The Expression Pattern and Prognostic Value of Human Epidermal Growth Factor Receptor-2 and Microvessel Density in Human Colorectal Cancer

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Abstract

In colorectal cancer, the selection of patients for adjuvant chemotherapy is currently based on the Dukes' stage. However, more prognostic markers are needed to allow better selection of patients whose risk of future recurrence is sufficiently high to warrant chemotherapy.

The aims of this study were to investigate the expression and prognostic value of Human Epidermal Growth Factor Receptor-2 (HER-2) and microvessel density (MVD) in patients with colorectal cancer and their relationship with clinico-pathological parameters. HER-2 is over-expressed in a number of epithelial cancers. The prognostic role of HER-2 has been established in breast cancer but remains controversial in colorectal cancer. The expression pattern and prognostic value of HER-2 was investigated in 170 patients with Dukes' B or C colorectal cancer, who underwent radical surgery between 1990 and 1998. The anti-HER-2 monoclonal antibody HM64.13 and a standard avidin-biotin peroxidase detection system were used. Overall, 87% and 41% of cases showed cytoplasmic and membranous HER-2 immunostaining, respectively.

In the Dukes' C cancers, a significant survival benefit was observed in those patients whose tumours showed cytoplasmic HER-2 (HR 0.46, CI95 0.24-0.87), with no association between membranous HER-2 over-expression and clinical outcome. The other independent prognostic factors in the Dukes' C group were apical node metastases, grade 3 tumours, and depth of invasion. No significant prognostic association was seen in the Dukes B patients.
Using the monoclonal antibody CD31, MVD did not seem to be a predictor of poor outcome in this study, and there was no association found between MVD and HER-2 overexpression.

These data suggest that there may be a correlation between cytoplasmic HER-2 overexpression and better prognostic tumours. Furthermore the high level of membranous HER-2 in colorectal cancer could make it an ideal target for monoclonal antibody-based therapy.
Acknowledgements

I am extremely grateful to my supervisors Dr Helmout Modjtahedi and Professor Hilary Thomas, without whom this thesis would not have been possible.

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This thesis is dedicated to
Gavin, Rohan, Nathan and Saskia
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>9</td>
</tr>
<tr>
<td>List of Tables</td>
<td>10</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>11</td>
</tr>
<tr>
<td><strong>Chapter One: Introduction</strong></td>
<td>13</td>
</tr>
<tr>
<td>1.1 Background</td>
<td>13</td>
</tr>
<tr>
<td>1.2 Colorectal Cancer</td>
<td>13</td>
</tr>
<tr>
<td>1.3 Current Chemotherapy used in Colorectal Cancer</td>
<td>14</td>
</tr>
<tr>
<td>1.4 Prognostic Factors in Colorectal Cancer</td>
<td>16</td>
</tr>
<tr>
<td>1.5 Tumourigenesis and Molecular Markers</td>
<td>20</td>
</tr>
<tr>
<td>1.6 Epidermal Growth Factor Receptor Family</td>
<td>22</td>
</tr>
<tr>
<td>1.7 Human Epidermal Growth Factor Receptor-2</td>
<td>25</td>
</tr>
<tr>
<td>1.7.1 Biological Significance</td>
<td>25</td>
</tr>
<tr>
<td>1.7.2 Indicators of Increased HER-2 Production</td>
<td>26</td>
</tr>
<tr>
<td>1.7.3 The Prognostic Role of HER-2 in Human Cancers</td>
<td>29</td>
</tr>
<tr>
<td>1.7.4 The Prognostic Role of HER-2 in Colorectal Cancer</td>
<td>30</td>
</tr>
<tr>
<td>1.8 Microvessel Density and Human Cancers</td>
<td>32</td>
</tr>
<tr>
<td>1.8.1 Microvessel Density and Colorectal Cancer</td>
<td>34</td>
</tr>
<tr>
<td>1.9 Molecular Targeting in Cancer</td>
<td>37</td>
</tr>
<tr>
<td>1.9.1 Signal Transduction Pathways</td>
<td>37</td>
</tr>
<tr>
<td>1.9.2 Immunotherapy</td>
<td>38</td>
</tr>
<tr>
<td>1.9.3 Anti-angiogenesis Therapy</td>
<td>39</td>
</tr>
<tr>
<td>1.9.4 Cyclo-oxygenase Type 2 (COX 2) Pathway</td>
<td>39</td>
</tr>
<tr>
<td>1.10 Aims of the Study</td>
<td>40</td>
</tr>
<tr>
<td><strong>Chapter Two: Materials And Methods</strong></td>
<td>42</td>
</tr>
<tr>
<td>2.1 Human Tumor Cell Lines</td>
<td>42</td>
</tr>
<tr>
<td>2.2 Cell Culture</td>
<td>42</td>
</tr>
<tr>
<td>2.2.1 Culture Media and Foetal Calf Serum</td>
<td>42</td>
</tr>
<tr>
<td>2.2.2 Passaging and Growth of Cell Lines</td>
<td>43</td>
</tr>
<tr>
<td>2.2.3 Long Term Storage of Cells</td>
<td>43</td>
</tr>
<tr>
<td>2.3 Antibodies</td>
<td>44</td>
</tr>
<tr>
<td>2.4 Other Chemical Reagents</td>
<td>45</td>
</tr>
<tr>
<td>2.5 Immunoprecipitation of S-Methionine-labelled Proteins</td>
<td>46</td>
</tr>
</tbody>
</table>
Chapter Five: Expression and Prognostic Significance of CD31 in Colorectal Cancer

5.1 Patient Details
5.2 Immunostaining of CD31
5.3 Prognostic Significance of CD31 Immunostaining
5.4 Summary

Chapter Six: Discussion and Conclusion

6.1 The Expression of HER-2 in Human Tumour Cell Lines
6.2 The Prognostic Role of HER-2 in Colorectal Cancer
6.3 Cytoplasmic HER-2 is Associated with a Better Survival
6.4 HER-2 Targeting
6.5 The Prognostic Role of CD31 Immunohistochemistry in Colorectal Cancer
6.6 The Co-Expression of HER-2 and MVD in Colorectal Cancer
6.7 Anti-Angiogenic Therapy
6.8 Future Considerations

References
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Differential binding of mAb HM64.13 to a panel of human tumour cell lines which express high or low levels of HER-2</td>
<td>66</td>
</tr>
<tr>
<td>2</td>
<td>Immunoprecipitation of p-185HER-2 by mAb HM64.13, using [^{35}S]-methionine-labelled proteins from SKBR3 cells</td>
<td>67</td>
</tr>
<tr>
<td>3</td>
<td>FACS histograms of anti-HER-2 mAb, HM64.13, and anti-EGFR mAb, ICR62, binding to human tumour cell lines</td>
<td>69</td>
</tr>
<tr>
<td>4</td>
<td>Level of expression of HER-2 and EGFR in a panel of human colorectal cell lines, using anti-HER-2 mAb HM64.13 and anti-EGFR mAb ICR16, respectively</td>
<td>71</td>
</tr>
<tr>
<td>5</td>
<td>Growth inhibition assay – The effect of anti-HER-2 and anti-EGFR mAbs, and EGFR tyrosine kinase inhibitor on the growth of human tumour cell lines</td>
<td>73,74</td>
</tr>
<tr>
<td>6</td>
<td>Kaplan-Meier curves for overall survival in 170 patients with colorectal cancer, stratified for stage Dukes' B or Dukes' C</td>
<td>78</td>
</tr>
<tr>
<td>7</td>
<td>Colorectal cancer section showing HER-2 immunostaining using mAb HM64.13</td>
<td>79, 80</td>
</tr>
<tr>
<td>8</td>
<td>HER-2 immunostaining of SKBR3 cells pellet, using mAb HM64.13</td>
<td>83</td>
</tr>
<tr>
<td>9</td>
<td>Kaplan-Meier plots of survival of 105 Dukes' C colorectal cancer patients, stratified for positive and negative cytoplasmic HER-2 immunostaining</td>
<td>85</td>
</tr>
<tr>
<td>10</td>
<td>Kaplan-Meier plots of overall survival of 65 Dukes' B colorectal cancer patients with high or low cytoplasmic HER-2 immunostaining</td>
<td>87</td>
</tr>
<tr>
<td>11</td>
<td>CD31 immunostaining of a colorectal cancer, using anti-CD31 mAb JC-70, showing a case of colorectal cancer with high microvessel density</td>
<td>91</td>
</tr>
<tr>
<td>12</td>
<td>Overall survival curves for 91 patients with colorectal cancer, stratified for low and high microvessel density tumours</td>
<td>93</td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>Overall survival of patients with colorectal cancer according to Dukes' stage</td>
<td>17</td>
</tr>
<tr>
<td>Table 2.1</td>
<td>Preparation of resolving and stacking gels for SDS-PAGE</td>
<td>51</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Clinico-pathological characteristics of 170 colorectal cancer patients by Dukes' staging</td>
<td>76</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Overall membrane and cytoplasmic HER-2 over-expression in Dukes' B and C colorectal tumours</td>
<td>82</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>Independent prognostic factors for 105 Dukes' C colorectal cancer patients</td>
<td>84</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>Median microvessel count in 91 patients with colorectal cancer</td>
<td>89</td>
</tr>
<tr>
<td>Table 6.1</td>
<td>Summary of studies looking at the over-expression and prognostic role of cytoplasmic HER-2 and membranous HER-2 in colorectal cancer</td>
<td>100</td>
</tr>
<tr>
<td>Table 6.2</td>
<td>Summary of studies looking at the prognostic significance of tumour vascularity in colorectal cancer</td>
<td>109</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADEPT</td>
<td>Antibody directed enzyme prodrug therapy</td>
</tr>
<tr>
<td>BV</td>
<td>Bevacizumab</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in colon cancer</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl-sulphoxide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagles medium</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunoadsorbent assay</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>F8RA</td>
<td>Factor VIII-related antigen</td>
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<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
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<tr>
<td>FPTase</td>
<td>Farnesyl protein transferase</td>
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<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
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<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate-conjugated</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
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<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
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<td>FCS</td>
<td>Foetal Calf Serum</td>
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<tr>
<td>FA</td>
<td>Folinic acid</td>
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<tr>
<td>GDP</td>
<td>Guanidine di-phosphate</td>
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<tr>
<td>GTP</td>
<td>Guanidine tri-phosphate</td>
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<tr>
<td>HER-2</td>
<td>Human epidermal growth factor receptor-2</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
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<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
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<td>-------------------------------------------------</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MVD</td>
<td>Microvessel density</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>pTYR-PROTEINS</td>
<td>Phosphorylated tyrosine kinase proteins</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>RCTs</td>
<td>Randomised controlled trials</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl-sulphate</td>
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<tr>
<td>TS</td>
<td>Thymidylate synthase</td>
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<tr>
<td>TGF-α</td>
<td>Transforming growth factor-α</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
</tbody>
</table>
Chapter One

Introduction

1.1 Background
Carcinogenesis is a complex, multistep process that leads to transformation of a normal cell into a cancerous phenotype. This process of transformation involves a number of prerequisite steps which may start with six or more independent mutational events [Fearon ER and Vogelstein B, 2001]. The mutated cells may then have a decreased responsiveness to the intercellular and intracellular signals that maintain their architecture and regulate their growth. These mutational changes result in dysregulation of the cell cycle, loss of tumour suppressor genes, genomic instability, and resistance to apoptosis. The potential result of all these genetic alterations is unregulated cell growth and proliferation.

1.2 Colorectal Cancer
Colorectal cancer is among the highest causes of cancer mortality in the Western world, being second only to lung cancer, and is responsible for some 400,000 deaths per year [Pisani P et al., 1993]. In 1998, in the United Kingdom (UK), there were 34,600 new cases of colorectal cancer [Office of National Statistics, 2001]. In the UK, colorectal cancer is the second most common cancer in females, after breast cancer, whilst it is the third most common cancer, after lung and prostate cancer, in males.

Overall, the incidence of colorectal cancer in men and women is the same, but colon cancer is more common in females whilst the incidence of rectal cancer is higher
amongst males. In 2000, there were 16,250 deaths from colorectal cancer in the UK, with higher mortality rates in men. The incidence trends for colorectal cancer in the UK differ in men and women. Whereas the incidence of colorectal cancer in men has increased by 20% since the late 1970's, female rates have remained fairly stable. Mortality is declining at similar rates for men and women [Office of National Statistics, 2001].

1.3 Current Chemotherapy used in Colorectal Cancer

Despite the majority of patients with colonic cancer apparently having complete microscopic clearance of their disease by resection, about half of these patients will recur at a distant site, which suggests that they had micrometastases present at diagnosis. Adjuvant chemotherapy (i.e. that given after surgery) is given to those patients who are considered at high risk of micrometastases to try and eradicate these circulating cancer cells before they become established. 5-Fluorouracil (5-FU) has been the principle drug of choice in a number of different schedules for the treatment of colorectal cancer since its introduction in the 1950s. It is a pro-drug which is converted intracellularly to various metabolites which inhibit the enzyme thymidylate synthase, thereby inhibiting DNA and RNA synthesis. However, 5-FU has modest activity as a single agent, and its activity is maximised by the addition of folinic acid (FA), which enhances the inhibitory effects of 5-FU on thymidylate synthase, and increases the tumour response rate, typically from 11% to 23% [Advanced Colorectal Cancer Meta-analysis Project, 1992].

When used in the adjuvant setting, 5-FU/FA chemotherapy results in approximately 6% increase in survival at 5 years [International Multicentre Pooled Analysis of Colon
Cancer Trials (IMPACT) investigators, 1995; O'Connell M et al., 1997. Therefore, new drugs have been developed with the aim of improving the outcome of those colorectal cancer patients who have undergone radical surgery. Oxaliplatin is one such chemotherapeutic agent. It is a platinum-based cytotoxic drug that forms DNA adducts, preventing replication and hence cell division, and in vitro studies show it to act synergistically with 5-FU in colorectal cell lines. A number of randomised controlled trials (RCTs) have shown improved tumour response rates (from under 23% to over 50%), and improved time to progression using this combined regimen of oxaliplatin/5-FU/FA in metastatic colorectal cancer, but they have failed to show an improved overall survival [De Gramont A et al., 2000; Giacchetti S et al., 2000; Levi FA et al., 1997]. An adjuvant study examining the benefit of 5-FU/FA/oxaliplatin (over 5-FU/FA alone) has reported a significant 3-year disease-free survival, but no overall survival benefit so far [De Gramont A, 2003]. Further follow-up data is probably needed before this regimen will become the standard adjuvant treatment in the UK.

Irinotecan is another drug which acts on a different molecular target. It is a topoisomerase I inhibitor, a DNA-unwinding enzyme essential for cell division. Irinotecan binds to the enzyme and stabilises the strand break. Multiple RCTs, in advanced colorectal cancer, using irinotecan and various schedules of 5-FU/FA have shown a statistically significant improvement in tumour response rate from around 20-30% with 5-FU/FA alone to approximately 40-50% with the addition of irinotecan [Douillard JY et al., 2000; Saltz LB and et al, 2000]. These same trials have shown a significant improvement in the time to progression and median survival by 2-3 months. The result of the adjuvant trial of irinotecan/5-FU/FA chemotherapy (ICCART) is still awaited, but on the basis of current data, this chemotherapy
regimen is licensed for adjuvant use already in a number of countries, including the United States of America.

Two oral pro-drugs of 5-FU, capecitabine and UFT (pro-drug fluorafur and uracil), are oral fluoropyrimidines that are licensed for use in the UK as alternatives to intravenous 5-FU, for use as first-line monotherapy in metastatic colorectal cancer. Currently, there are on-going studies investigating the substitution of infusional 5-FU for these new oral chemotherapies, in the adjuvant treatment of colorectal cancer.

Although a small proportion of patients with metastatic disease will be cured as a result of salvage resection of their metastases, the majority will die of their disease. So despite advances in surgical techniques, and chemotherapeutic strategies, colorectal cancer remains a major cause of cancer mortality. The modest improvements in survival seen with the newer chemotherapies, over the use of 5-FU/FA alone, suggests that there is not only a need to develop more effective drugs, but a need to combine the conventional chemotherapies with other agents that are active via alternative molecular pathways, for example receptor blockade and/or angiogenesis and enzyme inhibition.

1.4 Prognostic Factors in Colorectal Cancer

Currently, prognosis depends on pathologic staging of the disease. The Dukes' staging system is one of the frequently used systems in the UK (Table 1.1), and currently remains the most important independent prognostic factor [Dukes CE and Bussey HJR, 1958]. The five-year survival for colorectal cancer ranges from about 85% for Dukes' A patients to less than 5% for Dukes' D. However, the Dukes'
Table 1.1. Overall survival of patients with colorectal cancer according to Dukes' stage [National Health Service Executive, 1999]

<table>
<thead>
<tr>
<th>Dukes' Stage (modified)</th>
<th>Definition</th>
<th>Approximate frequency at diagnosis</th>
<th>5-year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Cancer localised within the bowel wall</td>
<td>10%</td>
<td>83%</td>
</tr>
<tr>
<td>B</td>
<td>Cancer which penetrates the bowel wall</td>
<td>35%</td>
<td>64%</td>
</tr>
<tr>
<td>C</td>
<td>Cancer spread to lymph nodes</td>
<td>26%</td>
<td>38%</td>
</tr>
<tr>
<td>D</td>
<td>Cancer with distant spread</td>
<td>30%</td>
<td>3%</td>
</tr>
</tbody>
</table>
staging system only allows stratification of the colorectal cancers into four (modified Dukes' system) prognostic groups, with significant tumour heterogeneity within some of these sub-groups and variable clinical outcomes. For example, Dukes' B cancers range from those which have only minimally breached the muscularis propria to those which have invaded into adjacent organs or other loops of colon. These tumours clearly will not have the same prognosis. However, when the difference between the depths of invasion of tumours is more subtle, the subjective opinions regarding their differences in prognosis become increasingly difficult.

The role of chemotherapy in the Dukes' B cancers remains unclear [International Multicentre Pooled Analysis of B2 Colon Cancer Trials (IMPACT B2) Investigators, 1999; Mamounas E et al., 1999], and currently there is no clear consensus on the routine use of adjuvant chemotherapy in this group of patients [International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators, 1995; International Multicentre Pooled Analysis of B2 Colon Cancer Trials (IMPACT B2) Investigators, 1999; Mamounas E et al., 1999]. The decision regarding adjuvant chemotherapy has often been based on the presence of factors indicative of a poor prognosis. Increasing age, poorly differentiated tumours and presence of lymphovascular invasion have been described as significant indicators of poor survival in colorectal cancer [Gastrointestinal Tumor Study Group, 1985]. Moreover, the presence of perforation or direct organ invasion has been shown to be an independent prognostic factor on multivariate analysis [Moertel CG et al., 1995b]. The work of Quirke's group demonstrated that involvement of the circumferential resection margin by residual microscopic disease was a significant indicator of local recurrence, independent of Dukes' stage [Quirke P et al., 1986; Quirke P and Dixon MF, 1988].
In contrast to the Dukes' B tumours, the role of adjuvant chemotherapy has been established in Dukes' C colorectal cancers in terms of conferring a significant survival benefit [Mamounas E et al., 1999]. However, the advantage of receiving adjuvant chemotherapy in Dukes' C colorectal cancer is such that approximately six patients will achieve a survival benefit for every one hundred patients given chemotherapy. Therefore, even in this group of patients there are a significant number of patients receiving chemotherapy for no survival benefit. Furthermore, it should be noted that in the Intergroup study of 929 patients with Dukes' C colorectal cancer, about 45% of patients treated with surgery alone were alive and disease-free after five years compared with approximately 65% who received adjuvant chemotherapy following surgery. This study indicated that nearly 50% of Dukes' C patients did not apparently require chemotherapy, whereas 45% of patients recurred in spite of the chemotherapy regimen given [Moertel CG et al., 1995a]. Thus, it is clear that even in the Dukes' C patients there is a great need for useful prognostic markers in order to identify those patients who need not be offered adjuvant chemotherapy. In the Dukes' B patients, the need for useful predictors of distant failure will help select those patients who may benefit from adjuvant therapy. The need for more individualised therapy becomes increasingly important with the advent of more toxic chemotherapeutic agents such as Irinotecan and Oxaliplatin. However, without improvements in the efficacy of chemotherapies, better selection of patients alone will not improve the survival rates of colorectal cancer patients.
1.5 Tumourigenesis and Molecular Markers

The development of colorectal cancer is thought to be dependent on multiple genetic events that involve alterations in specific genes - proto-oncogenes and tumour suppressor genes - that play an important role in the transition from normal mucosa through to the villous and more dysplastic adenoma, and then to frank carcinoma [Fearon ER and Vogelstein B, 2001]. The somatic alterations in these oncogenes include point mutations and alleleic losses on specific chromosomes. Gene amplification or re-arrangement does not seem to play a major role in oncogene activation in colorectal cancer [D'Emilia J et al., 1989].

A number of the genes involved in colorectal tumourigenesis have been identified – k-ras, FAP, DCC, and p53. Ras proteins belong to a family of 21 kilodalton (kDa) guanine nucleotide-binding proteins and are important in the control of cell growth and differentiation. The ras superfamily of proteins bind guanidine tri-phosphate (GTP) and hydrolyse it to guanidine di-phosphate (GDP), being active in the GTP-bound state and inactive in the GDP-bound state. Approximately 50% of carcinomas have been found to have k-ras gene mutations [Forrester K et al., 1987]. In contrast, such mutations are seen in fewer than 10% of adenomas less than 1 cm in size and the k-ras gene are thought to be an initiating event in a subset of colorectal cancers [Vogelstein B et al., 1988]. The loss of specific chromosomal regions (allelic loss) occurs frequently in colorectal cancer. This has been interpreted as evidence that these alleles carry tumour suppressor genes which are normally involved in regulating cell growth and differentiation such that when these genes are no longer present, the regulatory mechanism, and suppression of any neoplastic proliferation, is absent.
Chapter one

Introduction

An inherited predisposition to colorectal cancer occurs in familial adenomatous polyposis (FAP), in which hundreds of colorectal adenomas develop in an affected individual [Leppert M et al., 1987]. The locus linked to FAP has been mapped to chromosome 5q. The loss of a large portion of chromosome 17p has been seen in a large proportion of colorectal carcinomas, and this region contains the p53 gene, which is another tumour suppressor gene [Vogelstein B et al., 1988]; [Baker SJ et al., 1989]. The second most common region of allelic loss is chromosome 18q, which is lost in 70% of colorectal cancers [Vogelstein B et al., 1988]. The tumour suppressor gene identified in this region is the deleted in colon cancer (DCC) gene, which encodes a protein with significant homology to the cell adhesion family of proteins. It is thought that this gene is involved in the cell-cell and/or cell-matrix interactions, and that loss of this gene may affect these interactions.

Although the relative roles of these genes in colorectal tumourigenesis have been detailed [Fearon ER and Vogelstein B, 2001], currently there is still controversy over whether these molecular markers provide significant prognostic information prospectively, and whether this information is complimentary to that obtained by the pathologist. Another intracellular protein which has been extensively investigated for its prognostic role in colorectal cancer is thymidylate synthase (TS), which is a rate-limiting intracellular enzyme involved in the DNA synthesis pathway. Inhibition of TS has been the major focus of chemotherapeutic targeting in colorectal cancer, as it is the main intracellular target of 5-fluorouracil, the main chemotherapy drug used in the treatment of colorectal cancer. The majority of studies looking at TS overexpression in colorectal cancer have found it to be an independent prognostic factor [Johnston PG et al., 1994; Edler D et al., 2000; Lenz HJ et al., 1998]. Unfortunately, intratumoral levels of TS within individual tumours have been found to
be inconsistent, and in a subset of patients in whom multiple metastatic samples were taken, there was significant intra-patient heterogeneity of TS expression in the different metastatic sites [Aschele C et al., 1999]. Furthermore, in one recent large study, there was no association found between TS and survival in a group of Dukes' B patients, and so further evaluation is probably needed [Allegra CJ et al., 2002].

There are clearly a host of genes involved in the cancer phenotype. Identification of some of these genes whose protein products are either up-regulated or mutated in a high proportion of colorectal tumours may improve the current understanding of the tumour biology, and in turn may allow them to be used as prognostic markers. For example, changes in the expression or activity of various growth factors and growth factor receptors may also contribute to the pathogenesis of colorectal cancer [Salomon DS et al., 1992; Tahara E, 1990]. Investigating the overexpression of such growth factor receptors may help to determine whether they provide independent prognostic information. Furthermore, characterization of these receptors may also provide alternative therapeutic strategies such as antibody therapy or small molecule inhibitors targeting the receptor, either alone or in conjunction with conventional chemotherapies.

1.6 Epidermal Growth Factor Receptor Family

The epidermal growth factor receptor (EGFR) family, also known as the erbB family, are a family of structurally related tyrosine kinase receptors, and comprises epidermal growth factor receptor (EGFR or HER-1), Human Epidermal Growth Factor Receptor-2 (HER-2), HER-3, and HER-4. These four proteins are widely expressed in epithelial, mesenchymal and neural tissues and play an important role
during development [Gassman M et al., 1995; Sibilia M and Wagner EF, 1995]. They are involved in the complex signal transduction cascade that regulates normal cell growth, differentiation, and survival of cells [Hung MC and Lau YK, 1999; Yarden Y and Sliwkowski MX, 2001].

Overexpression of EGFR is a common feature of human epithelial tumours, and has been reported in squamous cell carcinoma of the head and neck [Stanton P et al., 1994], oesophagus [Itakura Y et al., 1994], non-small-cell lung cancer [Rusch V et al., 1993], and endometrial adenocarcinoma [Khalifa MA et al., 1994]. Furthermore, its overexpression has been associated with a poor prognosis in patients with carcinoma of the lung, bladder, ovary and pancreas [Gullick WJ, 1991; Neal DE et al., 1990]. In colorectal cancer also it has been reported that overexpression of EGFR may indicate advanced stage of disease [Gross ME et al., 1991] and may predict for metastatic potential [Radinsky R et al., 1995].

HER-2 is over-expressed to varying degrees in a number of human malignancies, including breast, ovary, bladder, prostate and stomach [Gullick WJ, 1990; Ménard S et al., 2001]. HER-2 is a 185 kilodalton transmembrane glycoprotein, also designated p185^HER-2, and is encoded by the c-erbB-2 gene, a proto-oncogene mapped to chromosome 17q21 [Coussens L et al., 1985]. HER-2 gene overexpression and/or amplification leads to continuous activation of HER-2 receptors, and is thought to play a pivotal role in transformation and tumourigenesis [Di Fiore PP et al., 1987; Hudziak RM et al., 1987].

The erbB receptors comprise three structural domains: an extracellular binding domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain. The
EGFR family signaling is complex and involves a large number of ligands. EGFR is activated by seven ligands: epidermal growth factor (EGF), transforming growth factor α (TGF-α), amphiregulin, heparin-binding EGF-like growth factor, betacellulin, epieregulin and the novel member epigen [Strachan L et al., 2001]. HER-2 shares close (50-70%) structural homology with the EGFR. However, unlike EGFR, no ligands that directly bind to HER-2 protein have been identified. It is thought that HER-2 is trans-activated through heterodimerisation with other members of the epidermal growth factor family of growth receptors [Olayioye MA et al., 2000]. HER-3 and HER-4 receptors bind heregulins, which are complex groups of proteins [Tzahar E et al., 1994; Pinkas-Kramarski R et al., 1997].

By binding to the extracellular domain of their respective receptors, the ligands induce receptor homodimers with the same receptor type (e.g. EGFR-EGFR), or heterodimers with a different receptor of the same family (e.g. HER-1-HER-2). Consequently, although HER-2 does not have a known ligand, this complex heterodimerisation between the various members of this family of cell surface receptors results in transactivation, with tyrosine phosphorylation of HER-2. HER-2 is thought to be the preferred dimerisation partner [Tzahar E et al., 1996]. Signaling through the EGFR family of receptors triggers a powerful network of down-stream cellular pathways, resulting in a range of responses including cell division, invasion, and cell death [Klapper LN et al., 2000; Yarden Y et al., 2001].
1.7 Human Epidermal Growth Factor Receptor-2

1.7.1 Biological Significance

Various animal and in vitro studies strongly indicate that HER-2 plays an important role in oncogenic transformation, tumourigenesis, and metastasis [Benz CC et al., 1993; Chazin VR et al., 1992; Hudziak RM et al., 1987]. Furthermore it has been shown that the growth of HER-2-positive human breast cancer cell lines and xenografts is inhibited by anti-HER-2 monoclonal antibodies [Harwerth IM et al., 1993].

HER-2 has no known ligands, but is activated by transactivation following HER homo- and heterodimer combinations. Furthermore, those dimerizations that contain HER-2 are long-lived and transmit strong signals, and are thus associated with malignant growth [Rubin I and Yarden Y, 2001]. The reasons for this increased potency are thought to be two-fold. First, the heterodimers are characterised by relatively slower ligand dissociation [Alroy I and Yarden Y, 1997], and second, HER-2 is a slowly internalising receptor compared to EGFR whose ligand-induced endocytosis is rapid [Baulida J et al., 1996; Pinkas-Kramarski R et al., 1996]. Thus activation of the cell signal transduction pathway via HER-2-containing heterodimers results in an enhanced signal. Transmembrane activation of HER-2 results in intrinsic protein tyrosine kinase activation of the intracellular domain. Consequently, tyrosine autophosphorylation of cytoplasmic signal proteins occurs and a cascade of different signalling pathways can be activated, transmitting signals across the cytoplasm to the nucleus. The complex system of heterodimerization of HER-2 gives a greater potential for diversification of the biologic messages and helps to explain how HER-2 plays a key role in oncogenic transformation in certain tumours.
1.7.2 Indicators of Increased HER-2 Production

Normal cells possess two copies of the HER-2 gene and have normal/low levels of HER-2 expression on the cell surface. HER-2 overexpression can occur through several different mechanisms:

1. Gene amplification - multiple copies of the HER-2 gene are generated in the nucleus due to unknown dysregulation of DNA replication
2. Increased transcription of HER-2 gene(s). This can occur following amplification of the gene resulting in additional copies of HER-2, or by post-transcriptional up-regulation of the mRNA.

The elevated levels of HER-2 DNA and mRNA in turn can result in the overexpression of HER-2 on the cell surface.

The need to establish the HER-2 status of a tumour has been particularly important in breast cancers, where its prognostic role has been established. But, its level of overexpression has been investigated in many other human tumours which have also looked at the association between HER-2 and clinicopathological parameters. The most practical application of HER-2 testing would be the ability to predict the response to treatment and to guide treatment decision-making. With the availability of trastuzumab (Herceptin™), the first monoclonal antibody (mAb) approved for treatment of patients with metastatic breast cancer, the HER-2 status of tumours has gained increased therapeutic importance. However, even in breast cancer, a consensus regarding the optimum method for HER-2 detection in standard practice has not yet been achieved [Larsimont D et al., 2002].
A number of different techniques exist to measure the level of HER-2 expression. These assays target different aspects of the HER-2 overexpression pathway: gene copy (fluorescence in situ hybridization [FISH] or polymerase chain reaction [PCR]), mRNA (PCR), cell surface protein (immunohistochemistry [IHC] or Enzyme-linked immunoadsorbent assay [ELISA]) and circulating receptor protein (ELISA). Each of these techniques has drawbacks.

Tissue-based detection of HER-2 overexpression by FISH and/or IHC has clear advantages over other techniques such as Western blot and PCR, which suffer from dilutional artefacts due to the mixture of normal and abnormal cells.

FISH is an assay that allows visualisation of HER-2 DNA in individual cells using a specific fluorescence-labelled probe. This assay can be used to look at gene amplification in individual tumour cells and this is one of its advantages.

The advantage of IHC and FISH is that they allow maintenance of the tissue architecture, and both can be carried out on formalin-fixed paraffin embedded tumour sections. IHC can additionally be performed on freshly frozen tissue. IHC and FISH assays both have their own level of diagnostic sensitivity and specificity. The FISH assay is said to be more convenient as it only requires small tissue samples. However, it is more time-consuming and costly, and necessitates particular expertise and equipment which may currently not be present in most histopathology departments [Jacobs TW, 2001; Jimenez RE et al., 2000].

Immunohistochemistry is the most frequently used assay to determine HER-2 overexpression in tumour tissue. The advantages are that the technique is simple
and cheap, and the tissue sample can be kept for reference, unlike the FISH assay which decays with time, leaving no permanent record. The disadvantage of IHC is that the reliability of the technique is dependent on a number of variables. Differences in the methodology used, fixation protocols, anti-HER-2 antibodies, and scoring systems can strongly influence the consistency and reproducibility of results [Press MF et al., 1994; van de Vijver MJ, 2001].

It is important to realise that the sequence of HER-2 gene amplification leading to increased HER-2 mRNA and protein overexpression is not necessarily linked causally or sequentially. For example, it has been shown that in a minority of breast tumours it is possible to have HER-2 overexpression in the absence of HER-2 gene amplification [Pauletti G et al., 1996]. It is precisely for this reason that in colorectal cancer, FISH may not be the most appropriate way of detecting HER-2 overexpression. Although the level of HER-2 overexpression in colorectal cancer is quoted as 20-35% in the literature (and may be higher), gene amplification in colorectal cancer is a relatively rare event, since it has been observed in a low percentage (about 7%) of cases [D'Emilia J et al., 1989]. Under these circumstances, FISH would not be the ideal method of detection of HER-2 overexpression in colorectal cancer, as it suggests that post-transcriptional up-regulation of the HER-2 gene may account for HER-2 overexpression in these tumours.

Furthermore, this discrepancy seen between IHC and FISH highlights a potential problem with using FISH to measure the level of overexpression of the receptor product of a gene. That is, gene overexpression does not necessarily translate into overexpression of the end protein product. It is possible for post-translational events
to prevent full translation of the gene copies into a fully functional receptor [Anderson L and Seilhamer J, 1997]. In addition, it is the over-expressed HER-2, and not its gene, which is ideal for the selection of suitable patients for mAb-based therapy.

In the UK, the current laboratory practice for HER-2 testing in breast cancer recommends that HER-2 overexpression is by IHC with HercepTest™ (DAKO, Glostrup, Denmark), which is the only standardised, US Food and Drug Administration (FDA) -approved test for HER-2 evaluation [Jacobs TW et al., 1999]. FISH is only recommended in cases with moderate staining after HercepTest™ (score of 2+), to confirm the HER-2 status [Larsimont D et al., 2002].

1.7.3 The Prognostic Role of HER-2 in Human Cancers

HER-2 gene amplification and/or protein overexpression has been described in a variety of human tumours including carcinoma of the breast [Slamon DJ et al., 1987], ovary [Slamon DJ et al., 1989], endometrium [Hetzel DJ et al., 1992], stomach [Yonemura Y et al., 1991] and bladder [Coombs LM et al., 1991]. Although the clinical significance of HER-2 overexpression is uncertain in many human tumours, its prognostic role has been established in breast carcinoma.

In breast tumours, HER-2 overexpression is reported in 40-60% of intraductal carcinomas [van de Vijver MJ et al., 1988] and in 20-30% of invasive cancers [Gullick WJ, 1990; Ménard S et al., 2001]. Studies have shown that a strong positive membranous HER-2 staining of the tumour is associated with an unfavourable prognosis in node-positive patients [Slamon DJ et al., 1987; Toikkanen S et al., 1992], and, furthermore, HER-2-positive tumours may be less responsive to
hormonal therapy [Newby JC et al., 1997]. This has led to the therapeutic use of anti-
HER-2 monoclonal antibody therapy in breast cancer patients with metastatic
disease, whose tumours are strongly HER-2 positive.

HER-2 overexpression has also been shown to be an independent prognostic
indicator in gastric cancer [Yonemura Y et al., 1991]. However, studies looking at the
prognostic utility of HER-2 overexpression in colorectal cancer have reached
conflicting conclusions, and an overview of the current data is given below.

1.7.4 The Prognostic Role of HER-2 in Colorectal Cancer

Several studies have investigated the prognostic role of HER-2 in colorectal
cancer, and these studies report different levels of HER-2 overexpression, and
furthermore, the localisation of the immunostaining (i.e. membranous or
cytoplasmic) varies. For example, moderate to strong membrane HER-2 expression
in these studies ranged from 1.4% to 81.9% [Kakani V et al., 2002; McKay JA et al.,
2002]. In addition, while membrane HER-2 was associated with a poor prognosis in
one study [Knosel T et al., 2002], others showed no such association [Benecke M et
al., 2002; Chamberlain NL et al., 1999; Kakani V et al., 2002; McKay JA et al.,
2002]. Paradoxically, a further study found membrane HER-2 overexpression to be
associated with a better prognosis in aneuploid tumours [Sun X-F et al., 1995].

Surprisingly, one study looking at HER-2 overexpression in Dukes' B colorectal
cancers, reported no evidence of membrane HER-2 overexpression in the 164
tumours examined. However, 33.5% of the cases in this study showed cytoplasmic
HER-2 immunostaining, and this was found to be an indicator of poor clinical outcome in Dukes' B cancers [Kay EW et al., 1994b].

McKay et al performed a semi-quantitative assessment of HER-2 overexpression, according to the FDA-approved scoring system [Jacobs TW et al., 1999]. They found no significant association between clinical outcome and positive membrane HER-2 in 249 colorectal tumours [McKay JA et al., 2002]. However, the FDA-approved scoring system makes no provision for cytoplasmic HER-2 assessment, and so its incidence in this study is not reported. Although membrane HER-2 alone has been shown to be of prognostic value in breast cancer, this may not be the only important criterion for assessing HER-2 overexpression in other tumours. There have been studies in breast [Zschiesche W et al., 1994] and thyroid cancer [Sugg SL et al., 1998] which have shown an association between good prognostic tumours and cytoplasmic HER-2.

Kapitanovic et al reported mixed cytoplasmic and membrane HER-2 immunostaining in their cohort of colorectal cancers, and found that those cases with moderate or strongly positive tumours had a significantly poorer survival than those with weakly positive tumours [Kapitanovic S et al., 1997]. In contrast, Chamberlain et al found no association between cytoplasmic or membrane HER-2, and survival in 96 tumours from an Australian cohort of patients [Chamberlain NL et al., 1999].

In colorectal cancers, the precise significance of membranous or cytoplasmic HER-2 immunostaining has yet to be fully elucidated. Current studies have yielded conflicting results as outlined above. A few investigators have suggested that cytoplasmic HER-2 overexpression is significant and may bear a correlation with
survival, but this is still controversial. The current study will help to define the potential prognostic role, if any, of HER-2 (whether cytoplasmic or membranous) in localised colorectal cancer.

1.8 Microvessel Density and Human Cancers

Angiogenesis, the formation of new vessels from pre-existing ones, is an essential normal process in embryogenesis, menstruation and wound healing. However, aberrant angiogenesis is an integral part of tumour growth, invasion, and ultimately metastatic spread of cancers [Folkman J, 1990]. In the absence of angiogenesis, the tumour enters a dormant phase, whereby the rate of cell proliferation is balanced by apoptosis. It is widely believed that this dormant phase forms a significant part of a tumour's lifespan. During this early period of tumour growth, the cancerous cells derive their oxygen and nutrition through processes such as simple diffusion. Beyond a certain size (1-2 mm), however, diffusion is no longer adequate and angiogenesis is needed for a number of reasons [Folkman J, 1990]. Firstly, neovascularisation provides the infrastructure to transport oxygen and other nutrients to the tumour for growth. Secondly, the formation and growth of microvessels within, and beyond the tumour into the surrounding tissues, provides the means of metastatic spread [Weidner N et al., 1991]. Finally, angiogenesis may also work through the paracrine effects of endothelial cells, with the release of growth factors that stimulate tumour growth and new blood vessel formation [Folkman J, 1994].

Several reports have demonstrated that colorectal cancers and other cancers produce a number of different angiogenic growth factors. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, seems to be one
of the major factors responsible for tumour angiogenesis in many cancers [Claffey KP and Robinson GF, 1996; Zhang HT et al., 1995]. VEGF is a 34-42 kDa multifunctional glycosylated dimeric protein, and is expressed in 4 isoforms derived by alternative mRNA splicing [Houck KA et al., 1991; Tischer E et al., 1991]. It binds to specific receptors, namely VEGF-receptor 1 (flt-1) and VEGF-receptor 2 (flk-1/kdr), which are tyrosine kinase receptors expressed on endothelial cells [Ferrara N and Davis-Smyth T, 1997]. VEGF expression can be determined by the analysis of mRNA levels or by immunohistochemistry with anti-VEGF antibodies, in fresh or paraffin-embedded tumour sections [Vermeulen PB et al., 1996]. However, other angiogenic factors have also been implicated, and these include basic fibroblast growth factor, platelet-derived growth factor (PDGF), angiogenin, and thymidine phosphorylase [Papamichael D, 2001].

The mechanism whereby some tumours remain locally invasive for long periods whereas others, of similar size, metastasize early is still not fully understood. However, it is clear that there is a well demarcated transformation from the pre-angiogenic phase of a tumour to the angiogenic phase. The pre-vascular phase is usually associated with limited tumour growth and a low probability of developing metastases. In the vascular phase, there is a clear transformation in the biological behaviour of the tumour with rapid growth, possible bleeding, and the potential for metastasis. This is well illustrated in studies looking at cancers of the cervix [Sillman F et al., 1981] and breast [Brem SS et al., 1977], and cutaneous melanoma [Srivastava A et al., 1988; Herlyn M et al., 1987;].

The first evidence that tumour vascularity could predict for the development of metastases was reported in cutaneous melanoma [Srivastava A et al., 1988]. Since
then, a number of studies in breast cancer have shown that a quantitative estimation of microvessel density (MVD) can be shown to be associated with a poor prognosis in both node positive and node negative disease [Weidner N et al., 1992; Giatromanolaki A et al., 1999]. MVD has also been shown to be of prognostic significance in non-small cell lung cancer [Macchiarini P et al., 1992], and stomach [Tanigawa N et al., 1996] and prostate [Weidner N et al., 1993] cancers.

Many of the quantitative studies investigating the prognostic role of MVD in tumours have been conducted by marking endothelial cells with specific antibodies, using immunohistochemical staining methods. In these studies, tumour vascularization, assessed by counting vessels immunostained for Factor VIII-related antigen (F8RA, also known as von Willebrand factor) or endothelial cells, CD31 or CD34, was correlated with the incidence of metastases and/or survival [Takebayashi Y et al., 1996; Tanigawa N et al., 1997; Vermeulen PB et al., 1999].

1.8.1 Microvessel Density and Colorectal Cancer

Over the last decade, a number of studies have attempted to investigate the potential prognostic role of angiogenesis in colorectal cancer, using various angiogenic markers as described above.

Although there have been a number of studies which have used anti-F8RA mAbs to quantitatively assess tumour vascularity, increasingly anti-CD31 and anti-CD34 mAbs are being utilised because of the increased specificity of the latter antibodies for endothelial cells when using paraffin sections [Vermeulen PB et al., 1996; Tanigawa N et al., 1996]. The adverse impact of tumour angiogenesis in colorectal
cancer in terms of relapse or prognosis has been investigated using one or more of these factors. These previous studies have however reached conflicting conclusions.

Four of these studies showed a significant association between microvessel count and survival [Choi HJ et al., 1998; Takebayashi Y et al., 1996; Tanigawa N et al., 1997; Vermeulen PB et al., 1999]. Although two other studies also showed an association between vessel counts and survival, this was only on univariate analysis [Frank R et al., 1995; Saclarides TJ et al., 1994].

Saclarides et al did not show any association between MVD and metastases. In this small series of only 48 patients with rectal cancer, they found significantly higher angiogenesis scores (mean±SD) in patients who died before five years (18.4 ± 5.2), compared to the MVD in tumours of survivors at this same time interval (14.7 ± 5.3) [Saclarides TJ et al., 1994]. Frank et al. had similar results with significantly higher angiogenesis scores for patients with recurrent disease, and there was also a better survival seen in patients with a MVD of less than 28. Another large study which evaluated 133 colorectal tumours (stages Dukes' A-D), found a significant association between MVD and overall survival, and the tumour vessel count was also the most important prognostic factor [Tanigawa N et al., 1997].

Takahashi et al demonstrated that VEGF expression in Dukes B (i.e. node negative) colon cancers was significantly associated with time to recurrence. However, but there was no association with survival, and only 27 patients were included in this study [Takahashi Y et al., 1997].
Chapter one

Introduction

MVD has also been demonstrated prospectively to be an independent prognostic factor in 116 Dukes' A-C colorectal cancers, using the JC-70 anti-CD31 mAb [Vermeulen PB et al., 1999]. No such association was found in the Dukes' D patients, and there was also no correlation between MVD and the presence of lymph node metastases detected at surgery. In another series of 133 patients, there was again a very strong association between MVD and both haematogenous spread of disease and survival [Tanigawa N et al., 1997]. In this study, MVD was not associated with lymph node or peritoneal metastases, suggesting a different mechanism in the development of these metastases.

Choi et al looked at the level of angiogenesis in 127 colorectal cancer specimens, using F8RA. They found a significant association between high MVD and liver metastases (p=0.0004), and MVD was also associated with many of the conventional pathological variables, such as histologic grade, lymphatic and vascular invasion, and Dukes' stage. On multivariate analysis, MVD was found to be an independent prognostic marker (p=0.0004). They concluded that MVD, as measured by F8RA immunostaining, did reflect the biological malignant potential of colorectal cancers [Choi HJ et al., 1998].

There were also two negative studies which did not show MVD to have any prognostic role in colorectal cancer [Cianchi F et al., 2002; Pietra N et al., 2000]. In one of these studies, looking at node-negative patients, the investigators found a significant association between high MVD and lymphocytic infiltration of the tumour [Cianchi F et al., 2002]. The presence of conspicuous lymphocytic infiltration in a tumour is considered to be an effective cell-mediated immune response against the tumour, and has been deemed a favourable pathological factor, and therefore may
have interfered with its predictive value. Nonetheless, a large study looking at 212 patients showed a completely opposite result from that previously reported. They demonstrated, on univariate analysis, a significantly longer survival in those patients whose tumours had a higher MVD [Lindmark G et al., 1996].

1.9 Molecular Targeting in Cancer

Tumour growth, invasion and progression to metastasis is a complex process which involves angiogenesis, and the induction of various growth factors such as the family of epidermal growth factors and vascular epidermal growth factors, and other stimulators of this process [Fox SB et al., 2001]. In the quest to improve the overall survival of colorectal cancer patients after radical surgery, there has been a recent significant focus on the development of agents which target the numerous pathways involved in tumour angiogenesis, invasion and metastasis.

1.9.1 Signal Transduction Pathways

The EGFR pathway is aberrant in up to 75% of colorectal cancers [Nicum S et al., 2003]. Thus, agents that target the extracellular ligand-binding regions and the intracellular tyrosine kinase domain have been investigated to try and abrogate the mitogenic signals mediated by this receptor. Cetuximab, a selective anti-EGFR mAb, has been shown to produce significant activity in combination with Irinotecan chemotherapy, in patients whose colorectal cancers were previously refractory to Irinotecan-based chemotherapy [Saltz L et al., 2001]. Cetuximab has now been approved by the US Food and Drug Administration for the treatment of metastatic
colorectal cancer in combination with chemotherapy (February 2004), and has entered phase III trials both in the adjuvant and metastatic setting in the UK.

Three very closely related proteins in the ras family, H-ras, K-rasB and N-ras, are primary regulators of cell growth. Mutations in the k-ras protein are found in about 50% of colorectal tumours and are believed to play an important part in tumourigenesis [Breivik J et al., 1994]. A wide variety of growth factors result in a rapid and transient elevation of active GTP-bound ras proteins. These include EGF and PDGF [Soler C et al., 1994]. Thus it seems that ras proteins are essential components of the tyrosine-mediated mitogenic signaling pathways.

Post-translational modification of the ras proteins is essential for its effective functioning, and the enzyme farnesyl protein transferase (FPTase) plays an important part in this process. Oncogenic ras proteins lose their transforming ability when farnesylation is prevented. Thus FPTase inhibitors have been investigated in metastatic colorectal cancer patients who have failed conventional chemotherapy. Unfortunately, the results so far have been disappointing [Verslype C et al., 2001].

1.9.2 Immunotherapy

In the adjuvant setting, a study looking at the effect of a monoclonal antibody 17-1A, that binds a tumour-specific antigen, in Dukes' C patients reported no advantage from the addition of 17-1A [Gambill BD, 2001].

Other methods of targeting tumour-associated antigens have included vaccination against carcinoembryonic antigen (CEA), an oncofetal antigen expressed in about
85% of patients with colorectal cancer. Trials are currently on-going in the adjuvant setting, as phase I trials have not demonstrated any significant toxicity [Cole DJ et al., 1996].

Antibody directed enzyme prodrug therapy (ADEPT) is another form of targeted therapy in which a prodrug is selectively activated at the tumour site via an enzyme which is conjugated to an antibody, targeted to the specific tumour. Initial trials have shown evidence of tumour response, and further studies are on-going [Francis RJ et al., 2002].

1.9.3 Anti-Angiogenesis Therapy
The ultimate target of anti-angiogenic therapy is the tumour blood vessel. They are attractive as therapeutic targets because they express proteins not expressed on normal blood vessels. Bevacizumab (BV), a humanized mAb that targets and binds VEGF, has been shown to prolong survival in a phase II clinical trial of colorectal cancer [Kabbinavar F et al., 2003]. This would seem to establish the validity of the anti-angiogenic approach in colorectal cancer. However, there is no evidence to suggest that tumour VEGF expression is predictive of clinical response. Furthermore, a consensus on the method of assessing tumour neoangiogenesis has yet to be agreed widely.

1.9.4 Cyclo-Oxygenase Type 2 (Cox 2) Pathway
Non-steroidal anti-inflammatory drugs (NSAIDs), such as sulindac, have been shown to have an anti-tumour effect, thought to be mediated through the COX 2
pathway [Gasparini G et al., 2003]. The COX 1 pathway is responsible for the gastrointestinal side-effects of this class of drugs. Selective COX 2 inhibitors have also been shown to inhibit angiogenesis and induce apoptosis in colorectal cancer cells [Elder DJ et al., 1997]. The selective COX 2 inhibitor, Rofecoxib is currently being assessed as an adjuvant treatment in a phase III double-blinded placebo-controlled trial in colorectal cancer patients. The aim of the study is to see if Rofecoxib, given for 2 or 5 years, improves the relapse-free and overall survival in Dukes' C colorectal cancer patients following completion of their conventional adjuvant treatments (i.e. chemotherapy and/or radiotherapy).

1.10 Aims of the Study

The aim of adjuvant chemotherapy in colorectal cancer is primarily to improve overall survival. Currently the selection of patients for whom chemotherapy offers a survival advantage is mainly determined by the presence of regional nodal metastases in the histological specimen (i.e. Dukes' stage). There is a need for better prognostic markers to enable selection of a poor prognostic group, and to avoid the over-treatment of a large number of patients.

HER-2 has been shown to be a significant prognostic marker in breast cancer, and may also predict response to therapy. It has therefore been used in breast cancer to tailor adjuvant therapy.

In colorectal cancer, a number of studies have investigated the role of HER-2 and microvessel density as prognostic markers, but they have had conflicting results. In view of the controversies, the aim was to investigate the expression and prognostic
significance of these two parameters in patients with colorectal cancer, and their association with clinico-pathological factors.
Chapter Two

Materials and Methods

2.1 Human Tumour Cell Lines

A panel of human colorectal, breast, and head and neck tumour cell lines were used in this study. Of these, the human colorectal tumour cell lines CCL218 (HT29), CCL225, CCL228, CCL244 were obtained from the American Tissue Culture Collection (ATCC), Massas, USA. The human colorectal cell line DiFi was a gift to Dr Helmout Modjtahedi from Dr Zhen Fan at the MD Anderson Cancer Centre, Houston, USA.

The following cell lines were used as controls in this study: Human breast (SKBR3) and ovarian (SKOV3) cancer cell lines that express high levels of HER-2, a head and neck (HN5) cell line which expresses high levels of EGFR, and a breast carcinoma (MCF-7) cell line that expresses low levels of EGFR and HER-2 [Cowley G et al., 1984; Modjtahedi H et al., 1998; Boente MP et al., 1998; Lewis GD et al., 1993; Modjtahedi H et al., 1993a].

2.2 Cell Culture

2.2.1 Culture Media and Foetal Calf Serum

Dulbecco's Modified Eagles medium (DMEM) and Penicillin-Streptomycin-Neomycin solution (100x) were obtained from Sigma- Aldrich Co. Ltd, Dorset, Uk. Foetal Calf Serum (FCS) was obtained from the PAA Laboratories Ltd (Somerset) and was heat inactivated at 56°C for 50 minutes before use. All the human tumour cell lines were
cultured routinely in DMEM supplemented with 10% FCS, penicillin (50 units per mL), streptomycin (50μg/mL) and neomycin 100μg/mL, as described previously [Modjtahedi H et al., 1998]. All cultures were incubated at 37°C in an atmosphere of 5% CO₂. Depending on the quantity of the cells required, they were cultured in large (175cm²), medium (75cm²) or small (25cm²) flasks.

2.2.2 Passaging and Growth of Tumour Cell Lines
Cells were cultured in 20-25 mL DMEM/10%FCS in 75cm² flasks, as described above. Once confluent, the cells were passaged weekly by first discarding the old medium. They were then incubated with 0.02% trypsin in phosphate-buffered saline (PBS), containing 0.04% Na₂EDTA, for 3-5 minutes, at 37°C. This trypsinisation time was variable depending on the cell line used, and sometimes needed extension of the incubation time, and/or an increase in the trypsin concentration (e.g. HN5 cells).

Once the cells were suspended, the trypsin was inactivated by the addition of 1.0 mL of FCS. The cell suspension was then pelleted by centrifugation for 3 minutes, at 1600-1800 rev/min. The cells were then re-suspended in fresh DMEM/10%FCS in a culture flask, and all cultures were incubated at 37°C, in an atmosphere of 5%CO₂. For growth inhibition assays, in order to reduce the effect of endogenous growth factors present in the FCS, the concentration of FCS was reduced to 2%.

2.2.3 Long Term Storage of Cells
Samples of human tumour cell lines were stored at -135°C in liquid nitrogen. Sub-confluent cells were trypsinised and centrifuged into a pellet as described above.
Chapter two

Materials and Methods

After discarding the supernatant, the cells were re-suspended in freezing medium, which constituted 93% of FCS mixed with 7% dimethyl-sulphoxide (DMSO). One millilitre aliquots of cells were then transferred to 1.5 mL freezing vials (Nunc, Gibco Europe Ltd), which were then frozen initially at -80°C for 24 hours, and then transferred to liquid nitrogen for long term storage.

When the cells were required again, the particular vial was removed from the liquid nitrogen, and thawed quickly at 37°C. The cells were then re-suspended gently, and diluted, in 20 mL of DMEM/10%FCS. After centrifugation, the cells were washed once more with 20 mL of fresh medium, and then plated into a 75 cm² flask.

2.3 Antibodies

The rat mAb ICR12 (IgG2a) was raised against the external domain of HER-2 using the human breast carcinoma BT474 as the source immunogen. This antibody was a kind gift from the late Dr Christopher Dean (The Institute of Cancer Research, Sutton, UK) to Dr Helmout Modjtahedi [Dean C et al., 1993; Styles JM et al., 1990]. The rat mAb HM64.13 (IgG2b) was raised against the external domain of HER-2 on the human breast carcinoma cell line SKBR3 by Dr Helmout Modjtahedi.

To assess microvessel density, the anti-CD31 monoclonal antibody (JC70) was obtained from DAKO (Glostrup, Denmark). The rat mAbs ICR16 and ICR62 are directed against the external domain of the EGFR and were produced and characterised as described previously [Modjtahedi H et al., 1993b; Modjtahedi H et al., 2003].
Chapter two Materials and Methods

The goat anti-rat IgG polyclonal antibody (Star 71), for use in radioimmunoassay, and the biotinylated rabbit anti-rat immunoglobulin, for use in the immunohistochemistry, were purchased from Serotec Ltd., (Oxford, UK) and DAKO respectively. For use in the radioimmunoassay, the Star 71 was also labelled with Iodine-125 to a specific activity of 10μCi/μg using the Iodogen procedure, as described previously [Modjtahedi H et al., 1993b]. The goat anti-rat IgG conjugated to Fluorescein Isothiocyanate isomer (FITC) (Star 69), for use in the fluorescence-activated cell sorter (FACS) analysis was obtained from Serotec Ltd., (UK).

Biotinylated goat anti-mouse and anti-rabbit secondary antibody was used in the CD31 immunohistochemistry procedure, and this was purchased from DAKO.

2.4 Other Chemical Reagents

The CD31 immunostaining was performed using the reagents recommended by the automated system, Dako TechMate™ 500 Plus. These were all ChemMate™ products (DAKO) and included the antibody diluent, the ‘negative control’ reagent, and the Washing Buffers 1-3, and Water Wash.

The EGFR tyrosine kinase inhibitor (TKI), gefitinib, was a gift from AstraZeneca to Dr H Modjtahedi.
Chapter two

Materials and Methods

2.5 Immunoprecipitation of $^{35}$S-Methionine-labelled Proteins

Specific cellular proteins were isolated from radiolabelled cell extracts, by precipitation with antibody, and analysed by electrophoresis in polyacrylamide gels containing sodium dodecyl-sulphate (SDS). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is a technique which is routinely used to determine the molecular weight of a particular protein. Because the migration of the protein is determined by its molecular weight, shape and electrical charge, the electrophoretic mobility of a particular protein through the polyacrylamide gel will be inversely proportional to its molecular weight. By using a standard marker of known molecular weight, it is therefore possible to determine the molecular weight of the target protein.

2.5.1 Preparation of Radiolabelled Cell Lysate

The human breast carcinoma cell line SKBR3, overexpressing the HER-2 antigen, were grown in 10% DMEM in large (175 cm$^2$) flask until they were sub-confluent. The cells were then washed once with 10mLs of Methionine-Cysteine free DMEM (Sigma) containing 10% FCS, and the medium then discarded. The cells in each flask were then incubated, at 37°C, with 10 mL of the same medium for 1 hour, prior to overnight incubation at 37°C in the presence of 500µCi of Tran$^{35}$S-Label$^\text{TM}$ (ICN).

After radiolabelling of the cells, the cell lysate was then prepared by adding 10 mL of lysing buffer (PBS azide containing 1% Triton X-100 and 1mM PMSF) for 30 minutes at 4°C. The cell lysate was then transferred to Ultra-Clear centrifuge tubes (Beckman) and centrifuged at 30,000 r/m for 30 minutes in a UltraCentrifuge
Chapter two Materials and Methods

(Beckman). The cell lysate was then collected and either stored at –80°C or used in
the immunoprecipitation study (see section 2.5.3).

2.5.2 Preparation of Immunoadsorbent Beads

Immunoadsorbent beads were prepared by linking the anti-HER-2 antibody to
activated Protein G Sepharose 4 Fast Flow at a concentration of 5μg of each mAb
per 100μl of Protein G (Amersham Pharmacia Biotech). Briefly, 400μl of Protein G
was mixed with 80μl of each primary antibody (80μg) or 80μl of PBS (control) in
1.5mL eppendorf tube and the total volume of each tube was adjusted to 1mL using
0.5% PBS azide. Following overnight incubation on a rotator at 4°C, the
immunoadsorbed beads were then centrifuged at 500 rpm for 1 min and the
supernatant discarded. After three washes with 1mL 0.5% PBSA, the
immunoadsorbent beads were re-suspended in 0.5% PBS-azide washing buffer for
use in immunoprecipitation study.

2.5.3 Immunoprecipitation

Immunoprecipitates were prepared by incubating 1 mL aliquots of radiolabelled cell
extract with 100 μl of antibody-linked beads in an eppendorf for 2 hours at room
temperature. Each tube was then centrifuged at 500rpm for 1 min, the supernatant
was discarded. The immunoprecipitates in each tube were then washed four times
with 500 μL of PBS azide and then mixed with equal volume of SDS-sample buffer
(see section 2.5.4), heated at 95°C for 5 minutes. 50 μL aliquots of the sample were
then loaded carefully into the individual wells of the SDS gel. The molecular weight
standard (MultiMark™), which is pre-reduced, was loaded into the first well for reference.

2.5.4 Materials for SDS-PAGE

Molecular Weight Marker:
Tricine MultiMark™ Multi-coloured Standard (Invitrogen LifeTech).

Acrylamide Stock Solution
30% Acrylamide (37.5:1) Bisacrylamide solution was obtained from Northumbria Biological Ltd. (UK).

Sample Buffer (reducing condition):
Stacking buffer 50 mL  
SDS 4 g  
Dithiotheritol 2 g  
Glycerol 10 mL  
PMSF (0.4M in ethanol) 5 mL  
H₂O: 35 mL  
Bromophenol blue: trace

Resolving Buffer pH 8.8:
Trizma base 181.6 g  
SDS 4.0 g  
Add de-ionized water to 1 L
Adjust with HCL to pH 8.8

Stacking Gel Buffer pH 6.8:
- Trizma base: 15.5 g
- SDS: 1.0 g
- Add H₂O to: 500 mL
- Adjust with HCL to pH 6.8

Running Buffer 10X Stock:
- Trizma base: 270 g
- Glycine: 1285.5 g
- SDS: 89.3 g
- H₂O to: 9000 mL

With the exception of sample buffers, which were stored frozen at -20°C, all other buffers were stored at -4°C.

2.5.5 Casting the SDS Gel

The SDS-page was formed using the method described by Laemmli (1970) using the BRL Vertical Gel Electrophoresis System V-16-2 (BRL, Paisley, Scotland). Before setting the gels, the glass plates, spacers and sample combs were washed thoroughly in distilled water and dried. The BRL system was then fitted together ensuring that a water-tight seal was achieved between paired glass plates.
Chapter two  

Materials and Methods

The appropriate volume of a 7.5% resolving gel was then made using the required concentration of polyacrylamide according to the values listed in Table 2.1. The acrylamide gel was then poured into the gap between the glass plates, leaving sufficient space for the stacking gel. A thin layer of water was then laid over the resolving gel using a Pasteur pipette to prevent diffusion of oxygen into the gel and inhibition of polymerisation, and also to obtain a flat surface for the polymerised gel.

After polymerisation was complete (30-60 minutes), the thin water layer was removed, using a Pasteur pipette. A 4.5% Stacking gel solution was made according to the values in Table 2.5, and this was then quickly poured onto the polymerised resolving gel. The plastic comb delineating the wells was inserted into the stacking gel at this stage. The SDS-page was then left to set overnight, at 4°C.

2.5.6 Electrophoresis and Autoradiography

Each sample was analysed by 7.5% SDS-Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out, in the running buffer, at a constant current of 30mA/gel. The electrophoresis was stopped when the bromophenol blue marker was about 1 cm from the bottom of the gel.

In order to analyse the immunoprecipitated proteins labelled with $^{35}$S-methionine, the gel needed to be dried. This is because $^{35}$S emits low energy $\beta$-particles, which are attenuated within a few millimetres of their source.

The gel was therefore fixed in 10% acetic acid for 1 hour, and then immersed, with gentle agitation, for 30 minutes in a fluorographic reagent, ‘Amplify’ (Amersham).
Table 2.1 Preparation of Resolving and Stacking Gels for SDS-PAGE

<table>
<thead>
<tr>
<th>GEL TYPE:</th>
<th>RESOLVING</th>
<th>STACKING</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACRYLAMIDE (%)</td>
<td>7.5</td>
<td>4.5</td>
</tr>
<tr>
<td>Acrylamide Stock(^a) (mL)</td>
<td>10.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Resolving Buffer (mL)</td>
<td>10.0</td>
<td>-</td>
</tr>
<tr>
<td>Stacking Buffer (mL)</td>
<td>-</td>
<td>9.6</td>
</tr>
<tr>
<td>Water (mL)</td>
<td>19.6</td>
<td>7.6</td>
</tr>
<tr>
<td>Ammonium</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Persulphate(^b) (mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMED(^c) (µL)</td>
<td>16.0</td>
<td>16.0</td>
</tr>
</tbody>
</table>

TOTAL VOLUME (mL) | 40.0 | 20.6 |
The gel was then transferred to filter paper, of appropriate-size, and dried under vacuum on a heated-bed gel dryer for 2 hours. After drying, the gel was exposed to Fuji RX film (Fuji Ltd., UK) within a light-proof metal cassette, at -70°C.

The autoradiograph was developed in Kodak developer for 10-30 seconds until the bands were clear, rinsed in water for 10 seconds, and then fixed in fixative solution for 20 seconds, before washing in tap water again, and then leaving to dry at 37°C.

2.6 Radioimmunoassay

The expression of HER-2 and EGFR on tumour cell lines was determined using radioimmunoassay, as described previously [Modjtahedi H et al., 1993b;Modjtahedi H et al., 1997;Styles JM et al., 1990]. Briefly, human tumour cell lines were grown to confluence in 200μl of DMEM containing 10%FCS in a 96-well plate. The cells were plated so as to have approximately the same number of cells seeded in each well at confluence. Following three washes of confluent cells with DMEM/2%FCS, triplicate 50 μl samples of each mAb (150 nM) in 2% DMEM, or medium alone, was added to the confluent monolayer of tumour cells and the plates were incubated for one hour on ice. Following three washes with 2% FCS/DMEM, the bound activity was measured by the addition of 125I–Star 71 (10^5 cpm/well) for 1 hour on ice. Following three more washes with 2% FCS/DMEM, the cells in each well were lysed by addition of 150 μl per well of 1M NaOH containing 1% Sarkosyl. The lysate was transferred to LP22 tubes (Elkay, Basingstoke, Hants) and the radioactivity present was determined in a LKB-WALLAC Clinigamma-1272 counter (Finland).
Chapter two

Materials and Methods

2.7 FACS Analysis

FACS analysis is a method used to measure cell surface levels of specific proteins, and was carried out as follows.

The human tumour cell lines were grown to near confluence in 75cm2 culture flask. Following trypsinisation, the cells were counted and then re-suspended at 10^6 cells per mL of DMEM/2%FCS. Then 1mL of each cell line was mixed with 10µg of each mAb (stock 1mg/mL) in a 1.5mL eppendorf tube, by rotation at 4°C for 1 hour. Control cells were treated with 10µl of medium alone. The cells were then washed twice before incubation with the secondary antibody. After each wash, the cells were centrifuged at 1800 rpm for 5 minutes and the supernatants were discarded. At the end of the second wash, the cells were re-suspended in 1mL of DMEM/2%FCS containing FITC F(ab')_2 fragment of goat anti-rat IgG (Star 69), and mixed on a rotator 4°C for 1 hour. Following three washes with DMEM/2%FCS to remove any unbound fluorochrome, the final pellet of cells were re-suspended in 2 mL of FACSFlow (Becton Dickinson) and analysed using a FACScan cell sorter (Becton Dickinson).

2.8 Growth Inhibition Studies in vitro

The effect of antibodies on the growth of human tumour cells, in vitro, was determined using the method described previously [Modjtahedi H et al., 1993b]. About 5 X 10^3 cells in 100 µL of 2% DMEM were seeded into each well of a 96-well plate. Following incubation for 4 hours at 37°C, 100 µL aliquots of each mAb (300nM), gefitinib (4 µM) were added to the wells in triplicate. Controls were set up
with wells that contained medium alone. The cultures were then incubated for about a further 5 days, until the cells incubated in medium alone were almost confluent. All the cells were then fixed by adding 20 μl of 0.25% glutaraldehyde (Sigma) to each well for 20 minutes, washed in water and then air dried. The cells were then stained by adding 100 μl of 0.05% methylene blue to each well for 15 minutes. After washing with tap water and air drying, 200 μl of 0.33N hydrochloric acid was added to each well, gently mixed and the absorbance was measured at 620nm ($A_{620}$) using the Labsystem Multiskan RC (Finland).

2.9 Patient Information

One hundred and seventy consecutive patients with Dukes’ B and C colorectal cancers who underwent radical surgery at the Royal Surrey County Hospital (Guildford, UK) between January 1990 and December 1998 were included in this retrospective study. Ethical approval for the study was given by the ethics committee at the Royal Surrey County Hospital. The names of two hundred and ten patients with a history of Dukes’ B or C colorectal cancer were initially obtained from the GUTS (Guildford Undetected Tumour Screening) database. Unfortunately, the information on the database for these patients was inadequate, and often incomplete, for the purposes of this study. Therefore, the clinical and pathological information for all 210 patients had to be obtained by reviewing their hospital notes (manually), and their histopathology reports. Therefore, a significant amount of time was consumed by tracking, collecting, and reviewing all 210 patient notes, so as to have accurate clinico-pathological, and follow-up details for each patient.
Chapter two Materials and Methods

Forty patients were eventually excluded from the study for various reasons. These included no follow-up data (n=9), mis-diagnosis on the database (e.g. in fact a Dukes' A cancer) (n=2), and incomplete histology (n=6). Any patient who had an incomplete excision on histopathological review was also excluded (n=2). Other reasons for patient exclusion included repeated poor immunohistochemistry (n=2) on the patient's tumour section, and an inability to find the tumour block (n=17). Patients who died within 30 days after surgery were excluded from this study to exclude bias (peri-operative death), as were those who had a second malignancy (n=2).

Detailed clinicopathological information was available for each patient. This included the tumour size, grade, presence of lymphovascular invasion, and the Dukes' stage. For the Dukes' C cases, the number of nodes involved by metastatic disease, and the involvement of the apical node was also documented. Information such as whether the patient had received radiotherapy or chemotherapy post-operatively was also obtained. None of the patients had received radiotherapy or chemotherapy before surgery.

Follow-up data was obtained by reviewing the patients' notes, and in collaboration with the Thames Cancer Registry (London). In a few cases, the patient's General Practitioner was contacted for follow-up information. Survival was measured from the date of the patients' operation until death or the end of June 2002, whichever came first. For those patients with recurrent disease, the date and site of relapse was obtained, in addition to their last observation (or date of death).
2.10 Immunohistochemistry

The main goal for the IHC technique in these studies was to achieve optimal specific immunostaining whilst also minimising interference from background staining. This optimisation process included the investigation of a series of mAbs, with varied dilutions and incubation times. It is well recognised that the epitope recognised by each antibody, incubation time and temperature, as well as antibody concentrations, are all tightly interwoven in their effect on the immunostaining. Inconsistent changes in these factors may cause significant variations in the intensity and overall quality of the immunostaining.

2.10.1 Optimisation of Immunostaining with mAb HM64.13

The optimisation of HM64.13 for use in immunohistochemical detection of HER-2 in paraffin embedded sections was performed as follows. Four human colorectal cancer specimens, which previously had been shown to be HER-2 positive, and a HER-2 overexpressing breast carcinoma cell line SKBR3 (positive control; see Section 2.10.2) were incubated with a 1:100, 1:200 and 1:500 dilutions of mAb HM64.13. The incubation period was also varied for each dilution, 30 minutes, 60 minutes and overnight incubation. The intensity of immunostaining was then detected using a standard IHC procedure (section 2.10.3), and the percentage number of cells in the tumour section exhibiting positive membranous or cytoplasmic immunostaining was determined.

To ensure that the IHC process was standardised, and the results consistent, a subgroup of 20 paraffin-embedded colorectal tumour sections were immunostained
using HM64.13 monoclonal antibody. This IHC method (section 2.10.3) was used subsequently for HER-2 immunostaining of a larger cohort of colorectal cancer sections.

2.10.2 Preparation of SKBR3 Cell Pellet for Immunostaining

The pellet for the human breast carcinoma cell line SKBR3 was prepared in the following way. Briefly, SKBR3 cells were grown in DMEM/10%FCS to near confluence in a large flask, as described above (section 2.2.2). The cells were then gently scraped off the flask and transferred (with medium) into a universal tube. Following centrifugation at 1600 rpm for 5 minutes, the supernatant was discarded. The pellet was then re-suspended in formalin for 45 minutes, and the cells fixed. The fixative was subsequently discarded after a further 5 minutes centrifugation. The cells were then washed twice in 5-10 mL of formaldehyde. After the final wash, and centrifugation, the formaldehyde was discarded, and the resultant pellet transferred to blotting paper. It was then embedded in paraffin wax, and after cooling, 3 μm sections were cut, and immunostained as below.

2.10.3 Immunostaining with mAb HM64.13

The overexpression of HER-2 in the tumour samples from 170 patients with Dukes' B and C colorectal cancers were assessed using the anti-HER-2 mAb, HM64.13 and a standard avidin-biotin-horseradish peroxidase system. This followed an initial standardisation process, as described above (section 2.10.1), to ensure reproducibility of the immunohistochemical staining.
Formalin-fixed paraffin-embedded tumour sections (3 μm) were cut from the blocks used for histopathological diagnosis, and mounted on slides. A particular tumour block was chosen, and the section of tumour initially stained with eosin and haematoxylin, to ensure that the cancer section was representative of the tumour. The tumour sections were initially dewaxed by placing the mounted slides in a slide rack, and immersing them in 2 separate xylene baths for 5 minutes each. The slides were then rehydrated by washing them for 1 minute, in each of three changes of industrial methylated spirit (IMS). No antigen retrieval was required. Endogenous peroxidase activity was blocked with a 3% hydrogen peroxidase solution for 5 minutes, followed by washing in distilled water, and then in Tris-buffered saline (TBS). Non-specific background staining, from secondary antibody binding, was blocked by applying 200 μL of normal rabbit serum (dilution 1:5) to each slide in a humidity chamber for 30 minutes. After blotting the excess rabbit serum, each tumour section was then incubated for 30 minutes with 200μl of the primary mAb, HM64.13, at optimal dilution (5μg/mL), and at room temperature. At the end of this incubation period, the slides were washed in TBS for 5 minutes, following which 150μl of secondary antibody, biotinylated rabbit anti-rat immunoglobulin (diluted 1:300 with TBS) (Dako Ltd, UK), was applied for a further 30 minutes.

Finally, after washing with TBS, the tumour sections were incubated with a horseradish peroxidase-labelled Avidin-Biotin complex (Dako Ltd, UK) for 30 minutes, and then rinsed again in TBS for 5 minutes. The bound primary antibody was then visualised by incubating the tumour sections for 5 minutes with liquid Diaminobenzidine Plus (dilution 1:50) (Dako Ltd).
After rinsing the slides in tap water, the tumour sections were counterstained with Mayer's haemalaum for 5 minutes, rinsed again in normal water and 'blued' in Scott's for 1 minute before mounting. To facilitate the latter process, the slides were immersed into 3 separate baths of IMS, for minute each, and then into 2 subsequent baths of xylene for a total of 10 minutes. The slides were then mounted in dibutyl phthalate in xylene, and the tumour sections were then ready for assessment using light microscopy.

The negative control tumour sections were incubated with TBS instead of the primary antibody, and the cell line SKBR3, known to over-express HER-2, was used as a positive control.

2.10.4 Scoring of HM64.13 Immunostaining

The tumour sections were assessed using light microscopy and a magnification of 200. The IHC staining was then scored by 2 independent observers, according to a defined protocol, and without prior knowledge of the patients' outcome. Two patterns of staining were looked at - cytoplasmic and membrane staining. Immunostaining was considered positive if > 10% of the tumour cells were stained by the antibody. The intensity of the immunostaining was also categorised as negative (0), weak (1+), and strong (2+). Any disparity in scoring was resolved by simultaneous evaluation of the tumour section by both observers.
2.11 Immunostaining with Anti-CD31 Antibody

Microvessels within the tumour can be identified using specific antibodies against CD31 antigen. CD31 is a platelet/endothelial cell adhesion molecule, which can be targeted with anti-CD31 antibodies to label vascular endothelial cells [Horak ER et al., 1992].

Because Dukes' B colorectal patients have a lower incidence of developing distant metastases, and therefore a better prognosis, only Dukes' C colorectal cancers were assessed for MVD. Ninety-one Dukes' C colorectal cancer sections were stained for CD31 antigen. The JC70 monoclonal antibody (DAKO, Copenhagen), which recognises the CD31 antigen, was used as it is known to stain positively for vascular endothelial cells in routinely fixed paraffin sections [Parums DV et al., 1990].

The tumour sections were again selected following review of the tumour blocks. The block with the greatest cross-section of tumour was selected. The tumour section from this block was initially stained with haematoxylin and eosin to ensure that a representative section of invasive carcinoma was being used for the purposes of the study.

The microvessel immunostaining was performed on 3-µm paraffin-embedded tumour sections using a standardised streptavidin-biotin process. The slides were de-waxed (as described in section 2.10.3), following which antigen retrieval was performed. For this, the slides were placed (in a rack) in a pressure-cooker, and immersed in antigen unmasking solution (Vector Lab., CA., USA) diluted 1:100 in distilled water, and then pressure-cooked for 2 minutes. The tumour sections were
then washed in TBS for 5 minutes, and then transferred to distilled water. They were then immunostained, using the anti-CD31 antibody, at a concentration of 1:100, diluted in ChemMate™ Antibody Diluent (Dako). A negative control was also performed for each tumour section, using a non-immune reagent (ChemMate™ Negative control, Dako). The immunostaining was performed on a Dako TechMate™ 500 Plus, strictly according to the manufacturer's recommendations. The immunostaining was via an automated system, and the washing of slides during this process was in specific Washing Buffers 1, 2, 3 and Water Wash. Washing Buffer 1 was used during the first part of the procedure, and the formulation of this buffer contributed to the blocking of non-specific binding of the immunological reagents to the tissue sections. Washing Buffers 2 and 3 were identical and were used for the remaining part of the procedure.

Briefly, after de-waxing and antigen retrieval, the sections were incubated with the primary antibody, JC70, for 25 minutes, following which they were washed in Washing Buffer 1 (ChemMate™ Buffer Kit, Dako), for about 5 minutes, and then incubated with the biotinylated goat anti-mouse and anti-rabbit secondary antibody for a further 25 minutes. After washing again in Washing Buffer 1 and then Buffer 2 for approximately 5 minutes, the slides were incubated with Peroxidase Blocking solution for 5 minutes. The sections were again washed in Washing Buffer 2 for 3-5 minutes, and then incubated in streptavidin peroxidase for 25 minutes. After washing the slides in Buffer 2 and then Buffer 3 for approximately 2 minutes, they were incubated in the chromogen, diaminobenzidine solution, diluted in HRP Substrate Buffer, for 10 minutes. After a washing period of about 5 minutes in Buffer 3, the sections were then counterstained with ChemMate™ Haematoxylin, and then washed in Water Wash at the end prior mounting as described previously.
Chapter two

Materials and Methods

2.12 Microvessel Density Determination

The CD31-stained tumour sections were assessed using light microscopy. Each section was initially scanned at low power (X40) to identify areas within the cancer with the highest areas of microvessel density ('hot spot'). The MVD was then determined by counting all vessels seen at a magnification of 200, in a set area of 0.2 mm². Five 'hot spot' areas within each tumour section were assessed for MVD, and the mean MVD calculated for each tumour.

To facilitate the counting of the microvessels, a digital camera (JVC TK-C1380) was used with Leica Qwin computer software, to capture the image of each 'hot spot' assessed. The area of assessment could then be accurately defined, and it was also possible to mark the counted vessel. This allowed more accurate counting as it eliminated the possibility of missing a vessel, and also prevented duplicate counting of a vessel (which could be clearly highlighted).

Initial attempts to use a computerised image analysis system to count the number of microvessels in 10 tumour sections, proved to be significantly less accurate than the method described above. The reason for this was the extremely low level of interference by non-microvessel structures allowed by the automated system.

Counting the intratumoral microvessels was carried out as previously described by Weidner et al. [Weidner N et al., 1991]. A microvessel was defined as any brown staining endothelial cell, or clusters of cells, that were distinct from others, and present in the invasive component of the tumour. A visible lumen was not necessary for the vessel to be included in the count. The assessment of the MVD was
performed without any knowledge of the patients' survival or the presence of distant metastases, or any other clinico-pathological variables.

2.13 Statistical Analysis

Statistical analyses were performed using the SPSS package (version 11.0). Correlation between clinico-pathological characteristics and the IHC scores was tested using $\chi^2$ tests, at a significance level of $p<0.05$.

Non-parametric analysis, using the kappa test, was used to determine the level of agreement of the IHC scores between observers.

The association between the IHC scores and survival was estimated using Cox proportional hazards regression analyses. The end-point for the survival analyses was overall survival. Other covariates considered for inclusion in the model were site and size of tumour, depth of tumour invasion (i.e. T stage), grade, lymphovascular invasion, and node stage (N1 or N2) and presence of apical node metastases (the latter two for Dukes' C cancers only). A Cox proportional hazards model was also used to assess the association of tumour variables, local relapse, site of relapse, distant metastases, and treatment with survival. Analyses were performed in which the survival analyses were stratified by Dukes staging, as this is currently the most important independent prognostic factor. Kaplan-Meier survival curves were computed, and the log rank test was used to test differences between the survival curves for the patient sub-groups. A p-value of less than 0.05 was considered significant.
Chapter Three

_in vitro_ Studies with Anti-HER-2 Antibodies in Human Colorectal Tumour Cell Lines

Overexpression of HER-2 has been demonstrated in a panel of human tumor cell lines of different origins including breast, ovarian and gastric cancer cell lines [Lewis GD et al., 1993]. In this chapter, following the characterisation of anti-HER-2 mAb HM64.13, the expression of HER-2 in a panel of human tumour cell lines was determined, and then the effect of HER-2 or EGFR blockade, by antibodies, on the growth of such cell lines in vitro was investigated.

3.1 Specificity of mAb HM64.13

Monoclonal antibody HM64.13 was raised against the external domain of HER-2 on the breast carcinoma cell line SKBR3. This antibody was developed by Dr Helmout Modjtahedi while working at The Institute of Cancer Research, Sutton and further characterisation of this antibody was conducted at The University of Surrey. The specificity of mAb HM64.13 (IgG2b) for HER-2 was determined by differential binding assay, immunoprecipitation studies, and FACS analysis. This antibody was subsequently used in the examination of human colorectal tumour specimens, to investigate HER-2 expression.

The binding of mAb HM64.13 to a panel of human tumour cell lines that express a high or low level of HER-2 was determined by in-direct radioimmunoassay. Human tumor cell lines were incubated in the presence of 150nM of each mAb or control...
medium for 1 hour, on ice. The bound antibodies were then detected using $^{125}$I-Star 71, as described in section 2.6 [Styles JM et al., 1990]. The differential binding of HM64.13 to human tumour cell lines that express a high (SKBR3, SKOV3), or low (HN5, MCF-7) level of HER-2 are shown in Figure 1. Indeed, the binding pattern of mAb HM64.13 to these cell lines was similar to that of the positive control anti-HER-2 mAb ICR12 [Dean CJ et al., 1993; Styles JM et al., 1990]. This is in agreement with previous studies which have shown high levels of HER2 overexpression in SKBR3 breast cancer cell line and SKOV3 an ovarian cancer cell line [Boente MP et al., 1998; Lewis GD et al., 1993]. In addition the binding of mAb ICR62, which is directed against the EGFR, was highest in HN5 cells which express the highest level of the EGFR [Modjtahedi H and Dean C, 1996].

The specificity of mAb HM64.13 for the product of the c-erbB-2 proto-oncogene was investigated further by immunoprecipitation studies, using the [$^{35}$S]-methionine-labelled proteins from SKBR3 cells. The immunoprecipitate was then analysed by SDS polyacrylamide gel electrophoresis, as described in section 2.5.

Fig. 2 shows the autoradiographs of the gel obtained following SDS-PAGE of immunoprecipitates prepared with antibodies HM64.13 and ICR12 (positive control). The results show that like anti-HER-2 mAb ICR12, mAb HM64.13 immunoprecipitated specifically the p185$^{\text{HER-2}}$ protein from Triton X-100 extracts of [$^{35}$S]-methionine labelled SKBR3 cells. The control medium, as expected, shows no immunoprecipitation.

The known differential expression of HER-2 and EGFR by SKBR3 and HN5 cells respectively, was also used to confirm the specificity of mAb HM64.13, using FACS
Figure 1. Differential binding of mAb HM64.13 to a panel of human tumour cell lines which express high (SKBR3 and SKOV3) or low (HN5, MCF-7) levels of HER-2. Confluent cultures of human tumour cell lines were incubated with 150nM of anti-HER-2 (HM64.13 or ICR12) or anti-EGFR (ICR62) mAb, or control medium, for 1 hour on ice. Then the bound antibody was detected by addition of 125I-goat anti-(rat IgG) polyclonal antibody (Star 71) (10^5 cpm/well), as described previously [Styles JM et al., 1990; Modjtahedi H et al., 1997].
Figure 2. Specificity of mAb HM64.13 for HER-2. Like positive control mAb ICR12, HM64.13 conjugated to protein G sepharose 4B immunoprecipitated the p-185\textsuperscript{HER-2} from \[^{35}\text{S}\]-methionine-labelled extract of SKBR3 cells [Styles JM et al., 1990; Modjtahedi H et al., 1993b].
analysis. In this study, the human colorectal cell line DiFi, which is known to overexpress EGFR (4.8 x 10^6 receptors/cell) [Gross ME et al., 1991] was also used. After mixing 10^6 cells with 10 μg of each mAb, HM64.13 (anti-HER-2) or ICR62 (anti-EGFR), at 4°C for 1 hour, the cells were washed twice prior to incubation with FITC goat anti-rat IgG antibody, again at 4°C for 1 hour. After further washing and centrifugation of the cells, the cell pellet was re-suspended in FACSFlow, and analysed in a FACScan.

The results of the FACS analysis are presented in Figure 3. As the results with mAb HM64.13 show, HER-2 expression is significantly higher in SKBR3 in comparison to both HN5 and DiFi cells. The latter two cell lines are both EGFR overexpressing cells [Cowley G et al., 1984; Gross ME et al., 1991], as was demonstrated by the higher level of binding by the anti-EGFR mAb ICR62.

These data show a distinct profile for the anti-HER-2 and anti-EGFR mAbs, and support the findings above which suggest that HM64.13 specifically recognizes p185^{HER-2}.

3.2 The Expression of HER-2 in other Human Colorectal Cell Lines

The level of expression of HER-2 in a panel of colorectal cell lines was investigated using mAb HM64.13 as the primary antibody, and the bound antibody was detected using an iodinated anti-rat polyclonal secondary antibody (STAR 71), as described in section 2.6. In this differential binding assay, the 4 additional human colorectal cell lines examined included CCL228, CCL244, CCL225, and CCL221, in addition to the
Figure 3. Fluorescence-activated cell sorting histograms of anti-HER-2 mAb HM64.13 and anti-EGFR mAb ICR62 binding to human tumour cell lines. The medium represents background fluorescence (binding of fluorescein-isothiocyanate-labelled goat anti-rat IgG in the absence of mAbs HM64.13 or ICR62). Cell lines analysed are: A) SKBR3 human breast carcinoma; B) HN5 head and neck carcinoma; C) DiFi colorectal carcinoma.
control cell lines, SKBR3 and HN5, which over-express HER-2 and EGFR respectively.

As the results in Fig. 4 show, using the anti-HER-2 mAb HM64.13, the expression of HER-2 in a panel of human colorectal cell lines was much lower than that in the human breast tumour cell line SKBR3. This is in agreement with Lewis et al who showed low levels of HER-2 overexpression in colorectal cell lines compared with breast tumour cell lines such as SKBR3 [Lewis GD et al., 1993].

Interestingly, in contrast to DiFi cells (Fig. 3) and HN5 cells, these colorectal cell lines were also found to express low levels of EGFR (Fig. 4).

3.3 Growth Inhibition Studies

Having shown that the level of expression of HER-2 in the colorectal cell lines examined above was not as high as that seen in breast cancer cell line SKBR3, next the effect of antibody-blockade of HER-2, antibody blockade of EGFR, or EGFR blockade using a small molecule tyrosine kinase inhibitor, gefitinib, was investigated.

Three human colorectal cell lines were used in this study – DiFi, CCL218, and CCL244. SKBR3 cells were also used. The cell lines were incubated with each of the anti-HER-2 antibodies, HM64.13 and ICR12, the anti-EGFR mAb ICR62, gefitinib, and EGF. After 4-7 days incubation, the proliferation of each cell line was compared with the untreated control.
Figure 4. Level of expression of HER-2 and EGFR in a panel of human colorectal cancer cell lines, using anti-HER-2 mAb HM64.13 and anti-EGFR mAb ICR16, respectively. Confluent cultures of colorectal cancer cell lines were incubated with 150nM of mAb or control medium for 1 hour on ice and then bound antibody was detected by the addition of $^{125}$I-Star 71(10^5 cpm/well), as previously described [Styles JM et al., 1990; Modjtahedi H et al., 1997].
Figures 5A – 5D illustrate the results of the in vitro growth inhibition studies. At a concentration of 150 nM, mAbs HM64.13 and ICR12 did not show any significant effect on the proliferation of the colorectal cell lines. However, in contrast to the cell lines CCL244 and CCL218, which showed no growth inhibition with gefitinib or the anti-EGFR mAb, DiFi showed significant inhibition of cell proliferation with the tyrosine kinase inhibitor and also with ICR62. The growth of SKBR3, which is known to express substantial levels of HER-2, was not affected by HM64.13 or ICR12. At a concentration of 2 μM, gefitinib showed 22.3% growth inhibition of SkBR3 cells. Inhibition of HER-2-overexpressing breast tumour cell lines by gefitinib has also been reported previously [Moulder SL et al., 2001].

3.4 Summary

The anti-HER-2 mAb HM64.13 was raised against the external domain of HER-2 on the breast carcinoma cell line SKBR3. Specific differential binding was shown by HM64.13 to cell lines known to express high (SKBR3 and SKOV3) and low (HN5 and MCF-7) levels of HER-2 (Figure 1). Like the positive control antibody ICR12, mAb HM64.13 immunoprecipitated the 185 kDa protein from the 35S-extract of the HER-2-overexpressing cell line SKBR3 (Figure 2). A panel of colorectal cell lines was shown to express low levels of HER-2, and antibody blockade of HER-2 did not affect their growth in vitro (Figures 4, 5). This may suggest that the HER-2 epitope being blocked by HM64.13 does not play a significant role in the growth signal transduction pathway in these colon cancer cells. Alternatively, the low level of expression of HER-2 in these cells might indicate that growth receptors, other than HER-2, play a more important role in their proliferation (please see discussion chapters).
Figure 5. The effect of 150 nM anti-HER-2 (HM64.13 and ICR12) and anti-EGFR (ICR62) mAbs, and 2 µM gefitinib (TKI) on the growth in vitro of human tumour cell lines is shown. 5 X 10^5 cells/well in 100 µL of 2% DMEM were seeded into a 96-well plate. Following 4 hours incubation at 37°C, 100 µL aliquots of each mAb (400nM) or gefitinib (4 µM) were added to the wells in triplicate. Controls were set up with medium alone. After 5 days incubation, the cells were fixed and stained with methylene blue. The absorbance (+1SD) of each well was measured at 620nm, and this is proportional to the number of cells present.
Figure 5 (cont’d). The effect of 150 nM anti-HER-2 (HM64.13 and ICR12) and anti-EGFR (ICR62) mAbs, and 2 μM gefitinib (TKI) on the growth in vitro of human tumour cell lines is shown. 5 X 10^5 cells/well in 100 μL of 2% DMEM were seeded into a 96-well plate. Following 4 hours incubation at 37°C, 100 μL aliquots of each mAb (400nM) or gefitinib (4 μM) were added to the wells in triplicate. Controls were set up with medium alone. After 5 days incubation, the cells were fixed and stained with methylene blue. The absorbance (+1SD) of each well was measured at 620nm, and this is proportional to the number of cells present.
Chapter Four

Expression Patterns and Prognostic Significance of HER-2 in Colorectal Cancer

Although the clinical significance of HER-2 overexpression is uncertain in many human tumours, HER-2 has been shown to be of prognostic value in breast cancer [Slamon DJ et al., 1987], and furthermore may predict for response to treatments [Newby JC et al., 1997]. A number of studies have examined the prognostic utility of HER-2 overexpression in colorectal cancer, and reached conflicting conclusions. The aim of this study was to examine the overexpression of HER-2 in colorectal cancer sections, using a standard avidin-biotin immunohistochemical technique and HM64.13 anti-HER-2 mAb. The localisation of this mAb is described and its prognostic significance discussed.

4.1 Patient Details

One hundred and seventy patients with Dukes’ B and C colorectal cancers were included in this study, and Table 4.1 shows the clinicopathological features of the study group. Eighty-one patients were male, and 89 were female. Their age ranged from 40 to 92 years (median 70 years; mean 70 years), and the median follow up time was 5.0 years. There were 65 Dukes’ B and 105 Dukes’ C cancers in this study cohort. Five Duke’s B patients and 63 Duke’s C patients had received adjuvant chemotherapy (mainly 5-fluorouracil-based), whilst 5 and 32 patients respectively
Table 4.1 Clinico-pathological characteristics of 170 colorectal cancer patients by Dukes' staging

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<thead>
<tr>
<th></th>
<th>NUMBER OF PATIENTS</th>
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<tbody>
<tr>
<td></td>
<td>DUKES' B N=65</td>
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<td>Male</td>
<td>26</td>
</tr>
<tr>
<td>Female</td>
<td>39</td>
</tr>
<tr>
<td><strong>Tumour site</strong></td>
<td></td>
</tr>
<tr>
<td>Right colon</td>
<td>28</td>
</tr>
<tr>
<td>Left colon</td>
<td>18</td>
</tr>
<tr>
<td>Rectum</td>
<td>19</td>
</tr>
<tr>
<td><strong>Size</strong></td>
<td></td>
</tr>
<tr>
<td>≤5cm</td>
<td>40</td>
</tr>
<tr>
<td>&gt;5cm</td>
<td>25</td>
</tr>
<tr>
<td><strong>Differentiation</strong></td>
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</tr>
<tr>
<td>Well</td>
<td>14</td>
</tr>
<tr>
<td>Moderate</td>
<td>36</td>
</tr>
<tr>
<td>Poor</td>
<td>14</td>
</tr>
<tr>
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<td>1</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Present</td>
<td>6</td>
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</tr>
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<td>-</td>
</tr>
<tr>
<td>T3</td>
<td>55</td>
</tr>
<tr>
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<tr>
<td><strong>Adjuvant chemotherapy</strong></td>
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<tr>
<td>Yes</td>
<td>5</td>
</tr>
<tr>
<td>No</td>
<td>60</td>
</tr>
<tr>
<td><strong>Radiotherapy</strong></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
</tr>
<tr>
<td>No</td>
<td>60</td>
</tr>
</tbody>
</table>
had received standard pelvic radiotherapy, as part of their post-operative management.

The mean (±SD) survival of the Dukes' B patients was 7.1 (± 3.7) years, whilst for the Dukes' C patients, it was only 4.6 (± 2.7) years. The Kaplan-Meier plots stratified for Dukes' staging (Fig. 6) show that there was a significant difference in the survival of the Dukes' B and C patients (p=0.00). Whereas the median survival of the Dukes' C patients was 6.9 years, the median follow-up of the Dukes' B patients had not yet been reached, at a maximum follow-up time of 11.9 years.

The significantly better survival seen in the Dukes' B patients, compared to the Dukes' C patients in this study, is consistent with the fact that Duke's stage is one of the most important prognostic factors in colorectal cancer currently.

4.2 Immunostaining of Colorectal Cancer Sections with HM64.13

The overexpression of HER-2 was examined immunohistochemically in 170 colorectal cancer sections, using the mAb HM64.13. There was heterogenous HER-2 immunostaining within the same tumour section. The predominant localisation of HER-2 immunostaining was cytoplasmic. Membrane HER-2 was seen with less frequency than cytoplasmic HER-2, and always in combination with cytoplasmic HER-2 on the tumour section.

Figure 7A illustrates a case of colorectal cancer showing strong cytoplasmic HER-2 immunostaining. Figure 7B shows the typical immunostaining seen in the majority of the tumour sections, with predominant cytoplasmic immunostaining and some
Figure 6. Kaplan-Meier curves for overall survival in 170 patients with colorectal cancer, stratified for stage Dukes' B or Dukes' C. There was a significant difference in the survival of these two groups of patients (p=0.000) (log rank test).
Figure 7. Colorectal cancer section showing HER-2 immunostaining using mAb HM64.13. (A) Shows a case of colorectal cancer showing strong cytoplasmic HER-2 immunostaining. (B) Illustrates a case of predominant cytoplasmic HER-2, and some membrane HER-2 immunostaining. (C) Shows the corresponding negative control, where the primary antibody has been omitted (magnifications: A-C, 200X)
Figure 7 (continued). Colorectal cancer section showing HER-2 immunostaining using mAb HM64.13 (A) Shows a case of colorectal cancer showing strong cytoplasmic HER-2 immunostaining. (B) Illustrates a case of predominant cytoplasmic HER-2, and some membrane HER-2 immunostaining. (C) Shows the corresponding negative control, where the primary antibody has been omitted (magnifications: A-C, 200X)
membranous HER-2 immunostaining, whilst Figure 7C shows the corresponding negative control where the primary antibody has been omitted.

There was very good agreement of the IHC scores between the 2 observers (kappa 0.88). The level of HER-2 overexpression in this cohort of Dukes' B and C cancer patients was quantified (Table 4.2). Eighty-seven percent of cases showed positive cytoplasmic HER-2, and over half of these exhibited strong intensity (2+) immunostaining. 41% of cases showed positive membrane HER-2, with 91% of positive cases exhibiting strong immunostaining.

Figure 8 shows the human breast tumour cell line, SKBR3, immunostained with anti-HER-2 mAb, HM64.13. This illustrates that the pattern of staining in this cell line includes strong membranous staining. This is similar to that seen in human breast tumour sections, immunostained for HER-2. As SKBR3 is known to over-express high levels of HER-2, SKBR3 pellet sections were used as a positive control for HER-2 immunostaining of the colorectal cancers.

4.3 Prognostic Significance of HER-2 Immunostaining

The data was stratified for Dukes' staging, as this is known to be the most significant prognostic factor in colorectal cancer currently. In the Dukes' C group, multivariate analyses showed tumour grade, depth of tumour invasion, positive apical node, and positive cytoplasmic HER-2 to be independent prognostic factors (Table 4.3). A significantly better overall survival was seen in those patients exhibiting positive cytoplasmic HER-2, with a Hazard Ratio (HR) of 0.46 (CI95 0.24-0.87, p=0.018) (Fig. 9).
Table 4.2. One hundred and seventy colorectal tumour sections were immunohistochemically stained, using anti-HER-2 mAb HM64.13 and a standard avidin-biotin horseradish peroxidase system. Using light microscopy, at 200X magnification, two patterns of immunostaining were assessed - membrane and cytoplasmic. The immunostaining was considered positive if > 10% of the tumour cells were stained by the antibody. The intensity of the immunostaining was also categorised as negative (0), weak (1+), and strong (2+). The scoring was performed by 2 independent observers.

<table>
<thead>
<tr>
<th>HER-2 Immunostaining</th>
<th>Membrane n (%)</th>
<th>Cytoplasmic n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER-2: Positive</td>
<td>71 (41.2)</td>
<td>147 (86.5)</td>
</tr>
<tr>
<td>Negative</td>
<td>99 (58.8)</td>
<td>23 (13.5)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Intensity of HER-2 Immunostaining</th>
<th>Membrane n (%)</th>
<th>Cytoplasmic n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>99 (58.2)</td>
<td>23 (13.5)</td>
</tr>
<tr>
<td>Weak (1+)</td>
<td>3 (1.8)</td>
<td>55 (32.4)</td>
</tr>
<tr>
<td>Strong (2+)</td>
<td>68 (40)</td>
<td>92 (54.1)</td>
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</table>
Figure 8. Immunostaining of the HER-2-overexpressing SKBR3 cell line pellet with anti-HER-2 mAb HM64.13 (A) or with no primary antibody (B) (Magnification 200X). SKBR3 cell line was grown to confluence and then fixed with formalin, following pellet formation, by centrifugation at 1600 rpm for 5 minutes. After two washes in formaldehyde, the pellet was re-formed and then embedded in paraffin wax. Cell line sections were immunostained using a standard avidin-biotin immunocytochemistry technique.
Table 4.3 Multivariate analyses of 105 Dukes' C colorectal cancer patients showed tumour grade, depth of tumour invasion, positive apical node, and positive cytoplasmic HER-2 to be independent prognostic factors. The end-point of the analyses was overall survival.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Number (n=105)</th>
<th>Hazard Ratio</th>
<th>95% Confidence Interval</th>
<th>P value</th>
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<tr>
<td><strong>Cytoplasmic HER-2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>17</td>
<td>Reference</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Positive</td>
<td>88</td>
<td>0.46</td>
<td>0.24 - 0.87</td>
<td>0.018</td>
</tr>
<tr>
<td><strong>Differentiation</strong></td>
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<tr>
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<td>6</td>
<td>0.43</td>
<td>0.12 - 1.54</td>
<td>0.196</td>
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<tr>
<td>Well</td>
<td>3</td>
<td>3.31</td>
<td>0.87 - 12.60</td>
<td>0.080</td>
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<tr>
<td>Moderate</td>
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<td>Reference</td>
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</tr>
<tr>
<td>Poor</td>
<td>40</td>
<td>1.80</td>
<td>1.06 - 3.08</td>
<td>0.031</td>
</tr>
<tr>
<td><strong>T stage</strong></td>
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<tr>
<td>T2</td>
<td>15</td>
<td>0.83</td>
<td>0.36 - 1.93</td>
<td>0.668</td>
</tr>
<tr>
<td>T3</td>
<td>64</td>
<td>Reference</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T4</td>
<td>26</td>
<td>2.31</td>
<td>1.28 - 4.19</td>
<td>0.006</td>
</tr>
<tr>
<td><strong>Apical node</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>56</td>
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<td>37</td>
<td>1.33</td>
<td>0.72 - 2.44</td>
<td>0.359</td>
</tr>
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</table>
Figure 9. Kaplan-Meier plots of overall survival of 105 Dukes' C colorectal cancer patients. Positive cytoplasmic HER-2 immunostaining was associated with a significantly better survival (p=0.018).
This improved survival was not significantly different between those patients who received and those who did not receive chemotherapy (p=0.25). On univariate analyses, strong intensity cytoplasmic immunostaining was associated with depth of tumour invasion, with T3 tumours showing a higher proportion of strong cytoplasmic immunostaining than the T4 tumours (p=0.013) (data not shown).

No significant association was seen between cytoplasmic HER-2 overexpression and clinical outcome in the Dukes' B cancers (Fig 10), but this may be due to the small number of deaths in this sub-group. In addition, no significant association was seen between membrane HER-2 and clinical outcome in either the Dukes' B or C cases.

4.4 Summary
Colorectal cancer expresses high levels of HER-2. In this study, it was found that the predominant pattern of HER-2 immunostaining seen in colorectal cancer is cytoplasmic, and that cytoplasmic HER-2 overexpression was associated with a significantly better prognosis in Dukes' C cancers. No such association was seen in Dukes' B cancers, but this may be as a result of the small number of events in this sub-group.

Membranous HER-2 overexpression was found in 41.2% of tumours. While there was no association between this factor and clinical outcome in either the Dukes' B or C colorectal cancers, the high level of membranous HER-2 in colorectal cancer could make it an ideal target for monoclonal antibody-based immunotherapy (please see discussion chapter).
Figure 10 Kaplan-Meier plots of overall survival of 65 Dukes' B colorectal cancer patients. There was no significant association between positive cytoplasmic HER-2 immunostaining and outcome.
Chapter Five

Expression and Prognostic Significance of CD31 in Colorectal Cancer

Tumour angiogenesis has been shown to have a significant association with prognostic outcome in a number of solid tumours, including breast, prostate, and lung [Macchiarini P et al., 1992; Weidner N et al., 1992; Weidner N et al., 1993]. In this study, the quantitative expression of angiogenesis in colorectal cancer was determined, and its association with clinico-pathological factors and survival is reported.

5.1 Patient Details

There were 91 patients in this study, and they were a sub-group of the HER-2 study population as described in section 2.9. The median age at diagnosis was 70, with a range from 40 to 86 years, and the median follow-up time was 4.2 years (range 0.32-10.86 years). There were 49 male and 42 female patients. Sixty-two patients had a tumour ≤5 cm in maximum dimension and 29 patients had a tumour of >5 cm.

Table 5.1 shows some of the important clinicopathological features of the 91 patients in this study, all of whom had a Dukes’ C colorectal cancer. Twenty-five patients had evidence of lymphovascular invasion in their tumour, and overall, 39 patients developed distant metastases, whilst 5 had local (either anastamotic or nodal) recurrences. Fifty-three and 28 patients received adjuvant chemotherapy and pelvic radiotherapy, respectively.
Table 5.1 Median Microvessel Count in 91 patients with colorectal cancer (non-parametric test Pearson $\chi^2$ test)

<table>
<thead>
<tr>
<th></th>
<th>Number of cases</th>
<th>Median microvessel count (25-75% quartiles)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size of tumour</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 5 cm</td>
<td>62</td>
<td>26 (13-37)</td>
<td></td>
</tr>
<tr>
<td>&gt; 5 cm</td>
<td>29</td>
<td>27 (14-44)</td>
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</tr>
<tr>
<td><strong>Depth of invasion</strong></td>
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<td></td>
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<tr>
<td>T2</td>
<td>11</td>
<td>33 (13-39)</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>57</td>
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<td>T4</td>
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</tr>
<tr>
<td>Positive</td>
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<td></td>
</tr>
<tr>
<td>unknown</td>
<td>35</td>
<td>18 (10-30)</td>
<td>0.60</td>
</tr>
<tr>
<td><strong>Lymphovascular invasion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>66</td>
<td>26 (13-42)</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>25</td>
<td>27 (7-32)</td>
<td>0.73</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>6</td>
<td>12 (4-20)</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>2</td>
<td>36 (14-57)</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>48</td>
<td>26 (13-35)</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>35</td>
<td>28 (13-42)</td>
<td>0.22</td>
</tr>
<tr>
<td><strong>Site of recurrence</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>47</td>
<td>26 (13-38)</td>
<td></td>
</tr>
<tr>
<td>Local</td>
<td>5</td>
<td>17 (13-36)</td>
<td></td>
</tr>
<tr>
<td>Distant</td>
<td>39</td>
<td>28 (11-42)</td>
<td>0.92</td>
</tr>
</tbody>
</table>
5.2 Immunostaining of CD31

CD31 immunostaining revealed heterogeneity of MVD in the colorectal cancer specimens. There were clear 'hot spots' of high intratumoral neoangiogenesis, and these areas were localised at low power (X40), using light microscopy. Five 'hot spot' areas were then assessed, at 200X magnifications, and for each 'hot spot' the number of microvessels in a set area of 0.2 mm² was counted, and the mean MVD (per 0.2 mm²) was calculated for each tumour.

Figure 11A shows a colorectal tumour stained immunohistochemically with anti-CD31 mAb, JC70. It illustrates the brown staining seen with this mAb highlighting the endothelial cells of the microvessels. Figure 11B shows the corresponding negative control slide, using non-immune anti-sera.

5.3 Prognostic Significance of CD31 Immunostaining

The median MVD for the 91 patients was 26 (range 0-100). As described previously by Vermeulen et al., [Vermeulen PB et al., 1999], and others [Takebayashi Y et al., 1996; Tanigawa N et al., 1997], 2 sub-groups were defined using the median MVD as the cut-off for the groups. The patients were therefore divided into those with low (≤ 25) and those with high (>25) MVD tumours.

In this study, MVD of colorectal carcinomas showed no association with the patients' age (p=0.53) or size of tumour (Table 5.1). No significant difference in tumour MVD was seen between patients with or without haematogenous spread of their disease. The median tumour microvessel count in those 39 patients who developed distant metastases was 28, in the areas of highest neovascularisation. In
Figure 11. CD31 immunostaining of a colorectal cancer, using anti-CD31 mAb JC-70, showing a case of colorectal cancer with high MVD (A), and the corresponding negative control slide where the primary antibody has been omitted (B) (magnifications: 200X).
those 47 patients who remained disease-free, the corresponding value was 26. Therefore, no difference in the degree of tumour neovascularisation was seen in these two groups (p=0.41). Furthermore, MVD did not predict for the type of recurrent disease (e.g. distant versus local) nor for the site of distant metastases (e.g. liver or lung).

The evaluation of MVD in relation to other clinico-pathological variables is shown in Table 5.1. No difference in the MVD was demonstrated in tumours of different nodal stage (p=0.38), or those with or without apical node metastases (p=0.60). Furthermore, those tumours which demonstrated vascular invasion histologically, showed no significant difference in their MVD compared to tumours with no vascular invasion (p=0.73). There was no association between depth of tumour invasion through the bowel wall and MVD (p=0.81). In addition, no association was found between tumour MVD and expression of membranous or cytoplasmic HER-2 (using mAb HM64.13) (data not shown).

On multivariate analysis (91 patients), the presence of apical node metastases was the only significant independent prognostic indicator (HR 5.1, 95% CI 2.2-11.8, p=0.000). The presence of a T4 tumour almost reached statistical significance (HR 3.3, 95% CI 0.9-12, p=0.07).

The survival rates for the 91 patients according to their MVD sub-group was calculated using the Kaplan-Meier method (Figure 12). There was no significant difference in survival between those patients with tumours with high MVD and those with low MVD tumours (p=0.349).
Figure 12. Overall survival curves for 91 patients with colorectal cancer. Survival of patients with low and high MVD tumours. There was no significance difference in the survival of the patients in these 2 groups using the log rank test (p=0.349).
5.4 Summary

Using mAb CD31, the microvessel density of ninety-one Dukes’ C colorectal cancers were assessed. There was no association seen between MVD and recurrence, and survival in this group of patients. There was also no association between MVD and HER-2 overexpression. In this study, MVD did not seem to act as a predictor for poor outcome.
Chapter Six

Discussion and Conclusion

Despite advances in new chemotherapies (e.g. oxaliplatin and irinotecan) used following surgery for colorectal cancer, the overall prognosis remains relatively poor, with a mean 5-year survival rate of approximately 50%. Currently, lymph node involvement and the number of positive nodes are considered amongst the most important prognostic indicators in colorectal cancer [Gastrointestinal Tumor Study Group, 1985; Wolmark N et al., 1986]. However, the lymph node status alone does not always predict the accurate outcome of the patient. A significant number (about 30%) of Dukes’ B colorectal cancer patients will develop progressive disease, and for this group, the decision regarding adjuvant chemotherapy remains difficult, using the prognostic indicators available to us at present. Large randomised studies have failed to show a definite survival advantage from adjuvant chemotherapy in Dukes’ B colorectal cancers [International Multicentre Pooled Analysis of Colon Cancer Trials (IMPACT) investigators, 1995; Mamounas E et al., 1999]. The recent report from the large randomized Quasar 1 study investigating the role of adjuvant chemotherapy in Dukes’ B cancers has disappointingly also failed to show a significant benefit (unpublished data). Furthermore, a subgroup of tumours from 647 Dukes’ B patients has been assessed with regard to pathological and histochemical variables. On multivariate analysis, vascular invasion (p=0.022) and serosal involvement (p=0.024) were the only significant prognostic variables.

However, there are an increasing number of studies in solid tumours indicating that protein markers may play a significant role in predicting recurrence and survival.
Consequently, there has been a shift towards looking at the molecular phenotype of cancer cells to see if this can help us to define molecular markers which may help predict prognosis more accurately so that we can better select those patients in a poorer prognostic group who should be offered adjuvant therapies. Furthermore, identification of these proteins may facilitate newer treatments. This strategy has been particularly successful in breast cancer patients where the role of HER-2 has been established in predicting not only outcome but response to treatments. Furthermore, in breast cancer, a specific monoclonal antibody directed against HER-2 (Trastuzumab™) has been developed and approved for clinical use in patients whose tumours over-express HER-2 [Slamon DJ et al., 2001].

The role of HER-2 has also been investigated in colorectal cancer, but the results have been inconsistent. The aim of the present study was to investigate the prognostic value of Human Epidermal Growth Factor Receptor-2 (HER-2) and microvessel density in colorectal cancer. Furthermore in vitro studies were also performed to investigate the expression pattern of HER-2 in a panel of human colon tumour cell lines and the effect of antibody blockade of HER-2 on the growth of these cell lines.

6.1 The Expression of HER-2 in Human Tumour Cell Lines

The anti-HER-2 mAb HM64.13, used in this study, was raised against the external domain of HER-2 on the human breast carcinoma cell line SKBR3 [Lewis GD et al., 1993]. The specificity of this mAb for HER-2 was then demonstrated by differential binding assay, immunoprecipitation studies, and FACS analysis, as shown in Figures 1-3.
Next, using anti-HER-2 mAbs HM64.13 and ICR12, the expression of HER-2 was examined in a panel of human colorectal cell lines. As the results presented in Figure 4 show, the level of expression of HER-2 on colorectal cell lines was much lower than on human breast carcinoma cell lines. This is in agreement with the findings of Lewis et al. who also found lower levels of p185HER-2 in most of the colorectal cell lines used [Lewis GD et al., 1993].

The effect of HER-2 blockade on the proliferation of human colorectal cell lines was then examined using HM64.13 and the control mAb ICR12. Figures 5A-5C show that anti-HER-2 mAbs HM64.13 and ICR12 did not have any significant inhibitory effect on the proliferation of the colorectal cell lines, at a concentration of 200 nM.

The low level of expression of HER-2 in colorectal cell lines may help to explain the lack of inhibition of cell growth seen with HM64.13 and the control mAb ICR12. With a lower number of receptors available for antibody-mediated blockade, any potential inhibitory effect may be of a smaller magnitude. However, interestingly, the breast cancer cell line SKBR3, which is known to over-express a high level of HER-2, also showed no significant growth inhibitory effect with HM64.13 and ICR12 (Figure 5D). Thus, in the current study, there was no relationship between p185HER-2 overexpression and antibody-mediated anti-proliferative effect. This phenomenon has previously been described for other tumour cell lines (e.g. gastric and colon cell lines) [Lewis GD et al., 1993], and it is unclear why some p185HER-2—overexpressing tumour cells show insignificant growth inhibition with anti-HER-2 monoclonal antibodies.
One hypothesis is that the functional activity of an anti-HER-2 antibody is more related to the epitope that it recognizes than to its antigen binding affinity. It has previously been shown that various anti-HER-2 mAbs inhibit cell growth *in vitro* with varying efficiency [Hudziak RM et al., 1989; Tagliabue E et al., 2004]. Furthermore, Xu *et al.* reported that anti-HER-2-mediated growth inhibition was via immunochemically and functionally distinct epitopes on the extracellular domain of the c-erbB-2 receptor [Xu F et al., 1993]. Thus, the particular HER-2 epitope being blocked by mAb HM64.13 may not necessarily play an instrumental part in the growth signaling pathway, in that particular tumour cell line. This may explain why no significant anti-proliferative effect was seen with HM64.13 in the colorectal cell lines used in this study. Furthermore, in contrast to the anti-HER-2 mAbs used by Lewis *et al.* (1993), it may explain why no growth inhibition of SKBR3 was seen with HM64.13 or ICR12. It would suggest that distinct epitopes of HER-2 facilitate different aspects of its function. For example, it has also been demonstrated that different anti-HER-2 immunotoxins targeted against different epitopes of the p185HER-2 protein exert different cytotoxic activity [Boyer CM et al., 1999].

Interestingly, when the part of the signaling pathway downstream to the extracellular domain, was blocked, using the tyrosine kinase inhibitor, gefitinib, some growth inhibition (22%) was seen in SKBR3 cells (Fig. 5D).

With the exception of the DiFi cell line, treatment of the other human colorectal cell lines with anti-EGFR mAb ICR62 or gefitinib showed no significant effect on their proliferation. DiFi is known to over-express EGFR (4.8 x 10⁶/cell) [Gross ME et al., 1991], and showed significant anti-proliferative effect with both ICR62 and gefitinib.
6.2 The Prognostic Role of HER-2 in Colorectal Cancer

In this study, the expression and prognostic significance of HER-2 was investigated in 170 colorectal cancer patients. Previous studies have revealed conflicting results in terms of the frequency and prognostic significance of HER-2 [McKay JA et al., 2002; Osaka T et al., 1998; Sun X-F et al., 1995]. Here, cytoplasmic HER-2 was found to be an independent prognostic marker in Dukes’ C cancers, where positive cytoplasmic HER-2 was associated with a significantly better survival. However, this survival advantage was not seen in the Dukes’ B cancers, but this might be explained by the small number of events in this sub-group. No significant association was seen between membrane HER-2 and clinical outcome in either Duke's B or C cases.

Previous studies investigating the prognostic role of HER-2 in colorectal cancer have given conflicting results, as illustrated in Table 6.1. However, the wide variability in the overexpression of HER-2 seen in these studies is not peculiar to colorectal cancer, and is well documented for other tumours including breast, ovary and bladder [Ménard S et al., 2001]. From Table 6.1, one can see that moderate to strong membrane HER-2 expression in these colorectal cancers ranged from 1.4% to 81.9%. In addition, while membrane HER-2 was associated with a poor prognosis in one study [Knosel T et al., 2002], others showed no such association [Benecke M et al., 2002; Chamberlain NL et al., 1999; McKay JA et al., 2002]. Also of note is that even when the same anti-HER-2 antibody is used, marked differences in the levels of HER-2 overexpression are reported. For example, the range of HER-2 overexpression seen in the three studies using anti-HER-2 antibody, NCL-CB11, was 33.5%-81.9% [Kay EW et al., 1994b; McKay JA et al., 2002; Sun X, 2001].
Table 6.1. Summary of studies looking at the overexpression of cytoplasmic HER-2 and membranous HER-2 in colorectal cancer, and their prognostic role.

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Number of patients</th>
<th>mAb</th>
<th>Dukes' staging</th>
<th>Reported Positive aCI and/or bMI</th>
<th>Hazard Ratio</th>
<th>p VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>McKay JA et al., 2002</td>
<td>249</td>
<td>NCL-CB11</td>
<td>A-D</td>
<td>MI 81.9% (CI nfa)</td>
<td>-</td>
<td>qNS</td>
</tr>
<tr>
<td>Sun X-F et al., 1995</td>
<td>293</td>
<td>NCL-CB11</td>
<td>A-C</td>
<td>MI 59% (CI nfa)</td>
<td>0.5</td>
<td>P=0.027</td>
</tr>
<tr>
<td>Kay EW et al., 1994b</td>
<td>164</td>
<td>NCL-CB11</td>
<td>B</td>
<td>CI 33.5% MI 0%</td>
<td>2.51</td>
<td>P=0.0005</td>
</tr>
<tr>
<td>Benecke M et al., 2002</td>
<td>74</td>
<td>Hercep Test Kit</td>
<td>D</td>
<td>MI 9% (CI nfa)</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Knosel T et al., 2002</td>
<td>45</td>
<td>Hercep Test Kit</td>
<td>Not specified</td>
<td>MI 49% (CI nfa)</td>
<td>MI associated with poorer survival</td>
<td>'significant' but p value not stated</td>
</tr>
<tr>
<td>Kakani V et al., 2002</td>
<td>71</td>
<td>Hercep Test Kit</td>
<td>B</td>
<td>MI 1.4% (CI nfa)</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Nathanson DR et al., 2003</td>
<td>139</td>
<td>Hercep Test Kit</td>
<td>A-D</td>
<td>MI 3.6% (CI nfa)</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Osaka T et al., 1998</td>
<td>146</td>
<td>Nichirei</td>
<td>A-D</td>
<td>MI 2.1% CI 66%</td>
<td>1.82</td>
<td>P=0.008</td>
</tr>
<tr>
<td>Kapitanovic S et al., 1997</td>
<td>155</td>
<td>Ab-3</td>
<td>A-C</td>
<td>Mixed CI and MI 84%</td>
<td>1.84</td>
<td>0.014</td>
</tr>
<tr>
<td>Chamberlain NL et al., 1999</td>
<td>96</td>
<td>Rabbit anti-HER2</td>
<td>A-D</td>
<td>MI 15.6% CI 9.4%</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>Lee JC et al., 2002</td>
<td>125</td>
<td>Triton</td>
<td>A-D</td>
<td>MI 4%</td>
<td>-</td>
<td>NS</td>
</tr>
</tbody>
</table>

a CI, cytoplasmic HER-2
b MI, membrane HER-2 (2-3+ in all cases except in the case of Chamberlain et al where percentage indicates those cases with a score of 1-3+)
c nfa, not formerly assessed
d NS: not statistically significant at p<0.05; point estimates and exact p-values not given.
e Only in aneuploid tumours with positive MI
Furthermore, there were also differences in the localization of the HER-2 immunostaining (i.e. membranous or cytoplasmic), even when the same mAb was used. Using anti-HER-2 mAb NCL-CB11, Kay and colleagues investigated the expression of HER-2 in 164 patients with Dukes’ B cancers [Kay EW et al., 1994b]. Whilst they reported no evidence of membrane HER-2 in their group of tumours, all 33.5% of the HER-2-positive cases showed cytoplasmic HER-2, and the latter was an indicator of poor clinical outcome in Dukes’ B cancers. In complete contrast to Kay’s study, Sun et al. reported a 59% incidence of membrane HER-2 overexpression in 293 patients with locally advanced colorectal cancer, using the same mAb NCL-CB11 [Sun X-F et al., 1995]. Overall, there was no significant association found between membrane overexpression of HER-2 and survival, but surprisingly, a favourable survival was seen on subgroup analysis in DNA aneuploid tumours. Cytoplasmic HER-2 was not quantified in this study. A third group, in Aberdeen, found no significant association between clinical outcome and positive membrane HER-2 in 249 colorectal tumours, again using NCL-CB11 [McKay JA et al., 2002]. They performed a semi-quantitative assessment of HER-2 overexpression, according to the United States Food and Drug Administration (USA FDA)-approved scoring system [Jacobs TW et al., 1999]. However, this scoring system makes no provision for cytoplasmic HER-2 assessment, and so its incidence in this study is not reported.

Although membrane HER-2 alone has been shown to be of prognostic value in breast cancer, the intuitive assumption that this is the only important criterion for assessing HER-2 overexpression in other tumours, including colorectal cancer, may not necessarily be valid. There have been studies in breast [Zschiesche W et al.,
1994] and thyroid [Sugg SL et al., 1998] cancer which have shown an association between good prognostic tumours and cytoplasmic HER-2. Cytoplasmic HER-2 has also been shown to be the predominant pattern of immunostaining in squamous cell carcinoma of the head and neck [Field JK et al., 1992].

6.3 Cytoplasmic HER-2 is Associated with a Better Survival

The current study, like others [Kay EW et al., 1994b; Osaka T et al., 1998], has found cytoplasmic HER-2 to be the predominant pattern of immunostaining in colorectal cancers (Table 4.2). However, to my knowledge, this is the first study in colorectal cancer to suggest a better survival associated with tumours showing positive cytoplasmic immunostaining (Figure 9). Furthermore, positive cytoplasmic HER-2 was found to be an independent prognostic marker (HR 0.46, p<0.02) in Dukes' C colorectal cancer, together with grade and depth of invasion of tumour, and apical node metastases (Table 4.3).

Investigators from Croatia reported mixed cytoplasmic and membrane HER-2 immunostaining in their cohort of colorectal cancer patients, and found that those cases with moderate or strongly positive tumours had a significantly poorer survival than those with weakly positive tumours [Kapitanovic S et al., 1997]. However, a median survival of only 2.3 or 0.5 years in patients whose tumours show moderate or strong HER-2 overexpression respectively is well below that expected in a cohort of patients with loco-regional disease. In another study, predominant cytoplasmic HER-2 immunostaining was described in the tumour specimens of 146 Japanese patients, and this was correlated with a poorer prognosis [Osaka T et al., 1998]. However, no prognostic significance was found for cytoplasmic or membrane HER-2 in 96
tumours from an Australian cohort of patients [Chamberlain NL et al., 1999](see Table 6.1).

The varying IHC techniques and conditions, the different anti-HER-2 antibodies, which differ in their binding affinity and epitope specificity, along with differences in the interpretation of HER-2 overexpression (including not reporting cytoplasmic staining) are probably the main reasons for the controversies surrounding the prognostic significance of HER-2 in colorectal cancer [Kay EW et al., 1994a; Press MF et al., 1994]. Any ethnic variability in the overexpression of HER-2 is unknown.

There is a definite need for a standardised system of reporting HER-2 overexpression in colorectal cancer if there is to be any valid comparison of studies. Some investigators have attempted to use the USA FDA-approved scoring system in colorectal cancer [Jacobs TW et al., 1999]. However, although this scoring system allows standardisation of the assessment and scoring of membrane HER-2, it is not designed to score cytoplasmic HER-2. The FDA-approved scoring system was developed to standardise the evaluation of HER-2/neu protein expression in breast cancers, using the HercepTest™ (DAKO, Glostrup, Denmark). In this context, where a decision regarding receptor-targeted antibody therapy (i.e. trastuzumab) is being considered, it is logical that the IHC scoring system assesses membrane immunostaining solely, as the antibody clearly has to bind the receptor to produce its effect. Any cytosolic form of HER-2, identified immunohistochemically, would be of no therapeutic value, in terms of antibody therapy, due to its reduced accessibility, and this would explain why scoring of the cytoplasmic HER-2 is not included in the therapy-focused FDA system. However, in attempting to identify a prognostic marker immunohistochemically, the criteria used to score the immunostaining need
not be limited to a membrane-localised molecular marker. The predominant pattern of HER-2 immunostaining seen in colorectal cancer is cytoplasmic, with membrane immunostaining seen to a lesser extent. Whilst the precise significance of cytoplasmic HER-2 remains under investigation, any assessment of HER-2 overexpression in colorectal cancer should ideally take into consideration cytoplasmic immunostaining of HER-2, as well as membrane HER-2.

In this study, it was found that the predominant pattern of HER-2 immunostaining seen in colorectal cancer is cytoplasmic. However, traditionally investigators have focused on the membranous overexpression of HER-2 [43][45]. The suggestion that the transmembrane position of HER-2 argues against it being detected in the cytoplasm, and that cytoplasmic immunostaining is an artefact, has probably contributed to under-reporting of cytoplasmic HER-2, and any association with outcome. However, there is data to suggest that the precise significance of cytoplasmic HER-2 should be fully elucidated, and that it should not be ignored. Cytoplasmic HER-2 has been correlated with gene amplification in bladder [Coombs LM et al., 1991] and colorectal cancers [D'Emilia J et al., 1989; Kapitanovic S et al., 1997]. The close correlation between cytoplasmic immunoreactivity and c-erbB-2 amplification argues that the cytoplasmic product represents some form of the HER-2 protein product, possibly a splice variant of the whole receptor. In the colorectal cancer studies, Western blotting techniques were used to characterize the molecular weight of the cytosolic protein, which was found to be 155kDa or 185kDa [Kapitanovic S et al., 1997; Osaka T et al., 1998]. Using immunoprecipitation studies, a 160-kD protein was found in a human gastric cell line, which over-expressed HER-2 that was pre-treated with an N-linked glycosylation inhibitor [Akiyama T et al.,
1986]. This protein was considered to be an incompletely glycosylated form of HER-2.

There are studies to suggest that the truncated form of HER-2 may also be of prognostic value. The full-length receptor p185HER-2 undergoes a slow proteolytic shedding of its ECD from the surface of HER-2-overexpressing tumour cells in culture [Lin YL and Clinton GM, 1991; Pupa S et al., 1993]. The HER-2 ECD has been detected in the serum of patients with advanced breast cancer where it was shown to correlate with recurrence [Brandt-Rauf PW, 1995]. Furthermore, elevated levels of circulating HER-2 ECD have been shown to correlate with increased resistance to chemotherapy, in patients with metastatic breast cancer [Colomer R et al., 2000]. It has also been reported that, in contrast to the full-length HER-2, the membrane-associated p95HER-2 fragment, which is missing its ECD, is more frequently expressed in lymph node metastatic tissue than in primary breast cancer tissues [Molina MA et al., 2002]. This could have both therapeutic and prognostic implications if indeed the truncated kinase p95 is shown to have a role in the development of metastasis.

The association between membranous overexpression of HER-2 and a poorer prognosis, as seen in the breast studies, can be explained by our knowledge of HER-2 and its role in the signal transduction pathway. It is less clear from previous studies why cytoplasmic HER-2 overexpression should be associated with a poorer survival in colorectal cancer [Kay EW et al., 1994b; Osaka T et al., 1998] as the mitogenic growth signal is presumably down-regulated under these circumstances. The survival benefit seen with positive cytoplasmic immunostaining, as reported in the current study, may represent internalization and possible modification of HER-2.
With the receptor no longer on the cell surface, it is unable to form homodimers, or heterodimers with other receptors of the same EGFR family, leading to down-regulation of the mitogenic growth signals, not only mediated by HER-2 itself, but also by EGFR, HER-3 and HER-4 [Gullick WJ, 1990; Rubin I and Yarden Y, 2001]. In vitro studies, using cell lines overexpressing HER-2, have shown a shift from membranous to cytoplasmic immunostaining, apparently reflecting internalization of HER-2, following activation of the receptor [Bacus SS et al., 1990; Bacus SS et al., 1992]. This process is accompanied by decreased proliferative activity of the tumour cells. This would explain the reduced potential for tumour growth and progression, as reflected in the improved survival in those patients whose tumours show cytoplasmic HER-2.

Future large studies in colorectal cancer are required to both standardise the reporting of membrane and cytoplasmic HER-2 overexpression, and to confirm their prognostic role. Finally, the high level of membranous HER-2 in this study (68 out of 170 cases) could make HER-2 an ideal target for monoclonal antibody-based immunotherapy in such patients.

6.4 HER-2 Targeting

The transmembrane position of HER-2 lends itself to being an ideal target for antibody-mediated therapy. In breast cancer, HER-2 has been shown to be of significant prognostic value [Slamon DJ et al., 1987], and this has led to the development of the first anti-HER-2 monoclonal antibody, transtuzumab (Herceptin™), being used in the treatment of metastatic breast cancer. This therapeutic option has necessitated the determination of HER-2 status in those
breast cancer patients in whom it is used, using their archival tumour specimens. The standardization of HER-2 immunostaining in breast tumour sections has been instrumental to identifying those patients for whom trastuzumab may be a therapeutic option.

There have been some reports suggesting that HER-2 may be of prognostic significance in other cancers, including those of the ovary, pancreas, stomach and colon. Most of these reports have looked at membranous HER-2 immunostaining, whilst others have acknowledged the presence of cytoplasmic HER-2, particularly in colorectal cancer, and its potential prognostic role in these tumours.

In the current study, there was no significant association between membrane HER-2 and survival in Dukes' B or C colorectal cancer patients. This was also true of the other large studies which assessed the prognostic role of membrane HER-2 in colorectal cancer [Benecke M et al., 2002; Chamberlain NL et al., 1999; McKay JA et al., 2002]. This does not necessarily suggest that anti-HER-2 antibody therapy would not have a significant role in the treatment of colorectal cancer. In addition, the interaction between HER-2 and other molecular proteins, via the signal transduction pathway, indicates that the association between HER-2 and clinical outcome may not be a linear one. Furthermore, since HER-2 does not have a known ligand of its own, it relies on heterodimerisation for its transactivation, and HER-2 is thought to be the preferred heterodimer partner in the HER signaling network [Klapper LN et al., 2000]. Consequently, in colorectal cancer, it may be necessary to look at the overexpression of HER-2 not in isolation, but in relation to the co-expression of other cellular receptors or proteins, either from the same (e.g. EGFR) or different (e.g. insulin-like growth factor-1 [IGF-1] receptor) family. Furthermore, the strategy of
specifically targeting HER-2 alone, with an anti-HER-2 mAb, may need to be reviewed in the context of the influence of other receptors known to play a significant role in the effectiveness of the mAb. Indeed, synergistic inhibition of growth in HER-2-overexpressing breast cancer cells has been demonstrated by co-targeting HER-2 and IGF-1 receptors [Camirand A et al., 2002]. Furthermore, recent experimental studies have indicated that the mechanism whereby tumour cells, previously sensitive to the anti-HER-2 mAb transtuzumab, subsequently develop a resistant phenotype is by up-regulation of other receptors such as IGF-1R [Albanell J and Baselga J, 2001].

6.5 The Prognostic Role of CD31 Immunohistochemistry in Colorectal Cancer

New tumour growth, and subsequent progression to metastases, is dependent on the formation of new blood vessels to provide the essential requirements for this process (e.g. nutrition and oxygen) [Folkman J, 1990]. The first suggestion that the number of microvessels in a tumour could be related to metastases and survival was described in malignant melanoma [Srivastava A et al., 1988]. Since then, the prognostic information from tumour vascularity has been described for other tumour sites, including breast [Weidner N et al., 1992], lung [Macchiarini P et al., 1992], stomach [Tanigawa N et al., 1996], and prostate [Weidner N et al., 1993].

In colorectal cancer, there is less known about the prognostic significance of tumour vascularity, and the studies to date reveal conflicting results, and are therefore by no means conclusive (Table 6.2).
Table 6.2. Summary of studies looking at the prognostic significance of tumour vascularity in colorectal cancer

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Number of patients</th>
<th>Dukes' stage</th>
<th>Factor assessed</th>
<th>Magnification of field</th>
<th>Cut-off for MVD groups</th>
<th>Results of multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saclarides TJ et al., 1994</td>
<td>48</td>
<td>A-D</td>
<td>F8RA</td>
<td>X100</td>
<td>39.5(^a) (no cut-off)</td>
<td>No multivariate analysis</td>
</tr>
<tr>
<td>Frank RE et al., 1995</td>
<td>105</td>
<td>B</td>
<td>F8RA</td>
<td>X100</td>
<td>28(^c)</td>
<td>No multivariate analysis</td>
</tr>
<tr>
<td>Bossi P et al., 1995</td>
<td>178</td>
<td>A-D</td>
<td>CD31</td>
<td>X200</td>
<td>115(^a)</td>
<td>P=NS</td>
</tr>
<tr>
<td>Lindmark G et al., 1996</td>
<td>212</td>
<td>A-D</td>
<td>F8RA</td>
<td>X125</td>
<td>&gt;10(^a)/field</td>
<td>Longer survival on univariate analysis (P=0.007)</td>
</tr>
<tr>
<td>Takebayashi Y et al., 1996</td>
<td>166</td>
<td>A-C</td>
<td>F8RA</td>
<td>X400</td>
<td>65(^b)</td>
<td>P=0.007</td>
</tr>
<tr>
<td>Tomisaki S et al., 1996</td>
<td>175</td>
<td>A-D</td>
<td>F8RA</td>
<td>X200</td>
<td>32(^a)</td>
<td>No multivariate analysis</td>
</tr>
<tr>
<td>Tanigawa N et al., 1997</td>
<td>133</td>
<td>A-D</td>
<td>CD34</td>
<td>X200</td>
<td>105(^b)</td>
<td>P=0.0002</td>
</tr>
<tr>
<td>Takahashi Y et al., 1997</td>
<td>27</td>
<td>B</td>
<td>F8RA</td>
<td>200X</td>
<td>26(^a)</td>
<td>High MVD correlated with time to recurrence</td>
</tr>
<tr>
<td>Choi HJ et al., 1998</td>
<td>127</td>
<td>A-D</td>
<td>F8RA</td>
<td>X200</td>
<td>55(^a)</td>
<td>P=0.0004</td>
</tr>
<tr>
<td>Vermeulen PB., et al., 1999</td>
<td>116</td>
<td>A-C</td>
<td>CD31</td>
<td>X200</td>
<td>74(^b)</td>
<td>P=0.006</td>
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<tr>
<td>Sternfeld T et al., 1999</td>
<td>146</td>
<td>A-C</td>
<td>CD31</td>
<td>X200</td>
<td>75(^a)</td>
<td>P=NS</td>
</tr>
<tr>
<td>Pietra N et al., 2000</td>
<td>119</td>
<td>B</td>
<td>CD31</td>
<td>X1000</td>
<td>254(^a) (no cut-off)</td>
<td>P=NS</td>
</tr>
<tr>
<td>Cascinu S et al., 2000</td>
<td>121</td>
<td>B</td>
<td>VEGF</td>
<td>'High power'</td>
<td>&gt;10% positive cells</td>
<td>Worse disease-free survival in VEGF positive cases (p=0.001)</td>
</tr>
<tr>
<td>Cianchi F et al., 2002</td>
<td>84</td>
<td>A-B</td>
<td>CD31</td>
<td>X250</td>
<td>45(^b)</td>
<td>P=NS</td>
</tr>
</tbody>
</table>

\(^a\)Mean MVD  
\(^b\)Median MVD  
\(^c\)median MVD at 66% percentile
In the current study, the mean (±SEM) MVD for those patients who remained
disease-free was 29 (± 3), whilst for those patients who developed a recurrence
(local or distant), the mean MVD was 27 (± 3). There was thus no significant
difference in the MVD seen in those tumours of patients with recurrence of their
disease when compared with those from patients who remained disease-free.
Furthermore, no association was found between MVD and other conventional
prognostic factors such as the number of nodes involved with metastases, vascular
invasion, depth of tumour invasion, and grade of tumour.

Two recent studies [Cianchi F et al., 2002; Pietra N et al., 2000] also found no
significant correlation between MVD and recurrence rates or survival. However, both
of these studies were in node negative colorectal cancers only, and therefore the
results cannot be compared to the current study which included only Dukes’ C
cancers. Node-negative colorectal cancer has a better prognosis than node positive
disease since the incidence of subsequent distant metastases in the former group is
significantly lower. Consequently, if angiogenesis does in fact have an independent
prognostic role, in predicting those patients who will relapse with distant metastases,
its may be more difficult to show in a multivariate analysis, particularly if the number
of events in the study population is low. This is reflected in the fact that two further
studies investigating the prognostic role of MVD in node-negative colorectal cancers
[Frank R et al., 1995; Takahashi Y et al., 1997] both failed to show MVD as an
independent prognostic marker for survival, and one of the studies [Frank R et al.,
1995] did not quote any multivariate analyses.

However, the current study which investigated Dukes’ C colorectal cancers failed to
show any association between recurrence of disease or survival and MVD, and there
may be a number of reasons for this. It is possible that MVD does not in fact play a
significant prognostic role in Dukes' C cancers. Neovascularization is essential for
tumour proliferation or the growth of tumour cells [Folkman J, 1990] but the formation
of new vessels, per se, may not necessarily have a causal relationship with
development of metastases in colorectal cancers. MVD may determine the speed of
tumour recurrence after the metastatic process has already been triggered by other
more important factors. This hypothesis was also suggested by Sternfeld et al who
also found no significant difference in overall survival between the two tumour groups
with high and low MVD, using the same methodology as the current study [Sternfeld
T et al., 1999]. However, in the sub-group of patients with recurrence, MVD was an
independent prognostic factor and a stronger predictor of overall survival and
relapse-free survival than conventional histological parameters. Their conclusion was
that because the correlation of MVD with the more aggressive tumours is
retrospective rather than prospective, it had no clinical value as a new prognostic
marker. MVD was an important predictor of poor outcome only in those tumours
where other, unknown mechanisms, had already triggered the metastatic process.

Ascertaining the density of microvessels in a tumour does not give any information
regarding the integrity of the microvessels. The process whereby blood vessels
become permeable to tumour cells, facilitating the metastatic process, is a complex
one. It involves numerous molecular pathways which include interactions with cellular
and humoral components of the immune/inflammatory system, coagulation
molecules, intercellular adhesion molecules, and the enzymes involved in the
integrity of surrounding connective tissue (e.g. proteases). Emerging characterization
of the signal transduction pathways in endothelial cells indicates that there are also a
number of endothelial growth factors which may act in concert in tumour
angiogenesis and affect the metastatic process [Barozzi C et al., 2002; Viloria Petit AM et al., 1997].

Recent studies also suggest that tumour micro-vascularity is not the only process that may affect prognosis. The degree of vascular maturation varies between tumours, and this is independent of MVD [Eberhard A et al., 2000]. Furthermore the ability to maintain the newly formed vessels also varies among tumours. Endothelial cell proliferation occurs predominantly at the tumour periphery, as shown by Fox et al. [Fox SB et al., 1993]. As the tumour grows, the new vessels, which are formed in the invading edge, are gradually incorporated by the growing tumour. The ability of these vessels to survive will play an important role in the vascular density of the tumour, and may, independently of MVD, also affect its potential for metastasizing. The importance of vascular survival ability was recently demonstrated in a study which assessed this parameter in a sample of 242 colorectal cancers [Giatromanolaki A et al., 2002]. Vascular survival ability was the most significant independent prognostic variable (p=0.0001) on a multivariate analysis, followed by vascular invasion and Dukes’ stage. They also noted that there was no association between vascular survival ability and the MVD in a tumour, suggesting that the biological pathways controlling angiogenesis are not identical to those controlling vascular survival. In the same study, the MVD was not different among stage A, B, and C tumours, suggesting that the angiogenic activity of a tumour reaches its maximum in the early stages of development of the disease in colorectal cancer.

The lack of association between MVD and Dukes’ stage was also reported by Bossi et al. [Bossi P et al., 1995] who looked at the association between MVD and outcome in 178 Dukes’ A-D colorectal cancer patients. They used the same anti-CD31 mAb
(JC-70) as in the current study, and also found no significant association between MVD and metastases or survival. They also found no association between MVD and stage of disease. However, they did demonstrate a significant progression in the MVD seen in normal bowel mucosa, adenomas and carcinomas of the colon, suggesting that although angiogenesis did not provide significant prognostic information in colorectal cancer patients, it was a critical step in colorectal tumourigenesis.

Of the 14 studies summarized in Table 6.2, nine performed multivariate analyses. Of these, four showed MVD to be an independent prognostic marker for survival, whilst four did not. In accordance with the former four studies, there is experimental evidence to show that as tumour neovascularization increases, the propensity for haematogenous spread of tumour cells also increases [Folkman J, 1994]. Furthermore, the association between increasing vascularity of a tumour and increased shedding of tumour cells into the vessels has been demonstrated [McCullough P et al., 1995]. However, one study, in 212 colorectal cancer patients, found the opposite to the findings in other studies. That is, a significantly longer survival time for patients who had tumours with a mean microvessel count of >10, as compared with those who had ≤5 [Lindmark G et al., 1996]. Although a surprising finding, it is difficult to ignore this study which is the largest series looking at MVD in colorectal cancer, and it highlights the current controversy about the precise prognostic role of angiogenesis in colorectal cancer.

The difficulty in interpreting these studies is that they have all used varying methodologies, although in some cases, the differences are subtle. In assessing the MVD, many have used the methodology initially described by Weidner et al.
[Weidner N et al., 1991], and subsequently recommended by the consensus paper [Vermeulen PB et al., 1996]. However, the differences in methodology between some of the studies make comparison of the results difficult. For example, although the magnification at which the microvessel count was performed was often the same (i.e. a magnification of 200X), some groups have deviated from this, and used field magnifications varying from 100X to 1000X to assess the vessel count, which means that the area of tumour over which the vessels are being counted are not comparable. Furthermore, there is no defined vessel count that automatically distinguishes a tumour with a high MVD from one with a low vessel count. Many investigators have used the median MVD, as the cut-off, to dichotomize the tumours into low and high MVD tumours, as recommended by the consensus paper [Vermeulen PB et al., 1996]. Others have used the mean microvessel count, or a random cut-off. Therefore the range of cut-offs for the low and high MVD groups from these studies vary from 28 to 254. Even in those studies which reported MVD to be an independent prognostic marker, the range of MVD used as the cut-off ranged from 55 to 105. The differences in the cut-offs is not accounted for by the different stages of disease in the various studies. Interpretation of all these results is difficult as there is significant overlap between the high and low MVD groups between the different studies.

The disparity seen among the different studies in terms of the prognostic role of MVD probably reflects the fact that the metastatic process is far too complex to look for one prognostic marker in isolation. In colorectal tumourigenesis, Fearon and Vogelstein proposed that colorectal cells must acquire between four and six molecular defects, including tumour suppressor gene inactivation and up-regulation of mutational oncogenes, and that it is the combination of these molecular events...
which culminates in the transition from adenoma to carcinoma [Fearon ER and
Vogelstein B, 2001]. Likewise, the continuing evolution of a tumour’s molecular
make-up and the proliferation-apoptosis balance are influenced by a number of
factors, which not only includes tumour angiogenesis but also includes cell-cell and
cell-matrix adhesion, cancer cell motility and migration, and the host immune
response. It is the interaction of all these factors, and others that will determine the
metastatic potential of a tumour.

6.6 The Co-expression of HER-2 and MVD in Colorectal Cancer

In the current study, no significant association was seen between the overexpression
of HER-2 and MVD in Dukes’ C colorectal cancer. It is thought that HER-2, through
its mediation of the signal transduction pathways, may up-regulate the expression of
one or more growth factors that function as stimulators of angiogenesis [Bouck N et
al., 1996; Rak J et al., 1994]. One such factor is vascular endothelial growth factor,
which is currently regarded as the major angiogenesis stimulator in human cancers
[Ferrarra N, 1995; Folkman J, 1996].

The co-expression of HER-2 and angiogenic markers in tumours may yield more
information regarding prognosis. There has been some work done on this in breast
tumours, where again the results are variable. For example, in one report of ninety-
nine invasive ductal cancers, MVD was significantly associated with p53, the other
gene that is thought to be involved with VEGF expression. In this study, there was no
association with HER-2 [Lee JS et al., 2002]. In another recent study looking at 77
node-negative breast cancers, high MVD was significantly associated with HER-2
overexpression, and high MVD was independently linked to relapse-free survival
[Koukourakis MI et al., 2003].
In colorectal cancer, there have been few studies investigating the co-expression of MVD and HER-2. Berney et al looked at the significance of the combination expression of five protein markers in fifty-eight patients with Dukes' A-C colorectal cancer. The five proteins were those believed to play a role in the development of colorectal cancer - c-erbB-2 protein product, the two tumour suppressor genes p53 and nm23, and VEGF and urokinase-like plasminogen activator. They found that although co-expression of these five protein markers seemed to be related to an increased risk of developing liver secondaries in colorectal cancer, nm23 was the only independent prognostic marker [Berney CR et al., 1999].

Another group of investigators also looked at the relevance of a broad panel of biological markers, including VEGF and HER-2, in colorectal cancer patients [Barozzi C et al., 2002]. They found that the expression levels of HER-2 and VEGF were significantly higher in tumours which had metastasised, compared to those of patients who had achieved a disease-free survival of greater than six years.

The correlation between the various tumour protein markers must be diverse, and the interaction of these markers in the metastatic cascade equally complex. The use of microarray techniques and proteinomics to look at the co-expression of these proteins will hopefully facilitate more of these studies which may help to select out those protein markers that are an absolute pre-requisite to the metastatic process. If successful, this will ultimately allow the clinician to improve the selection of those patients who are at increased risk of developing metastatic disease. The recent work of Van't Veer et al has shown an excellent example of how molecular profiling can accurately predict the outcome of breast cancer patients [van 't Veer LJ et al., 2002].
They used gene-expression microarrays, and were able to show that collectively 70 marker genes could accurately predict whether a young node-negative breast cancer patient would relapse within 5 years. If in fact this type of molecular profiling does indeed improve prediction of recurrence of disease, its use in other tumour sites, such as colorectal cancer will be a great advantage not only in the selection of patients for adjuvant treatments, but in particular the development of more specific treatments to target some of these proteins.

However, the limitations of such microarray techniques must be borne in mind. With the high throughputs of such techniques, the quality control may suffer. For example, to generate a cDNA microarray, there are many error-prone steps – PCR amplification, purification of DNA, and transfer of DNA into 384-well plates for printing. It is thought that for greater than 10% of cDNA arrays, the DNA on the microarray is not what the label says it is. Furthermore, the high cost of microarrays has tended to prohibit replication of experiments to confirm results. Other considerations include tumour heterogeneity, resulting in different genetic profiles within the same tumour, and the final interpretation of the mound of data from genomics and proteomics to biologically meaningful information [Zhang W et al., 2001]. After all, the detection of a specific protein within a tumour may be of prognostic value, but may be of limited value as a therapeutic target.

6.7 Anti-Angiogenic Therapy

Targeting the endothelial cells that line the tumour blood vessels is a new anticancer strategy. Bevacizumab is an inhibitor of vascular endothelial growth factor. It has been combined with conventional chemotherapy (5-FU/FA) in a phase II randomized
trial in metastatic colorectal cancer patients. This combination has shown significant results with improved response rates and longer median survival in the 5-FU/FA alone arm [Kabbinavar F et al., 2003]. The ability of this anti-VEGF mAb to prolong survival in this trial has given credence to this anti-angiogenic approach, but there are still unanswered questions regarding the selection of patients for such treatments, and the method of determining MVD in tumours if this is deemed necessary. Despite the continued uncertainty of the prognostic significance of tumour MVD, bevacizumab has now (February 2004) been approved to be used in combination with intravenous 5-FU-based chemotherapy as a treatment for patients with previously untreated metastatic colorectal cancer.

6.8 Concluding Remarks and Future Considerations

Tumour heterogeneity means that combination therapies are needed to prevent sub-optimal treatments. However, the current low improvement in survival (approximately 6% at 5 years) seen with conventional adjuvant combination chemotherapies is strong evidence that more molecular targets with a significant role in the proliferation, invasion and metastatic behaviour of tumours are needed. The explosive development of molecular genetic techniques has led to the identification of key proteins that regulate normal biological processes. These proteins may give us further vital information about the expected behaviour of a particular cancer and improve the selection of patients for whom adjuvant treatment has a definite survival advantage. The difficult task is in deciphering the relative importance of these cellular proteins in the mitogenic process. The therapeutic goal is to capitalise on this new information and translate it into more tumour selective therapies that will demonstrate not only greater efficacy, but also less toxicity than current ones.
Chapter Six

Discussion and Conclusion

It will be important to validate the prognostic role of cytoplasmic HER-2 in a prospective study. Standardization of the HER-2 immunohistochemistry method and assessment is of vital importance in colorectal cancer if this question is to be answered. In addition, the relative contribution of other growth factor receptors to the malignant behaviour of human colorectal cancer warrants further investigation. Indeed, an assessment of other growth factor receptors is currently underway in the same group of colorectal cancer patients. The receptors being investigated include wild-type EGFR, activated (phosphorylated) EGFR, EGFR VIII, phosphorylated tyrosine kinase proteins (pTyr-proteins), and IGF-1R. There are few reports of tyrosine phosphorylation and the biological characteristics of a tumour. In lung cancer, it has been shown that overexpression of pTyr-proteins is associated with a poor disease-free survival in spite of the absence of correlation with clinico-pathological factors [Gong Y et al., 2002]. The aim is to examine the level of co-expression of all these receptors in colorectal cancer, and their prognostic role. In addition, the effect of simultaneous blockade of these various growth factors on the malignant behaviour of such tumours will be examined. Such studies, in turn, will also help to unravel whether simultaneous targeting of such receptors is more effective in the treatment of colorectal cancer patients.


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