prince Charles Hospital, ext 8581.

Yours sincerely

Dr D Cassidy
Deputy Medical Director &
R&D Chair

Prince Charles Hospital/Ysbyty Tywysog Siarl
Merthyr Tydfil CF47 9DT/Merthyr Tudful CF47 9DT
Tel/Ffôn: 01685 721721
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☐ Aberdare Hospital/Ysbyty Aberdâr
Aberdare CF44 0RF/Aberdâr CF44 0RF
Tel/Ffôn: 01685 872411
Minicom: 01685 872411

☐ Community Mental Health Team, Tîm Iechyd Meddwl Cymunedol
Seymour Berry Centre, Canolfan Seymour Berry
Victoria Street, Dowlais/Dowlais Canolfan Iechyd
Merthyr Tydfil CF48 3RL/Merthyr Tudful CF48 3RL
Tel/Ffôn: 01685 721721

St Tydfil's Hospital/Ysbyty Santes Tudful
Merthyr Tydfil CF47 0SJ/Merthyr Tudful CF47 0SJ
Tel/Ffôn: 01685 723244

☐ Mountain Ash Hospital/Ysbyty Aberpennar
Mountain Ash CF45 4DE/Aberpennar CF45 4DE
Tel/Ffôn: 01685 872411

☐ Hollies Health Centre/Canolfan Iechyd Hollies
Swan Street/Stryd yr Alarch
Merthyr Tydfil CF47 8ET/Merthyr Tudful CF47 8ET
Tel/Ffôn: 01685 384023

Appendix D

Clinical data

Age	Sex
Date of operation	Type of operation (AR or APR)*
Tumour type	Tumour size
Tumour differentiation	Lymphatic invasion
Extramural vascular invasion	Perineural invasion
Lymph nodal involvement	Mesorectal tumour deposits
No of lymph nodes examined	No of positive lymph nodes for tumour
Distal resection margins	Circumferential margins
Involvement of adjacent organs	Sphincter invasion
TNM Staging	Dukes staging
Tumour location	Apical lymph node involvement
Follow-up duration	Pre-operative chemotherapy
Pre-operative radiotherapy	Post-operative chemotherapy
Post-operative Radiotherapy	Follow-up to date or time to death
Local recurrence	Systemic recurrence
Site of systemic recurrence	Liver involvement
Pulmonary metastases	Bone involvement

Brain metastases	Other sites metastases
Time to local recurrence	Time to systemic recurrence
Developed both systemic and local recurrence?	How local recurrence detected?
How systemic recurrence detected?	Micrometastases or Isolated tumour cells detected in the mesorectum using immunocytochemistry
Cause of death: Cancer related death/ other.	Micrometastases or isolated tumour cells detected at the circumferential margin using immunocytochemistry

Appendix E

H&E staining module

1.	XYLENE	30 seconds
2.	XYLENE	30 seconds
3.	XYLENE	30 seconds
4.	I.M.S	30 seconds
5.	I.M.S	30 seconds
6.	I.M.S	30 seconds
7.	Running Water	30 seconds
8.	Mayers Haematoxylin	30 seconds
9.	Mayers Haematoxylin	30 seconds
10.	Mayers Haematoxylin	30 seconds
11.	Mayers Haematoxylin	30 seconds
12.	Mayers Haematoxylin	30 seconds
13.	Mayers Haematoxylin	30 seconds
14.	Mayers Haematoxylin	30 seconds
15.	Running Water	30 seconds
16.	Scotts Tap Water Substitute	30 seconds
17.	Running Water	30 seconds
18.	1% Eosin (aqueous)+ 20ul glacial acetic acid	30 seconds
19.	1% Eosin (aqueous) +20ul glacial acetic acid	30 seconds
20.	Running Water	30 seconds
21.	I.M.S	30 seconds
22.	I.M.S	30 seconds
23.	I.M.S	30 seconds
24.	I.M.S	30 seconds
25.	I.M.S	30 seconds
26.	I.M.S	30 seconds
27.	Xylene	30 seconds

<u>Mayers Haematoxylin:</u>

Haematoxylin	2g
Aluminium Potassium Sulphate	100g
Sodium iodate	4g
Citric acid	2g
Chloral Hydrate	100g
Distilled water	2litres

<u>Eosin:</u>

Eosin Yellowish	10g
Distilled water	1litre

Appendix F

Reagents of the DAB detection kit

1. Vantana medical systems / VIEW DAB detection kit.
2. Inhibitor solution: 3.0% Hydrogen peroxide solution.
3. Biotinylated Ig G secondary antibody: Affinity purified anti-goat -anti-mouse antibody(120 µg/ml).
4. Streptavidin Horseradish peroxidase (< 250 µg/ml) in protein stabilizer.
5. Hydrogen peroxide solution 0.04-0.08% H2O2 in a stabilizing solution.
6. Substrate solution Diaminobenzidine (DAB) 2gm/L in a stabilizer solution and preservative.
7. Copper sulphate 5gm/L in a buffered solution and preservative.

Appendix G

Immunoassaying procedure

1. Inhibitor solution has been optimised to remove the endogenous peroxidase activity. This step takes 4 minutes at 37 deg C.
2. The slides were rinsed; the optimized specific antibody is applied and incubated for 4-32 minutes at 37 deg C.
3. The slides were rinsed; universal biotinylated secondary antibody has optimised for most specific antibodies. This step takes 8 minutes at 37 deg C.
4. The slides were rinsed; Streptavidin-HRP step has been optimised for 8 minutes at 37 deg C.
5. The slides were rinsed; the VIEW DAB solution is mixed with hydrogen peroxide solution on the specimen slide. This reaction has been optimised for 8 minutes at 37 deg C.
6. The slides were rinsed; Copper DAB enhancer is applied with mixing for 4 minutes at 37 deg C.
7. Counter stain was then applied to the slides and incubated with mixing for 4 minutes at 37 deg C.

Appendix H

Pathology Proforma

Data collection for rectal cancer micrometastases project

Patient's identification Number ..... Pathology slides seen by

DOB.....

Age..... Pathology slide number

Gender.....

Level 1

Micrometastases

MNF 116		
CK5D3		
H&E		

Isolated Tumour Cells

MNF 116		
CK5 D3		
H&E		

Level 2

Micrometastases

MNF 116		
CK5D3		
H&E		

Isolated Tumour Cells

MNF 116		
CK5D3		
H&E		

Level 3

Micrometastases

MNF 116		
CK5D3		
H&E		

Isolated Tumour Cells

MNF 116		
CK5D3		
H&E		

Comments

Incidence in Wales 2002

MALE

	ICD10 code	Age Band																		CR	EASR
		Under 5	5-9	10-14	15-19	20-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	80-84	85+	All Ages	
Oral Cavity	**C01-C06**	0	0	0	0	1	0	0	0	1	3	12	13	14	13	13	5	5	2	**82**	**5.81**	**4.94**
Lip, Oral Cavity & Pharynx	**C00-C14**	0	0	0	0	3	0	0	3	6	6	22	24	29	28	29	19	15	6	**190**	**13.47**	**11.22**
Head & Neck	**C00-C14,C30-C32**	0	0	0	1	3	0	0	3	11	14	33	46	45	44	52	31	23	8	**314**	**22.26**	**18.56**
Oesophagus	**C15**	0	0	0	0	0	0	0	2	1	9	13	30	32	30	43	44	29	26	**259**	**18.36**	**14.39**
Stomach	**C16**	0	0	0	0	0	0	0	3	1	8	10	25	27	51	59	70	52	36	**342**	**24.24**	**18.18**
Colon	**C18**	0	0	0	0	0	0	1	3	9	10	30	41	58	88	111	118	84	45	**598**	**42.38**	**32.14**
Rectum	**C19-C21**	0	0	0	0	0	0	0	3	6	8	33	35	54	69	83	76	37	35	**439**	**31.12**	**24.57**
Colorectal	**C18-C21**	0	0	0	0	0	0	1	6	15	18	63	76	112	157	194	194	121	80	**1037**	**73.51**	**56.71**
Pancreas	**C25**	0	0	0	0	0	0	0	0	6	3	12	17	24	26	37	39	27	14	**205**	**14.53**	**11.16**
Larynx	**C32**	0	0	0	0	0	0	0	0	4	8	10	17	16	14	20	10	5	2	**106**	**7.51**	**6.34**
Trachea, Bronchus & Lung	**C33-C34**	0	0	0	2	0	0	2	3	3	22	39	65	138	182	197	206	135	84	**1078**	**76.42**	**58.38**
Bone	**C40-C41**	0	0	1	5	0	1	0	0	1	1	1	0	1	0	2	0	0	0	**13**	**0.92**	**0.91**
Malignant Melanoma	**C43**	0	0	0	0	1	2	4	8	8	11	18	18	14	18	15	20	13	9	**159**	**11.27**	**9.58**
Prostate	**C61**	1	0	0	0	0	0	0	0	0	7	38	100	177	254	322	332	259	223	**1713**	**121.43**	**90.61**
Testis	**C62**	1	0	0	6	13	9	14	11	12	0	6	7	0	3	1	0	1	0	**84**	**5.95**	**6.16**
Kidney	**C64-C66,C68**	0	0	0	0	0	0	1	1	6	7	14	25	38	38	39	35	16	21	**241**	**17.08**	**13.85**
Bladder	**C67**	0	0	0	0	0	0	1	2	5	14	20	56	67	89	105	138	78	62	**637**	**45.16**	**34.38**
Brain & CNS	**C70-C72**	5	3	2	0	1	1	2	3	6	12	8	25	7	14	15	14	12	6	**136**	**9.64**	**8.34**
Thyroid	**C73**	0	0	0	0	0	2	2	4	1	0	2	6	3	3	2	3	0	1	**29**	**2.06**	**1.87**
Hodgkins Disease	**C81**	0	3	0	4	1	3	3	7	3	2	4	0	2	4	1	1	0	1	**39**	**2.76**	**2.74**
Non Hodgkins Lymphoma	**C82-C85,C96**	1	2	1	6	0	5	0	4	6	12	17	18	29	25	36	33	25	14	**234**	**16.59**	**13.62**
Multiple Myeloma	**C90**	0	0	0	0	0	0	0	0	1	8	12	11	8	12	19	21	23	18	**133**	**9.43**	**7.30**
Leukaemia	**C91-C95**	5	2	8	6	1	2	1	4	6	2	7	19	29	31	34	45	38	20	**260**	**18.43**	**14.56**
All exc NMSC	**All C exc C44**	19	11	12	35	21	29	38	71	105	163	349	579	823	1056	1285	1365	949	696	**7606**	**539.17**	**419.29**

CR = Crude Rate per 100,000 population
EASR = European Age Standardised Rate per 100,000 population

Appendix I

Incidence in Wales 2002

FEMALE

	ICD10 code	Age Band																		CR	EASR
		Under 5	5-9	10-14	15-19	20-24	25-29	30-34	35-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75-79	80-84	85+	All Ages		
Oral Cavity	C01-C06	0	0	0	1	0	0	2	0	1	2	3	6	6	5	9	9	6	6	56	3.71	2.61
Lip, Oral Cavity & Pharynx	C00-C14	0	0	0	1	0	1	4	2	1	5	6	13	10	14	13	13	13	11	107	7.10	5.14
Head & Neck	C00-C14,C30-C32	0	0	0	1	0	1	4	2	3	5	9	15	16	18	15	21	17	13	140	9.28	6.68
Oesophagus	C15	0	0	0	0	0	0	0	0	0	2	7	7	15	19	41	31	41	27	190	12.60	7.34
Stomach	C16	0	0	0	0	0	0	0	1	0	4	8	8	15	20	32	39	35	36	198	13.13	7.69
Colon	C18	0	0	0	0	0	0	3	2	9	11	26	35	43	53	83	105	104	105	579	38.39	22.99
Rectum	C19-C21	0	0	0	0	0	1	2	7	6	7	19	18	36	26	40	47	42	42	293	19.43	12.85
Colorectal	C18-C21	0	0	0	0	0	1	5	9	15	18	45	53	79	79	123	152	146	147	872	57.82	35.84
Pancreas	C25	0	0	0	0	0	1	0	1	0	0	7	13	20	24	34	37	42	39	218	14.46	8.49
Larynx	C32	0	0	0	0	0	0	0	0	2	0	2	2	4	2	1	4	3	2	22	1.46	1.04
Trachea, Bronchus & Lung	C33-C34	0	0	0	0	0	1	1	4	5	15	30	63	61	88	117	141	99	68	693	45.95	29.86
Bone	C40-C41	0	0	1	1	0	0	0	1	1	0	0	0	1	0	1	0	3	1	10	0.66	0.47
Malignant Melanoma	C43	0	0	0	1	5	6	14	20	14	18	16	22	20	23	19	21	17	14	230	15.25	12.70
Female Breast	C50	0	0	0	0	1	3	27	73	113	179	281	275	287	205	223	194	166	177	2204	146.15	117.26
Cervix Uteri	C53	0	0	0	0	3	8	16	14	12	13	13	11	13	7	16	6	10	7	149	9.88	8.75
Corpus Uteri	C54	0	0	0	0	0	0	2	3	9	11	25	42	46	42	41	34	35	21	311	20.62	15.41
Ovary	C56-C57	1	0	0	2	3	7	4	11	11	20	37	50	46	57	51	41	38	24	403	26.72	20.90
Kidney	C64-C66,C68	4	2	1	0	0	0	0	2	3	5	12	9	15	13	21	37	18	14	156	10.34	7.20
Bladder	C67	0	0	0	0	0	0	1	4	3	8	8	9	15	27	40	49	45	49	258	17.11	10.08
Brain & CNS	C70-C72	3	3	3	2	3	3	2	3	4	4	11	12	4	14	17	18	8	5	119	7.89	6.44
Thyroid	C73	0	0	0	0	5	0	5	5	5	4	7	5	3	6	6	1	3	2	57	3.78	3.42
Hodgkins Disease	C81	0	0	1	3	1	3	2	4	2	2	2	0	4	3	2	1	1	0	31	2.06	2.01
Non Hodgkins Lymphoma	C82-C85,C96	1	0	0	0	1	2	3	5	5	12	15	18	20	18	31	25	28	23	207	13.73	9.80
Multiple Myeloma	C90	0	0	0	1	0	0	0	0	2	3	3	7	9	17	18	21	17	11	109	7.23	4.66
Leukaemia	C91-C95	8	3	2	2	2	2	1	1	4	4	6	21	15	18	19	27	29	27	191	12.67	8.95
All exc NMSC	All C exc C44	22	9	8	15	27	42	94	169	223	344	583	699	761	768	944	999	921	870	7498	497.21	355.23

CR = Crude Rate per 100,000 population
EASR = European Age Standardised Rate per 100,000 population

Appendix J

Appendix K

The mean and standard deviation of the Welsh data for the year 2002

Class interval	X (mid-point	m	f	xf (males)	xf (females)	xxf	xxf
35-39	37	3	7	111	259	4107	9583
40-44	42	6	6	252	252	10584	10584
45-49	47	7	8	329	376	15463	17672
50-54	52	33	19	1716	988	89232	51376
55-59	57	35	18	1995	1026	113715	58482
60-64	62	54	36	3348	2232	207576	138384
65-69	67	69	26	4623	1742	309741	116714
70-74	72	83	40	5976	2880	430272	207360
75-79	77	76	47	5852	3619	450604	278663
80-84	82	37	42	3034	3444	248788	282408
85-89	87	35	42	3045	3654	264915	317898
Total	438	291	30281	20472	2144997	1489124	
Mean age		**69.1347**	**70.3505**		**117.915**		**168.6491**
Standard deviations		**10.85889**	**12.9865**				

RESULTS

2.7 Rectum *(ICD10: C19-C21)*

Appendix L

TRENDS IN INCIDENCE : 1992-2001

MALES

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	Total
Under 5	0	0	0	0	0	0	0	0	0	0	**0**
5-9	0	0	0	0	0	0	0	0	0	0	**0**
10-14	0	0	0	0	0	0	0	0	0	0	**0**
15-19	0	0	0	0	0	0	0	0	0	0	**0**
20-24	0	0	0	0	0	0	0	0	0	0	**0**
25-29	0	0	1	1	0	1	1	0	1	1	**6**
30-34	1	0	2	0	3	3	0	2	2	0	**13**
35-39	5	2	1	5	1	5	3	2	0	2	**26**
40-44	5	3	2	6	3	5	4	11	7	7	**53**
45-49	20	14	11	10	20	14	12	19	10	12	**142**
50-54	35	23	17	21	28	25	21	31	30	28	**259**
55-59	46	41	49	44	35	38	40	32	35	37	**397**
60-64	59	65	47	54	65	62	68	63	71	55	**609**
65-69	74	88	86	72	85	77	56	74	64	66	**742**
70-74	94	74	90	79	77	90	86	84	84	85	**843**
75-79	78	45	53	55	63	79	81	95	81	78	**708**
80-84	50	30	47	47	43	54	45	46	53	47	**462**
85+	25	23	24	27	32	29	28	28	23	22	**261**
All Ages	**492**	**408**	**430**	**421**	**455**	**482**	**445**	**487**	**461**	**440**	**4521**
Crude Rate	**35.35**	**29.26**	**30.79**	**30.12**	**32.56**	**34.43**	**31.73**	**34.72**	**32.85**	**31.34**	**32.32**
EASR	**30.68**	**25.41**	**25.90**	**25.48**	**27.46**	**28.25**	**25.81**	**28.05**	**26.17**	**24.71**	**26.76**
WASR	**20.48**	**17.36**	**17.29**	**17.09**	**18.58**	**18.95**	**17.24**	**18.90**	**17.62**	**16.63**	**17.99**

FEMALES

	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	Total
Under 5	0	0	0	0	0	0	0	0	0	0	**0**
5-9	0	0	0	0	0	0	0	0	0	0	**0**
10-14	0	0	0	0	0	0	0	0	0	0	**0**
15-19	0	0	0	0	0	0	0	0	0	0	**0**
20-24	1	0	0	0	0	0	1	0	0	0	**2**
25-29	0	1	1	1	0	0	1	0	0	0	**4**
30-34	0	1	0	2	1	2	0	2	2	0	**10**
35-39	2	2	1	3	4	1	1	4	3	2	**23**
40-44	5	5	4	6	3	4	7	4	5	1	**44**
45-49	5	9	10	6	17	10	6	15	11	8	**97**
50-54	17	12	8	7	9	16	15	18	15	11	**128**
55-59	18	18	19	22	22	21	20	13	25	21	**199**
60-64	39	25	22	29	15	26	28	26	23	17	**250**
65-69	39	40	44	42	48	44	43	35	38	39	**412**
70-74	42	48	53	42	42	43	37	51	33	54	**445**
75-79	48	50	44	44	43	60	54	54	51	50	**498**
80-84	50	38	46	33	56	43	41	36	45	45	**433**
85+	50	45	43	47	48	43	43	51	50	45	**465**
All Ages	**316**	**294**	**295**	**284**	**308**	**313**	**297**	**309**	**301**	**293**	**3010**
Crude Rate	**21.28**	**19.77**	**19.82**	**19.09**	**20.68**	**21.01**	**19.92**	**20.72**	**20.11**	**19.55**	**20.19**
EASR	**14.05**	**12.96**	**12.66**	**12.68**	**13.16**	**13.71**	**13.05**	**13.43**	**13.01**	**12.14**	**13.08**
WASR	**9.43**	**8.70**	**8.46**	**8.61**	**8.84**	**9.20**	**8.85**	**9.12**	**8.76**	**8.01**	**8.80**

EASR: Age Standardised Incidence Rate per 100,000 population (European Standard Population)
WASR: Age Standardised Incidence Rate per 100,000 population (World Standard Population)

Classification Table[a,b]

Observed			Predicted		
			RECURRENCE YES/NO		Percentage Correct
			0	1	
Step 0	RECURRENCE YES/NO	0	52	0	100.0
		1	28	0	.0
	Overall Percentage				65.0

a. Constant is included in the model.

b. The cut value is .500

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I.for EXP(B)	
								Lower	Upper
Step 1[a]	circmm	2.406	1.124	4.582	1	.032	11.087	1.225	100.33
	Constant	-.796	.251	10.052	1	.002	.451		
Step 2[b]	circmm	2.557	1.128	5.135	1	.023	12.895	1.413	117.71
	mesomm	1.641	.907	3.270	1	.071	5.158	.871	30.52
	Constant	-.947	.270	12.288	1	.000	.388		

a. Variable(s) entered on step 1: circmm.

b. Variable(s) entered on step 2: mesomm.

Appendix N

Classification Table[a,b]

Observed			Predicted		
			ALIVE OR DEAD AT ANALYSIS		Percentage Correct
			ALIVE	DEAD	
Step 0	ALIVE OR DEAD AT ANALYSIS	ALIVE	56	0	100.0
		DEAD	24	0	.0
	Overall Percentage				70.0

a. Constant is included in the model.

b. The cut value is .500 .

Variables in the Equation

		B	S.E.	Wald	df	Sig.	Exp(B)	95.0% C.I.for EXP(B)	
								Lower	Upper
Step 1[a]	circitc	2.672	1.127	5.620	1	.018	14.474	1.589	131.8
	Constant	-1.063	.266	15.954	1	.000	.345		
Step 2[b]	circitc	2.959	1.136	6.789	1	.009	19.286	2.082	178.6
	mesomm	2.959	1.136	6.789	1	.009	19.286	2.082	178.6
	Constant	-1.350	.300	20.260	1	.000	.259		

a. Variable(s) entered on step 1: circitc.

b. Variable(s) entered on step 2: mesomm.

Appendix O

16th April 2003

Our ref: DRPC/240303/04(i)/ps

Mr G Harinath
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Cardiff
CF23 8AT

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Head of School/
Pennaeth yr Ysgol
Professor Donna M Mead
Athro Donna Mead

Dear Mr Harinath,

Registration for MPhil at the University of Glamorgan – Title of Research Project "Local recurrence of Rectal Cancers: Role of Immunocytochemistry in the Evaluation of Circumferential Margins of Rectal Cancers'

I am writing to confirm that at its meeting on the 24th March 2003, the School of Care Sciences' Departmental Research Programmes Committee (DRPC) approved your registration for the award of MPhil.

Please note that this registration takes effect from the 1st January 2003 and is subject to ethical approval being granted by the School of Care Sciences' Ethics Committee. I enclose a copy of the University of Glamorgan Research Student Handbook for your information.

Your supervision team was approved as:

Dr J Ameen (Director of Studies)
Dr Ann-Marie Coll
Prof. P N Haray

If you have any queries about the Committee's decision, please do not hesitate to contact me.

Yours sincerely,

pp Paula Samuel
School Registrar & DRPC Secretary

cc: Dr J Ameen
Dr Ann-Marie Coll
Prof. P N Haray