Molecular and biochemical markers in colorectal cancer

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Introduction

Colorectal cancer (CRC) is the second most common cause of cancer death in Europe. In 1999 there were an estimated 150,000 new cases of colorectal cancer and 60,000 deaths which accounts for approximately 10% of all cancer deaths in Europe. In the post-operative setting, there is clear evidence that offering adjuvant chemotherapy significantly improves survival in stage III (Dukes’ C) colorectal cancers, and 5-fluorouracil (5-FU)-based chemotherapy is the standard of care for these patients. However, the management of stage II (Dukes’ B) patients remains an area of debate as the 5-year survival for these patients is approximately 75%, indicating that the majority are cured by surgery alone and therefore would not benefit from chemotherapy. The identification of those patients who are at high risk of relapse has traditionally depended on pathological features such as disease stage, perforation, adherence or invasion of adjacent organs. However, recent studies have begun to use biochemical and molecular markers to identify patients at increased risk of relapse. In addition, these prognostic markers may allow identification of those patients who have a higher likelihood of response or benefit from chemotherapy.

The first clues to the genes involved in the genesis of colorectal cancer came when Vogelstein et al. [1] demonstrated that activation of certain oncogenes, such as \( \text{ras} \), and mutation of tumour suppressor genes, such as \( \text{p53} \), were consistently found in colorectal tumours. Their results supported the concept of an accumulation of genetic alterations over time in the progression from colonic adenoma to invasive carcinoma. The majority of colorectal cancers had allelic deletions, the four most common of which were localised to chromosomes 5, 8, 17 and 18, and the most common single abnormality is mutation of the \( \text{K-ras} \) oncogene on chromosome 12.

K-ras oncogene

K-ras gene mutations are believed to be among the earliest events in colorectal tumorigenesis, present in approximately 70% of cancers and 40% of adenomas. K-ras mutations are thought to occur as relatively early events in colorectal tumour progression and are associated with uncontrolled proliferation in primary rodent fibroblasts. The \( \text{ras} \) oncogene encodes a 21 kDa membrane-bound protein involved in signal transduction, which is part of the GTP family of proteins. The majority of \( \text{ras} \) mutations are found in K-ras on the short arm of chromosome 12, and typically at codons 12, 13 and 61. Point mutations at these codons produce an aberrant \( \text{p21} \) protein, which then continually activates downstream signal transduction pathways.

A number of studies have tried to determine whether the presence of K-ras mutations correlates with response to adjuvant therapy or survival (Table 1), but to date have yielded conflicting results. It is becoming increasingly recognised that different K-ras mutations have different impacts on outcome, even when the mutations occur at the same locus on the gene. The RASCAL II study (The Kirsten \( \text{ras} \) in Colorectal Cancer Collaborative Group) collected data on genotype and outcome in 3439 patients worldwide with K-ras mutations [2]. This analysis demonstrated that of the 12 possible mutations on...
codons 12 and 13 of K-ras, only one mutation on codon 12, glycine to valine (found in 8.6% of all patients), had a statistically significant impact on failure-free survival (P = 0.004) and overall survival (P = 0.008). This mutation appeared to have a greater impact on outcome in Dukes’ C (stage III) cancers (failure-free survival, P = 0.008; overall survival, P = 0.02) than in Dukes’ B tumours (failure-free survival, P = 0.46; overall survival, P = 0.36).

An interesting study by the Southwest Oncology Group (SWOG) attempted to relate K-ras mutational status not only with survival but also with response to adjuvant chemotherapy [3], and analysed stage II and III patients separately. In stage II patients, K-ras mutations were associated with significantly worse 7-year survival (86% versus 58%, P = 0.007), whereas the presence of K-ras mutations in stage III patients was associated with a lack of benefit from adjuvant 5-FU-based therapy. K-ras mutations may therefore indicate which stage II patients are at greatest risk of relapse and which stage III patients may not respond to 5-FU therapy. However, other studies examining the clinical importance of K-ras mutations have not reached the same conclusions and, as with other genetic markers, its clinical relevance needs to be addressed in larger randomised clinical studies.

<table>
<thead>
<tr>
<th>Reference</th>
<th>n</th>
<th>Stage</th>
<th>Frequency (%)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bell et al. (1993) [73]</td>
<td>100</td>
<td>A/B/C</td>
<td>24%</td>
<td>No significant correlation with survival and K-ras or p53 mutation separately, but worse survival when both mutations present (13 versus 33 months, P &lt;0.004)</td>
</tr>
<tr>
<td>Finkelstein et al. (1993) [74]</td>
<td>413</td>
<td>A/B/C/D</td>
<td>39%</td>
<td>Significantly higher rate of K-ras mutations in visceras metastases or transcoelomic implants (P&lt;0.01)</td>
</tr>
<tr>
<td>Andersen et al. (1994) [75]</td>
<td>100</td>
<td>A/B/C</td>
<td>40%</td>
<td>No significant correlation between K-ras mutations and survival, stage or histological grade</td>
</tr>
<tr>
<td>Morrin et al. (1994) [76]</td>
<td>52</td>
<td>A/B/C</td>
<td>36%</td>
<td>Mutation of K-ras or p53 did not correlate with Dukes’ stage, tumour differentiation or survival</td>
</tr>
<tr>
<td>Tanaka et al. (1994) [77]</td>
<td>62</td>
<td>A/B/C</td>
<td>38%</td>
<td>K-ras mutation correlated with vascular invasion (P &lt;0.01) and metastases (P &lt;0.01)</td>
</tr>
<tr>
<td>Ahnen et al. (1998) [3]</td>
<td>229</td>
<td>II/III</td>
<td>41%</td>
<td>Significantly worse 7-year survival in stage II tumours with K-ras mutation (58% versus 86%, P = 0.007), but not stage III (P = 0.06). Trend towards worse survival in stage III tumours with K-ras mutations after 5-FU chemotherapy</td>
</tr>
<tr>
<td>Kressner et al. (1998) [78]</td>
<td>191</td>
<td>B/C</td>
<td>32%</td>
<td>No significant correlation with K-ras mutations and survival</td>
</tr>
<tr>
<td>Tortola et al. (1999) [15]</td>
<td>140</td>
<td>A/B/C</td>
<td>41%</td>
<td>No significant correlation between K-ras mutation and overall survival. Significant correlation between p53 mutation and survival (P = 0.005)</td>
</tr>
<tr>
<td>Thebo et al. (2000) [79]</td>
<td>20</td>
<td>B2</td>
<td>49%</td>
<td>Dukes’ B tumours with K-ras mutations had an increased risk of recurrence (37.5% versus 0%)</td>
</tr>
<tr>
<td>Esteller et al. (2001) [80]</td>
<td>119</td>
<td>A/B/C</td>
<td>38%</td>
<td>No significant correlation with K-ras mutations and survival</td>
</tr>
<tr>
<td>Andreyev et al. (2001) [2]</td>
<td>3439</td>
<td>C</td>
<td>34%</td>
<td>Only mutations involving codon 12 (glycine to valine) (8.6% of patients) were significantly associated with worse survival (P = 0.008) in stage III patients</td>
</tr>
</tbody>
</table>

Table 1. K-ras mutations in colorectal cancer

An interesting study by the Southwest Oncology Group (SWOG) attempted to relate K-ras mutational status not only with survival but also with response to adjuvant chemotherapy [3], and analysed stage II and III patients separately. In stage II patients, K-ras mutations were associated with significantly worse 7-year survival (86% versus 58%, P = 0.007), whereas the presence of K-ras mutations in stage III patients was associated with a lack of benefit from adjuvant 5-FU-based therapy. K-ras mutations may therefore indicate which stage II patients are at greatest risk of relapse and which stage III patients may not respond to 5-FU therapy. However, other studies examining the clinical importance of K-ras mutations have not reached the same conclusions and, as with other genetic markers, its clinical relevance needs to be addressed in larger randomised clinical studies.

**Loss of 5q allele**

Allelic losses of chromosome 5q have been observed in 20–50% of sporadic colorectal cancers and in 30% of adenomas [1]. The adenomatous polyposis coli (APC) gene has been mapped to chromosome 5q21 and inherited mutations of this gene result in the familial adenomatous polyposis syndrome (FAP). This syndrome is characterised by the presence of hundreds of colonic adenomas which appear when patients are in their twenties and is associated with an increased risk of colonic carcinomas (mostly left-sided). In sporadic colon cancers inactivation of the APC gene is one of the earliest events in carcinogenesis. The APC protein is normally involved in the degradation of β-catenin [4]. β-Catenin, in conjunction with the DNA-binding protein TCF-4, functions as a transcriptional activator of an ever increasing array of genes including MMP-7, e-myc, cyclin D, CD44, gastrin, COX-2 and PPARβ [5]. Mutational inactivation of the APC gene results in accumulation of β-catenin, which enters the nucleus, complexes with transcription factor TCF-4, and up-regulates the aforementioned genes. Given its early role in tumorigenesis, there have been surprisingly few studies examining the role of APC as a prognostic marker in CRC; however, there is increased interest in whether up-regulated APC target genes like MMP-7 and COX-2 may be important therapeutic targets.

**Loss of 8p allele**

Loss of material from the short arm of chromosome 8 is found in approximately 50% of cases of colorectal cancer. The introduction of the short arm of chromosome 8 into colon cancer...
cell lines decreases their tumorigenicity [6]. There may be at least two tumour suppressor genes located on 8p and these have been mapped to 8p11 and 8p21–22 [7]; however, their physiological role is unknown at present. Loss of heterozygosity in 8p in colorectal cancer has been shown to correlate with later stages of disease and also the presence of micrometastases [8]. Based on this, Halling et al. [9] analysed the allelic imbalance in 508 Dukes’ B2 and C colorectal cancers and correlated their findings with 8-year survival. 8p Allelic imbalance was an independent prognostic indicator for decreased survival (P = 0.002) and for time to recurrence (P = 0.002). This is the first large study exploring the role of 8p allelic imbalance in prognosis of resected colorectal cancers.

**Loss of 17p allele**

Loss of material from the short arm of chromosome 17 is found in up to 75% of cases of colorectal cancer [1] and this segment contains the tumour suppressor gene p53. The p53 gene encodes a nuclear protein that functions as a transcription factor. p53 functions as a tetramer, therefore, only one allele may need to be deleted as the remaining wild-type p53 protein may be inactivated due to a dominant negative effect of the mutated p53 gene product. p53 is regarded as one of the most important tumour suppressor genes, with a number of key cellular functions such as DNA damage repair, initiation of programmed cell death and cell cycle checkpoint control. Increased p53 expression is triggered by DNA strand breaks such as those caused by radiation or chemotherapy [10]. Two protein kinases, ATM and ATR, are involved in ‘sensing’ DNA damage and phosphorylating proteins including p53 [11]. Phosphorylation of p53 stabilises the protein and results in transcriptional activation of a variety of genes including p21, which causes cell-cycle arrest, and genes involved in mediating apoptosis including Fas/CD95 and the pro-apoptotic Bcl-2 family members. It is clear that p53 mutations which interfere with its function have the potential to confer a significant survival advantage to any tumour, by preventing cell-cycle arrest or apoptosis.

Missense mutations of the p53 gene result in production of an abnormal p53 protein with an increased half-life which accumulates in the nucleus and cytoplasm, and which can be detected by immunohistochemistry (IHC). Detection of increased p53 protein expression by IHC has been used as a surrogate for p53 mutation in many studies attempting to correlate p53 mutation with prognosis. Studies of at least 100 patients with CRC using p53 monoclonal antibodies have generally found that p53 overexpression was associated with worse prognosis; however, some studies have failed to find a correlation. There is a significant body of in vitro work linking p53 status with cellular sensitivity to chemotherapeutic agents [12]. This has been borne out in several clinical studies examining the relationship between p53 overexpression and benefit from adjuvant 5-FU-based chemotherapy, but not in all (Table 2). Detection of accumulated p53 protein does not always equate to a p53 mutation because cancers with wild-type p53 can develop increased levels of the protein due to mutations in other proteins such as MDM-2. In one study of

<table>
<thead>
<tr>
<th>Reference</th>
<th>n</th>
<th>Stage/Dukes</th>
<th>Outcome</th>
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</thead>
<tbody>
<tr>
<td>Sun et al. (1992)</td>
<td>293</td>
<td>A–C</td>
<td>24% showed increased nuclear p53 staining and 10% in cytoplasm. 15% had both. Only cytoplasmic staining for p53 protein significantly associated with worse survival (P&lt;0.0001)</td>
</tr>
<tr>
<td>Bosari et al. (1994)</td>
<td>206</td>
<td>A–D</td>
<td>Nuclear and cytoplasmic staining. Worse overall (P = 0.02) and disease-free survival (P = 0.004) in tumours showing p53 positivity</td>
</tr>
<tr>
<td>Tollema et al. (1998)</td>
<td>238</td>
<td>A–C</td>
<td>Nuclear p53 and Bcl-2 expression analysed. No significant association between either and survival</td>
</tr>
<tr>
<td>Ahnen et al. (1998)</td>
<td>229</td>
<td>II (66) III (163)</td>
<td>K-ras mutations and p53 overexpression analysed. K-ras mutations in 41% of tumours and p53 overexpression in 63%. Stage III patients with either K-ras mutation or p53 overexpression failed to show any benefit from 5-FU/LEV adjuvant therapy</td>
</tr>
<tr>
<td>Kaklamanos et al. (1998)</td>
<td>224</td>
<td>A–C</td>
<td>Cytoplasmic Bcl-2 and nuclear p53 expression by IHC. Significantly worse survival in patients with tumours demonstrating p53 overexpression (P = 0.01). No association between bcl-2 and survival. Unknown how many patients received adjuvant chemotherapy</td>
</tr>
<tr>
<td>Buglioni et al. (1999)</td>
<td>171</td>
<td>A–D</td>
<td>Analysed p53, Bcl-2, Ki67 and DNA ploidy. p53 positivity and Bcl-2 negativity were significantly associated with Dukes’ C and D tumours. Both were significantly associated with worse survival</td>
</tr>
<tr>
<td>Diez et al. (2000)</td>
<td>174</td>
<td>I–III</td>
<td>Serum CEA and p53 expression analysed. 47% of tumours demonstrated p53 overexpression. No information on whether adjuvant chemotherapy administered or not. p53 positivity was significantly associated with worse disease-free survival and overall survival</td>
</tr>
<tr>
<td>Paradiso et al. (2000)</td>
<td>108</td>
<td>D</td>
<td>Cytoplasmic TS and nuclear p53 IHC analysed. Response to 5-FU chemotherapy significantly correlated with TS negative status of tumours (30% versus 15%; P = 0.04), but not overall survival. No correlation between response to chemotherapy and p53 protein overexpression</td>
</tr>
</tbody>
</table>

CEA, carcinoembryonic antigen; 5-FU, 5-fluourouracil; IHC, immunohistochemistry; LEV, levamisole; TS, thymidylate synthase.
316 breast cancer specimens, IHC failed to detect 33% of p53 mutations confirmed by cDNA sequencing [13]. In another study of 191 frozen colorectal cancer specimens the concordance between IHC and cDNA sequencing was only 74% [14]. Recently, investigators have used DNA sequencing instead of IHC to determine the relationship between p53 mutations and prognosis. While there is some disagreement in the IHC studies about the significance of p53 overexpression, the majority of DNA sequencing studies have shown a significantly shorter survival time in patients with p53 mutations [15–19].

Loss of 18q allele

Loss of material from the long arm of chromosome 18 is found in approximately 70% of cases of colorectal cancer and 50% of adenomas. The 18q segment contains three candidate tumour suppressor genes: DCC (deleted in colon cancer gene) and Smad 2 and Smad 4 (initially described as DPC4) genes [20]. DCC is a transmembrane protein with considerable homology to neural-cell adhesion molecules. Smad proteins are transcription factors involved in the TGF-β signalling pathway. These proteins are involved in signalling from TGF-β receptors and regulate transcription of key target genes (c-myc, CBFA1, FLRF and furin). Activation of Smads results in their translocation from the cytoplasm into the nucleus, where together with transcription factors they regulate target gene expression. Targeted disruption of Smad genes in mice has revealed their importance in embryonic development, and a tumour suppressor role for Smad genes in human cancers has been described. Ras overexpression is associated with increased ubiquitin-proteasome degradation of Smad 4. O’Connell et al. [21] found that allelic loss on chromosome 18q was associated with poorer prognosis in Dukes’ B and C tumours. Subsequently, Jen et al. [22] studied a larger number of Dukes’ B and C patients and confirmed their findings. The finding that the absence of DCC protein in tumour cells from stage II or III cancers was associated with worse survival pointed to this tumour suppressor gene as potentially playing a significant role in the biology of CRC. Indeed, in the study by Jen et al. [22], patients with stage II cancers and undetectable DCC protein had a similar survival to those patients with stage III cancers with detectable DCC protein. Watanabe and colleagues analysed tumour from 148 patients with stage III tumours treated with 5-FU-based therapy. They noted loss of heterozygosity at 18q in 49% of cases and this was associated with a significantly increased risk of recurrence and death (P < 0.001) [23]. In a study by Sun et al. [24], the prognostic significance of absence of DCC protein by IHC was confined to DNA diploid tumours and those with a low S-phase fraction on flow cytometry. The authors argued that both these features indicated early-stage tumour development and where loss of DCC expression was significantly associated with probability of survival (P = 0.02).

Microsatellite instability

Human DNA contains repetitive di-, tri- and tetrانucleotide repeats (or microsatellites) which are frequently located between genes and in the past were referred to as ‘junk’ DNA. Microsatellite instability (MSI) refers to variations in the number of repetitive units in each microsatellite. It is caused by failure of the DNA mismatch repair system to repair errors which occur during replication. Mismatch occurs when DNA polymerase inserts incorrect nucleotide bases during DNA synthesis [25]. These mistakes are usually corrected by the mismatch repair genes which include hMLH1, hMSH2 and hMSH6. In the autosomal dominant inherited condition hereditary nonpolyposis colorectal cancer (HNPCC) >90% of cases have mutations in these genes [26]. This condition accounts for only 1–5% of all cases of colorectal cancer, but 10–15% of sporadic cases also have mutations in mismatch repair genes [27]. In 1997, the National Cancer Institute convened a workshop to address the issue of an internationally accepted definition for high-frequency MSI. This led to the publication of criteria for the detection of MSI in colorectal cancer, defining high-frequency MSI as the presence of instability at two or more markers when compared with adjacent normal tissue (Table 3) [28]. This definition will make publication of any data regarding MSI more uniform and reproducible. Recent studies examining the importance of MSI in CRC have shown a significant survival advantage for patients with high-frequency MSI. Thus, while mutations in mismatch repair genes are involved in the evolution of some colorectal cancers, they may also predict for a less aggressive

<table>
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<tr>
<th>Criteria for interpretation</th>
<th>Interpreta</th>
<th>on</th>
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<tbody>
<tr>
<td>No. of markers exhibiting instability length changes</td>
<td>≥2</td>
<td>≥30–40%</td>
</tr>
<tr>
<td>1</td>
<td>&lt;30–40%</td>
<td>MSI-L</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>MSS or MSI-L</td>
</tr>
</tbody>
</table>

MSI, microsatellite instability; MSI-H, high-frequency MSI; MSI-L, low-frequency MSI; MSS, microsatellite stable.
clinical course. The reasons for this are currently poorly understood; however, some studies have suggested that there are fewer mutations in the APC and p53 tumour suppressor genes in patients with high-frequency MSI, which may partly account for the less aggressive phenotype of some MSI tumours [29]. As early as 1993 MSI was significantly correlated with right-sided colonic tumours, increased patient survival and inversely correlated with allelic loss on 5q, 17p and 18q [30].

Studies to date have looked at relatively small numbers of colorectal cancers with high-frequency MSI (Table 4). These studies have shown a potential role for MSI as an independent prognostic indicator; however, randomised prospective trials are required to determine whether the presence, absence or the degree of MSI will be of prognostic or predictive value for patients with CRC. The PCR techniques used in previous studies to detect MSI do not lend themselves readily to screening large numbers of patients and studies to determine whether immunohistochemical analysis of hMLH1 and hMSH2 can be used as a surrogate for the presence of the mismatch repair deficiency are currently being tested. While many of the studies to date have demonstrated a survival advantage for MSI tumours, it is often unclear from these studies which patients received chemotherapy and therefore whether tumours with high-frequency MSI respond better to chemotherapy.

### Chemotherapeutic targets

The most active combinations of chemotherapeutic drugs in colorectal cancer still only achieve response rates of 40–50% [31]. Thus, the ability to predict for chemotherapy resistance would avoid needlessly treating patients with a low likelihood of response and would permit alternate, possibly more targeted treatments to be used. The drugs most commonly used in colorectal cancer are 5-FU, irinotecan and oxaliplatin. Newer agents include epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, matrix metalloproteinase inhibitors, COX-2 inhibitors and farnesyl transferase inhibitors (FTI). These newer agents target specific molecules and have readily identifiable potential predictive markers.

### Thymidylate synthase

Thymidylate synthase (TS) is an essential enzyme required for DNA synthesis as it provides the only de novo source of thymidylate, catalysing the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate. Thymidylate synthase represents an important chemotherapeutic target for fluoropyrimidines such as 5-FU and folate-based inhibitors such as tomudex (TDX), the multitargeted antifolate (MTA) and ZD9331.
TS protein overexpression predicted for response to 5-FU/methotrexate

IHC (cytoplasmic)

Technique

100 patients received adjuvant 5-FU therapy: 86% of those relapsing had

Rectal cancer analysed with TS monoclonal antibody. High TS levels

significantly associated with worse disease-free survival (P < 0.01)

and significantly associated with Dukes’ stage (P < 0.01)

DIE

IHC (cytoplasmic)

Patients treated with surgery (D, B2, C)

High TS β-actin mRNA ratio determined from biopsies. High TS β-actin ratio

significantly associated with resistance to 5-FU chemotherapy (P = 0.004)

PCR, western

All patients received 5-FU chemotherapy. TS β-actin mRNA ratio and

TS protein staining demonstrated good correlation. TS β-actin ratio and

TS protein expression significantly higher (P < 0.01) in non-responders
to chemotherapy

Leichman et al.

(1995) [95]

26

D

PCR

High TS β-actin ratio significantly associated with resistance to 5-FU

chemistry (P = 0.003) and worse OS (P = 0.002). p53 mutation
(by IHC or cDNA sequencing) not associated with response to
chemotherapy or OS

Edler et al.

(2002) [35]

862

B, C

IHC

Patients treated with surgery (n = 442) or surgery plus 5-FU-based

chemotherapy (n = 420). TS expression is independent prognostic factor

for OS (P = 0.05) but TS expression had no prognostic value with regard
to survival in the 5-FU treated group (P = 0.4). No benefit seen in overall
survival in surgery alone arm versus surgery plus 5-FU chemotherapy
(P = 0.8)

Table 5. Thymidylate synthase expression and outcome

<table>
<thead>
<tr>
<th>Reference</th>
<th>n</th>
<th>Stage</th>
<th>Technique</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johnston et al. (1994) [94]</td>
<td>294</td>
<td>A–D</td>
<td>IHC (cytoplasmic)</td>
<td>Rectal cancer analysed with TS monoclonal antibody. High TS levels significantly associated with worse disease-free survival (P &lt; 0.01) independent of Dukes’ stage. TS protein level significantly associated with Dukes’ stage (P &lt; 0.01)</td>
</tr>
<tr>
<td>Johnston et al. (1995) [33]</td>
<td>21</td>
<td>Colon (D), gastric (operable)</td>
<td>PCR, western</td>
<td>All patients received 5-FU chemotherapy. TS β-actin mRNA ratio and TS protein staining demonstrated good correlation. TS β-actin ratio and TS protein expression significantly higher (P &lt; 0.01) in non-responders to chemotherapy</td>
</tr>
<tr>
<td>Leichman et al. (1995) [95]</td>
<td>26</td>
<td>D</td>
<td>PCR</td>
<td>TS β-actin mRNA ratio determined from biopsies. High TS β-actin ratio significantly associated with resistance to 5-FU chemotherapy (P = 0.004)</td>
</tr>
</tbody>
</table>
| Lenz et al. (1998) [34] | 36  | D     | PCR (TS), IHC (p53)            | High TS β-actin ratio significantly associated with resistance to 5-FU

chemistry (P = 0.003) and worse OS (P = 0.002). p53 mutation
(by IHC or cDNA sequencing) not associated with response to
chemotherapy or OS |
| Aschele et al. (1999) [96] | 48  | D     | IHC (cytoplasmic)              | TS protein overexpression predicted for response to 5-FU/methotrexate

chemotherapy in patients (67% versus 24%, P = 0.03) and median survival time (18.4 versus 15.4 months, P = 0.02) |
| Paradiso et al. (2000) [97] | 108 | D     | IHC (cytoplasmic)              | Response to 5-FU chemotherapy significantly correlated with TS negative status of tumours (30% versus 15%; P = 0.04) but not OS. No correlation between response to chemotherapy and p53 protein overexpression |
| Cascini et al. (2001) [98] | 150 | III   | IHC (cytoplasmic)              | 100 patients received adjuvant 5-FU therapy: 86% of those relapsing had TS overexpressing tumours at diagnosis and 69% of those patients disease free at follow-up had TS negative tumours at diagnosis (P < 0.001) |
| Allegre et al. (2002) [37] | 465 | B2, C | IHC                            | Patients treated with surgery (n = 151) or surgery plus 5-FU-based chemotherapy (n = 314). No statistically significant interaction between treatment and TS intensity |
| Edler et al. (2002) [35] | 862 | B, C  | IHC                            | Patients treated with surgery (n = 442) or surgery plus 5-FU-based chemotherapy (n = 420). TS expression is independent prognostic factor for OS (P = 0.05) but TS expression had no prognostic value with regard to survival in the 5-FU treated group (P = 0.4). No benefit seen in overall survival in surgery alone arm versus surgery plus 5-FU chemotherapy (P = 0.8) |

5-FU, 5-fluorouracil; OS, overall survival; TS, thymidylate synthase.

Pre-clinical studies have demonstrated that TS expression is a key determinant of 5-FU sensitivity [32] and multiple clinical investigations have demonstrated an improved response to 5-FU-based therapy in patients with low TS expression in their tumours [33, 34]. In addition, several studies have demonstrated that patients with high tumour TS expression have significantly worse clinical outcome than those with low TS, irrespective of response to treatment; therefore, TS may also be a valuable prognostic marker [35, 36]. The majority of previous studies have used either reverse transcription–polymerase chain reaction (RT–PCR) or immunohistochemistry (IHC) techniques to assess for TS overexpression and have consistently shown that TS overexpression predicts for resistance to 5-FU chemotherapy and worse overall and disease-free survival (Table 5). However, two recent studies found either no significant association between TS expression and response to 5-FU chemotherapy [37] or that low-TS expressing primary tumours actually had a worse outcome with adjuvant 5-FU therapy [35]. It is pertinent to note that the latter study failed to demonstrate any benefit with adjuvant 5-FU therapy, a finding inconsistent with the vast majority of the literature. A meta-analysis of all studies examining the relationship between TS expression and response to 5-FU therapy is currently underway and will address these issues of the validity of TS as a predictive marker. Two studies [38, 39] assessed TS status in patients who had not received 5-FU chemotherapy and still found that TS overexpression was significantly associated with a worse survival, independent of Dukes’ stage. The TS gene promoter is polymorphic with either two (TSER*2) or three (TSER*3) tandem repeats of 28 base pairs [40]. In vitro studies have demonstrated that three copies of the tandem repeat generated approximately 2.6-fold higher TS mRNA expression than two copies. Recently, preliminary studies have suggested that stage III CRC patients with the TSER*3/TSER*3 genotype do not receive the survival benefit from adjuvant 5-FU treatment observed in patients with the TSER*2/TSER*2 or TSER*2/TSER*3/TSER*3 genotypes [40]. To date, TS remains the most convincing molecular marker for prognosis in both early and advanced colorectal cancer. Recent studies have found that TS levels measured in primary tumours do not correspond with those measured in the corresponding metastasis [41, 42]. For
example, in matched samples of primary and metastatic tumours from the same patient, TS mRNA expression in lymph node and pulmonary metastases was significantly higher than in the corresponding primary tumour, whereas liver metastases expressed lower levels of TS than primary tumours [41]. These results may explain why TS expression in primary tumour samples may not predict the response of metastases to treatment [43].

**Dihydropyrimidine dehydrogenase**

Dihydropyrimidine dehydrogenase (DPD) is a key rate-limiting enzyme in 5-FU catabolism and is normally involved in the breakdown of endogenous pyrimidines. Over 80% of 5-FU is catabolised by DPD in the liver. Between 3% and 5% of the normal population have reduced DPD activity and manifest more severe gastrointestinal and haematological toxicity when exposed to 5-FU. There are now a number of in vitro and in vivo studies showing an inverse correlation between tumour DPD expression and sensitivity to 5-FU [44, 45]. Furthermore, the measurement of DPD levels along with TS levels improves the ability to predict responsiveness to 5-FU-based chemotherapy regimens. Moreover, the development of DPD inhibitors has excited interest in whether sensitivity to fluoropyrimidines may be restored by treatment with these inhibitors in conjunction with 5-FU [46].

**Thymidine phosphorylase**

Thymidine phosphorylase (TP) is an enzyme involved in pyrimidine nucleotide metabolism and is identical to platelet-derived endothelial cell growth factor. It has been implicated in angiogenesis and increased TP expression in colorectal cancer tissue has been observed relative to normal tissues [47]. More significantly, it is a key enzyme in the conversion of the oral fluoropyrimidine capecitabine to its active form 5-FU, and therefore it would be expected that elevated TP expression in a tumour would increase the efficacy of this therapy. Nishimura et al. [48] compared the ratio of TP to DPD expression in 88 patients with stage II or III CRC treated for two years with oral 5′-DFUR (an intermediate metabolite of capecitabine). They found that patients with high TP and low DPD had the best survival after 4 years of follow-up and conversely those with low TP and high DPD the worst survival. These results suggest that TP expression levels may indeed predict tumour response to capecitabine.

**ERCC1**

The *ERCC1* gene encodes a 297 amino acid protein which functions in complex with the protein XPF. The ERCC1–XPF complex functions as a nuclease with a central role in nucleotide excision repair. Proteins of the nucleotide excision repair pathway are thought to repair DNA damage caused by platinum chemotherapy agents. Oxaliplatin is a platinum-based chemotherapy agent containing a 1,2-diaminocyclohexane (DACH) ring which causes DNA damage through interstrand cross-links and DNA–protein cross-links [49]. Oxaliplatin, unlike cisplatin or carboplatin, is not dependent on a mismatch repair-proficient phenotype [50], but does appear to be dependent on ERCC1 levels for activity. Shirota et al. [51] analysed TS and *ERCC1* mRNA from 50 metastatic CRC tumour samples in patients receiving oxaliplatin and 5-FU combined chemotherapy. Both *ERCC1* and TS gene expression were found to be independent predictors of survival (*P* = 0.008 and 0.002, respectively), ERCC1 may therefore have a role to play as a predictive marker for response to oxaliplatin.

**Topoisomerase I**

DNA topoisomerase I is a nuclear enzyme involved in DNA replication. It causes a single strand break upstream of double strand separation which is essential for DNA polymerase function. Topoisomerase I inhibitors, such as irinotecan (CPT-11), cause double strand breaks of topoisomerase I-linked DNA that subsequently cause G2 arrest and death. DNA damage occurs during the S-phase of the cell cycle, but immediate induction of chromatin breaks independent of DNA synthesis occurs also [52]; therefore, topoisomerase I inhibitors have S-phase-dependent and -independent mechanisms of action. Both p53-dependent and -independent apoptosis has been demonstrated in cell lines treated with topoisomerase I inhibitors [53], which is important given the finding that up to 75% of colorectal cancers have mutated *p53* genes. CPT-11 is the most extensively studied topoisomerase I inhibitor. In clinical studies CPT-11 has demonstrated single-agent activity in advanced CRC with response rates of 20–25%. In combination with an infusional regimen of 5-FU, CPT-11 is the most active treatment available with response rates of 41% and improved median survival compared with 5-FU alone (17.4 versus 14.1 months, *P* = 0.03) [54]. Topoisomerase I expression in cell lines and human tumour specimens is being investigated as a potential predictive marker for response to CPT-11. Immunohistochemistry for topoisomerase I expression in 44 patients with advanced CRC demonstrated expression in 32% of cases, but this did not correlate with overall survival. It is still disputed whether levels of topoisomerase I protein or topoisomerase I–DNA complexes correlate with response to topoisomerase I inhibitors [55–57], and this remains an ongoing area of research.

**Vascular endothelial growth factor**

At present, several angiogenesis inhibitors are in clinical trials for patients with advanced metastatic cancer, and seven are in phase III trials. The eventual role of these agents is likely to be in combination with conventional cytotoxic agents and in early stage disease to prevent metastases from occurring. One of the major anti-angiogenic targets is vascular endothelial growth factor (VEGF). It is a homodimeric glycoprotein that exists in four isoforms due to alternative splicing of the
primary transcript. The isoforms are designated VEGF(121), VEGF(165), VEGF(189) and VEGF(205), according to the number of amino acids that each protein contains. The receptors for VEGF are expressed almost exclusively on endothelial cells and it has been shown to induce angiogenesis. Increased VEGF expression is associated with colon cancer metastases [58]. In 121 patients with successfully resected stage II colon cancers, the recurrence rate in VEGF-positive tumours was significantly higher than that in patients with VEGF-negative tumours (50% versus 11.7%, P = 0.001) [59]. VEGF mRNA expression by northern blotting in primary colorectal cancers has also been shown to be associated with the presence of lymph node metastasis and decreased survival [60].

**Epidermal growth factor receptor**

The epidermal growth factor receptor (EGFR) is a 170-kDa membrane-spanning glycoprotein with an intracellular tyrosine kinase domain. Six growth factors bind to EGFR: epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin, heparin-binding EGF-like growth factor, betacellulin and epiregulin. EGF mRNA has been detected in 55% of primary or metastatic human colorectal cancers compared with 22% of normal colon mucosa and is associated with a worse prognosis and a more aggressive clinical course [61]. Activation of the receptor causes autophosphorylation and activation of downstream signalling pathways including the Ras/Raf/MEK/ERK pathway. Mutations of the EGFR gene are also commonly found in many human tumours with deletions in the extracellular domain being the most frequent. These deletions result in constitutive phosphorylation of the receptor and thus continuous activation of downstream signalling cascades. Increased activation of these pathways is therefore the result of either overexpression of the receptor or activating mutations. In both cases the tyrosine kinase domain is overactive and as such represents an important therapeutic target. Several tyrosine kinase inhibitors are in development. The tyrosine kinase inhibitor ZD-1839 (Iressa) has demonstrated activity in colorectal, ovarian, prostate and non-small-cell lung cancer [62, 63]. Overexpression of EGFR by immunohistochemistry may help identify those patients who would potentially benefit from this therapy.

**Matrix metalloproteinase inhibitors**

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases regulating the turnover of the extracellular matrix and have been implicated in tumour invasion and metastasis. The matrix metalloproteinase matrilysin (MMP-7) was found to be overexpressed in 80–90% of colorectal cancers [64] and excited interest as a possible therapeutic target. Its overexpression is the result of mutational inactivation of the APC gene, which is seen in the majority of colorectal cancers and results in the accumulation of the protein β-catenin. Several matrix metalloproteinase inhibitors have been developed and entered in clinical trials in advanced cancer; however, the results to date have been disappointing [65, 66]. In retrospect it may have been better to perform these trials in early stage disease and in tumours manifesting increased expression of MMPs. If MMPs are important in the development of metastases, then they may be less relevant as targets once metastases have already occurred. More recent studies have also focused on their use in combination with conventional cytotoxic agents in cancers where MMPs have been shown to be important [67].

**COX-2**

COX-2 is overexpressed in 40% of colorectal adenomas and 85–90% of carcinomas [68, 69]. This may again be due to mutational inactivation of the APC gene resulting in β-catenin accumulation and transcriptional activation of COX-2 expression. Indeed, analysis of 38 colorectal cancers demonstrated an association between APC mutations and increased COX-2 expression [70]. There is now a significant body of evidence from epidemiological studies that use of non-steroidal anti-inflammatory drugs (NSAIDs) reduces the risk of colorectal cancer and adenoma by approximately 40–50% [71]. NSAIDs inhibit the enzymes COX-1 and COX-2. Use of the selective COX-2 inhibitor celecoxib in animal models reduced the incidence of colon tumours by 93% [72]. Furthermore, in a colon cancer cell line exhibiting high COX-2 expression the selective COX-2 inhibitor etodolac was cytotoxic and suppressed the invasive property [69]. This suggests that COX-2 inhibitors may have a role to play not only in chemoprevention of colon cancers but also in treatment of tumours.

**Conclusion**

Advances in our understanding of colorectal cancer biology have progressed rapidly over the last decade. This has been paralleled by a major increase in the number of drugs available to treat this disease in both the adjuvant and metastatic disease settings. These treatments have resulted in significant improvement in the disease-free survival and survival of patients with colorectal cancer. The prognosis for patients with colorectal cancer is based on clinical and pathological staging, but the majority of these will develop recurrent disease and die from metastatic disease. The explosion in our knowledge of colorectal cancer biology has led to the identification of molecular markers that may prove not only to have prognostic or predictive value, but may also be important screening tools and therapeutic targets. The next generation of adjuvant and metastatic disease studies will need to address the importance of these biological properties in defining risk of recurrence for individual patients and the likelihood of response to a given chemotherapeutic treatment or combination of treatments.
References

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