Prognostic molecular markers for planning adjuvant chemotherapy trials in Dukes’ B colorectal cancer patients: how much evidence is enough?

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The benefit of postoperative adjuvant chemotherapy in patients with Dukes’ B colorectal cancer is still uncertain and its routine use is not recommended. Prognostic biomarkers may be useful for identifying high-risk patients with resected, node-negative disease, and this stratification may represent an innovative strategy for designing adjuvant chemotherapy trials. Featured prognostic molecular markers can be divided into the following categories: cell proliferation indexes (Ki-67, Mib-1, proliferating cell nuclear antigen); oncogenes/tumor suppressor genes [p53, K-ras, Deleted in Colorectal Cancer (DCC), Bcl-2, c-erbB2]; DNA repair (microsatellite instability); markers of angiogenesis (vascular count, vascular endothelial growth factor); markers of invasion/metastasis (plasminogen-related molecules, matrix metalloproteinases); and biochemical markers (thymidylate synthase). Studies that have investigated their prognostic role in Dukes’ B colorectal cancer patients are reviewed here. Current data do not provide sufficient evidence for the incorporation of available prognostic biomarkers into clinical practice. However, a biomarker-based approach could be an effective strategy for improving results of postoperative adjuvant treatments in high-risk Dukes’ B colorectal cancer patients. Markers of altered DCC function have shown promising prognostic role and sufficient prevalence in retrospective investigations and they deserve further assessment in prospective studies.

Key words: colorectal cancer, Dukes’ B, prognosis, tumor markers

Introduction

Colorectal cancer remains a significant health care problem worldwide, and the tumor–node–metastasis (TNM) system represents the main tool for identifying prognostic differences among patients with early-stage disease [1]. After surgery, fluorouracil-based adjuvant chemotherapy is indicated in patients with node-positive, stage Dukes’ C disease. Although up to 40% of patients with Dukes’ B colorectal cancer will develop recurrent disease during their lifetime, the role of adjuvant chemotherapy in this setting is still unclear [1].

The identification of categories of patients with high-risk colorectal cancer would be of great help for improving treatment strategies in the node-negative disease, and probably, patients’ outcome [2]. A growing burden of data suggests that several prognostic molecular markers might be useful in defining individual patients’ risk after radical surgery and determining which patients might benefit most from adjuvant chemotherapy [3, 4]. However, since it is unlikely that all featured prognostic biomarkers will be investigated in large, prospective trials, time and effort should be given to address, in hypothesis-generating retrospective studies, those that show sufficient prevalence in colorectal carcinomas and have promising prognostic roles.

In this perspective, our aim was to evaluate current results of molecular prognostic molecular markers in Dukes’ B colorectal cancer and data in support of their validation in future prospective studies.

Materials and methods

Studies on prognostic molecular markers in early-stage colorectal cancer were searched for in peer-review journals; the search was restricted to English-language publications. Investigations published in abstract form only were excluded. Where results were reported or updated in more than one publication, only the most recent publication was used. The content terms colon, rectal or colorectal with prognosis, prognostic and survival were used for every featured biomarker in colorectal cancer. The CancerLit and Medline databases were used.

In the next step, each study was evaluated and those including patients with Dukes’ B disease were selected. These studies were assessed for methodological quality according to a six-point checklist [5] (Table 1), and only studies that fulfilled these quality features were selected for the review. The assessment of evidence was limited to and performed in studies that investigated the prognostic role of the biomarker in the subgroup of patients with Dukes’ B disease (Tables 2–8). In these studies, adjustment with multivariate analysis for all important clinico-pathological features and post-operative treatments like adjuvant chemotherapy had to be provided.
The following categories of prognostic biomarkers have been considered: cell proliferation indices [Ki-67, Mib-1, proliferating cell nuclear antigen (PCNA)]; oncogenes/tumor suppressor genes [p53, K-ras, Deleted in Colorectal Cancer (DCC), Bcl-2, c-erbB2]; DNA repair (microsatellite instability); markers of angiogenesis [vascular count, vascular endothelial growth factor (VEGF)]; markers of invasion/metastasis [plasminogen-related molecules, matrix metalloproteinases (MMPs)]; and biochemical markers [thymidylate synthase (TS)].

### Proliferation indices

Antibodies that recognize nuclear proteins associated with tumor cell proliferation can be determined by immunohistochemistry and have represented an attractive alternative to the analysis of cell proliferation as determined by flow cytometry. Currently, PCNA and Ki-67, and its epitope Mib-1, are the most popular methods that have been investigated as prognostic factors in colorectal cancer. PCNA is a DNA polymerase accessory protein that complexes with cyclin D and cyclin-dependent kinases. The specific antibody recognizes PCNA protein, which is maximally elevated in late G1 and S phase of proliferating cells. The Ki-67 antigen and its epitope Mib-1 are expressed in all phases of the cell cycle except G0.

Among prognostic studies of PCNA and Ki-67 in early-stage colorectal cancer [3, 4, 6–13], eight studies investigated survival of Dukes’ B patients, and their data are summarized in Table 2 [6–13]. In the largest series of 293 carcinomas, which included 101 Dukes’ B patients, PCNA results did not correlate with survival [6]. In addition, Ki-67 analysis in two large series of 255

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### Table 1. Methodological features for evaluating quality of prognostic studies

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Feature required</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Sample of patients</td>
<td>Inclusion criteria defined, sample selection explained, adequate description of diagnostic criteria, clinical and demographic characteristics fully described, representative, assembled at common (usually early) point in course of disease, complete (all eligible patients included)</td>
</tr>
<tr>
<td>(2) Follow-up of patients</td>
<td>Sufficiently long</td>
</tr>
<tr>
<td>(3) Outcome</td>
<td>Objective, unbiased (for example, assessment blinded to prognostic information), fully defined, appropriate, known for all or high proportion of patients</td>
</tr>
<tr>
<td>(4) Prognostic variable</td>
<td>Fully defined, including details of measurement methods if relevant, precisely measured, available for all or high proportion of patients</td>
</tr>
<tr>
<td>(5) Analysis</td>
<td>Continuous predictor variable analyzed appropriately, statistical adjustment for all important prognostic factors</td>
</tr>
<tr>
<td>(6) Treatment subsequent to inclusion in cohort</td>
<td>Fully described, standardized or randomized and included in the multivariate analysis</td>
</tr>
</tbody>
</table>

*Adapted from Altman DG [5].

### Table 2. Characteristics of studies on the prognostic role of proliferation indexes

<table>
<thead>
<tr>
<th>Ref.</th>
<th>No. pts</th>
<th>Stage</th>
<th>Method</th>
<th>Marker</th>
<th>% positive</th>
<th>Cut-off</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sun [6]</td>
<td>293</td>
<td>A–C</td>
<td>IHC</td>
<td>PCNA</td>
<td>41</td>
<td>&gt;25</td>
<td>No prognostic role of high PCNA</td>
</tr>
<tr>
<td>Allegra [8]</td>
<td>465</td>
<td>B–C</td>
<td>IHC</td>
<td>Ki-67</td>
<td>23</td>
<td>0–40</td>
<td>No prognostic role of Ki-67 determinations</td>
</tr>
<tr>
<td>Palmqvist [9]</td>
<td>56</td>
<td>B</td>
<td>IHC</td>
<td>Ki-67</td>
<td>62</td>
<td>&gt;25</td>
<td>High Ki-67 determined at the invasive margin correlated with worse survival: HR = 12.1; 95% CI 1.1–1.33; P = 0.04</td>
</tr>
<tr>
<td>Guerra [10]</td>
<td>108</td>
<td>A–C</td>
<td>IHC</td>
<td>PCNA</td>
<td>35</td>
<td>Score &gt;25</td>
<td>No prognostic role of high PCNA</td>
</tr>
<tr>
<td>Chen [11]</td>
<td>70</td>
<td>B–C</td>
<td>IHC</td>
<td>Ki-67</td>
<td>40</td>
<td>&gt;45</td>
<td>No prognostic role of high Ki-67 but higher risk of recurrence in patients with Dukes’ B disease</td>
</tr>
<tr>
<td>Bhavadekar [12]</td>
<td>98</td>
<td>B–C</td>
<td>IHC</td>
<td>Ki-67</td>
<td>25</td>
<td>Score &gt;1</td>
<td>No prognostic role of high Ki-67</td>
</tr>
</tbody>
</table>

*Unless otherwise indicated, cut-off indicate the percentage of positively malignant nuclei (mean value or median value).

*Mean value of the ratio between the positive area and the staining intensity.

*Score 1+ is defined as faint expression in <10% of tumor cells.

PCNA, proliferating cell nuclear antigen; IHC, immunohistochemistry; HR, hazard ratio; CI, confidence interval.
In particular, Allegra et al. [8] failed to demonstrate any significant prognostic role of Ki-67 in 465 colorectal cancer patients, with 220 patients being Dukes’ B2 disease. The only positive finding was reported by Palmqvist et al. [9], who found an independent prognostic role of Ki-67 in 56 stage Dukes’ B patients when determined at the invasive margin.

Available data do not support routine evaluation of cell proliferation indices in early stage colorectal cancer. In addition, the lack of a consistent role of Ki-67 as a prognostic marker suggests that other markers or combinations of markers may be more useful in early-stage disease.
of positive prognostic indications in Dukes’ B disease does not support their validation in prospective trials.

In recent years, new molecules involved in the cell cycle regulation, such as cyclines or the putative tumor suppressor gene $p_{27}$, seem to be more promising prognosticators than PCNA and Ki-67 [14, 15]. However, these promising data are still limited and have not been confirmed in large series of patients with node-negative disease.

### Angiogenesis

Angiogenesis plays a key role in tumor growth and metastasis. This phenomenon may have prognostic relevance and it can be assessed by the vascular density in the tumor and/or by the analysis of angiogenesis promoting molecules. VEGF is a glycoprotein similar to platelet-derived growth factor, and it is considered to be the main angiogenic stimulator [16]. Microvessel counts in human tumors are performed by marking endothelial cells with specific antibodies (CD34, CD31, anti-VIII factor). VEGF expression can be determined by the analysis of mRNA levels or by immunohistochemistry with anti-VEGF antibodies, in fresh or paraffin-embedded tumor tissues [3].

The prognostic role of angiogenesis features in early stage colorectal cancer has been investigated in retrospective series of patients [12, 17–32]. Seven studies [12, 17–21, 23] showed the

<p>| Table 5. Characteristics of studies on the prognostic role of TS analysis |
|---------------------------|----------------|----------------|-----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Ref.</th>
<th>No. pts</th>
<th>Stage</th>
<th>Method</th>
<th>% positive cases</th>
<th>Cut-off</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Johnston [54]</td>
<td>294</td>
<td>B–C</td>
<td>IHC</td>
<td>69</td>
<td>High TS for grade 2–3 staining intensity</td>
<td>High TS predicted worse survival ($P &lt;0.01$) in the whole group and in patients with Dukes’ B disease $P &lt;0.01$ (HR and CI not reported)</td>
</tr>
<tr>
<td>Lenz [57]</td>
<td>45</td>
<td>B</td>
<td>IHC</td>
<td>36</td>
<td>High TS for grade 2–3 staining intensity</td>
<td>High TS expression was associated with poor survival ($P = 0.004$) and its prognostic role was retained after adjustment for clinicopathological features (HR and CI not reported)</td>
</tr>
<tr>
<td>Edler [60]</td>
<td>862</td>
<td>B–C</td>
<td>IHC</td>
<td>72</td>
<td>High TS for grade 2–3 staining intensity</td>
<td>In surgery alone patients, high TS correlated with poor survival: $P = 0.001$; HR = 2.0; 95% CI 1.5–2.7; and this prognostic role was confirmed in Dukes’ B patients ($P = 0.04$). No prognostic role of TS in patients treated with adjuvant chemotherapy</td>
</tr>
<tr>
<td>Allegra [8]</td>
<td>465</td>
<td>B–C</td>
<td>IHC</td>
<td>47–74$^a$</td>
<td>Staining intensity and staining pattern$^b$</td>
<td>No prognostic role of TS expression</td>
</tr>
</tbody>
</table>

$^a$Patients with rectal cancer.

$^b$See text for more details on methods for TS assessment and results.

IHC, immunohistochemistry; HR, hazard ratio; CI, confidence interval; TS, thymidylate synthase.

<p>| Table 6. Characteristics of studies on the prognostic role of p53 analysis |
|---------------------------|----------------|-----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Ref.</th>
<th>No. pts</th>
<th>Stage</th>
<th>Method</th>
<th>% positive cases</th>
<th>Cut-off</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buglioni [69]</td>
<td>94</td>
<td>B</td>
<td>IHC</td>
<td>2</td>
<td>Any positivity</td>
<td>p53 overexpression correlated with poor survival: $P = 0.02$; HR = 5.4; 95% CI 1.26–23.8</td>
</tr>
<tr>
<td>Allegra [8]</td>
<td>465</td>
<td>B–C</td>
<td>IHC</td>
<td>60</td>
<td>&gt;10%</td>
<td>p53 overexpression did not show independent prognostic role</td>
</tr>
<tr>
<td>Gervaz [68]</td>
<td>126</td>
<td>B</td>
<td>PCR-SSCP IHC</td>
<td>31</td>
<td>p53 mutations &gt;10%</td>
<td>p53 overexpression correlated with poor survival: $P = 0.01$; HR = 5.4; 95% CI 1.12–4.11. p53 molecular analysis did not show independent prognostic role</td>
</tr>
<tr>
<td>Giatromanolaki [17]</td>
<td>106</td>
<td>B–C</td>
<td>IHC</td>
<td>44</td>
<td>&gt;10%</td>
<td>p53 overexpression did not show independent prognostic role</td>
</tr>
<tr>
<td>Soong [70]</td>
<td>995</td>
<td>B–C</td>
<td>PCR-SSCP</td>
<td>39</td>
<td>p53 mutations</td>
<td>p53 molecular analysis did not show independent prognostic role</td>
</tr>
<tr>
<td>Bhatavdekar [12]</td>
<td>98</td>
<td>B–C</td>
<td>IHC</td>
<td>25</td>
<td>&gt;10%</td>
<td>p53 overexpression did not show independent prognostic role</td>
</tr>
<tr>
<td>Bouzourene [71]</td>
<td>122</td>
<td>B</td>
<td>PCR-SSCP IHC</td>
<td>47</td>
<td>&gt;10% p53 mutations</td>
<td>p53 overexpression correlated with poor survival: $P = 0.02$; HR = 2.16; 95% CI 1.12–4.11. p53 molecular analysis did not show independent prognostic role</td>
</tr>
</tbody>
</table>

PCR-SSCP, polymerase chain reaction-single strand conformation polymorphism; IHC, immunohistochemistry; HR, hazard ratio; CI, confidence interval.
Table 7. Characteristics of studies on the prognostic role of DCC analysis

<table>
<thead>
<tr>
<th>Ref.</th>
<th>No. pts</th>
<th>Stage</th>
<th>Method</th>
<th>% positive cases</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Font [79]</td>
<td>77</td>
<td>B</td>
<td>Molecular analysis with 3 microsatellite markers</td>
<td>39% with LOH</td>
<td>LOH in stage Dukes’ B disease was associated with worse prognosis: P = 0.006 (HR and CI not reported)</td>
</tr>
<tr>
<td>Shibata [86]</td>
<td>132</td>
<td>B-C</td>
<td>IHC</td>
<td>50% with loss of DCC expression(^1)</td>
<td>Loss of DCC expression was prognostic: HR = 3.15; 95% CI 1.7–5.8; P &lt;0.001. Worse survival in Dukes’ B patients with DCC-negative tumors (P &lt;0.001)</td>
</tr>
<tr>
<td>Jernvall [87]</td>
<td>195</td>
<td>A-D</td>
<td>Molecular analysis with 7 microsatellite markers</td>
<td>52% with LOH</td>
<td>LOH showed prognostic role: HR = 3.9 95% CI 0.9–9.1; P = 0.04. Worse survival in Dukes’ B patients with LOH (P values not reported)</td>
</tr>
<tr>
<td>Jen [88]</td>
<td>145</td>
<td>B-C</td>
<td>Molecular analysis with 10 microsatellite markers</td>
<td>67% with LOH</td>
<td>LOH showed prognostic value: HR = 2.46; 95% CI 1.06–5.71; P = 0.036. Worse survival in Dukes’ B patients with LOH: P = 0.006</td>
</tr>
<tr>
<td>Ogunbiyi [89]</td>
<td>126</td>
<td>A-C</td>
<td>Molecular analysis with 5 microsatellite markers</td>
<td>53% with LOH</td>
<td>LOH showed prognostic value: HR = 2.0; 95% CI 1.26–3.86; P = 0.003. Worse disease-specific survival in Dukes’ B patients with LOH: P = 0.01</td>
</tr>
<tr>
<td>Lanza [90]</td>
<td>118</td>
<td>B-C</td>
<td>Molecular analysis with 3 microsatellite markers</td>
<td>53% with LOH</td>
<td>LOH showed prognostic value: HR = 7.1; 95% CI 2.1–23.9; P &lt;0.001. Worse survival in Dukes’ B patients with LOH: P = 0.004</td>
</tr>
<tr>
<td>Carethers [91]</td>
<td>70</td>
<td>B</td>
<td>Molecular analysis with 5 microsatellite markers</td>
<td>43% with LOH</td>
<td>LOH did not show a prognostic role</td>
</tr>
<tr>
<td>Martinez-Lopez [92]</td>
<td>144</td>
<td>A-C</td>
<td>Molecular analysis with 3 microsatellite markers</td>
<td>45% with LOH</td>
<td>LOH showed prognostic value: HR = 4.39; 95% CI 1.2–16.0; P = 0.01. Worse survival in Dukes’ B patients with LOH: P = 0.008</td>
</tr>
<tr>
<td>Sun [93]</td>
<td>195</td>
<td>A-D</td>
<td>IHC</td>
<td>28% with loss of DCC expression(^1)</td>
<td>Loss of DCC expression was prognostic in diploid tumors: HR = 2.3; 95% CI 1.3–8.1; P = 0.008. The DCC status did not predict survival in individual Dukes’ stages</td>
</tr>
<tr>
<td>Watanabe [98]</td>
<td>121</td>
<td>B(^a)</td>
<td>Molecular analysis with 6 microsatellite markers</td>
<td>49% with LOH</td>
<td>LOH did not show prognostic role</td>
</tr>
</tbody>
</table>

\(^1\)A cut-off value was unnecessary since DCC staining resulted an ‘all or nothing’ phenomenon.
\(^a\)Watanabe et al. [98] performed a separate analysis in stage Dukes’ B and stage Dukes’ C patients. Both groups had received adjuvant chemotherapy.

LOH, loss of heterozygosity of chromosome 18q; IHC, immunohistochemistry; HR, hazard ratio; CI, confidence interval.

prognostic analysis in stage Dukes’ B patients, and their data are summarized in Table 3. In this group, three investigations found an independent prognostic role of vascular count [12, 18, 19], and one investigation with VEGF found worse disease-free survival in Dukes’ B patients whose tumors showed expression in >10% of tumor cells [23].

Surprisingly, Lindmark et al. [22] found that patients whose tumors had a mean of >10 microvessels per five fields had significantly longer survival than patients with less vascularized tumors. The authors did not report the prognostic analysis of the angiogenic feature in the node-negative disease, and this study has not been included in Table 3; however, it is still the largest prognostic analysis of microvessel counts in colorectal cancer and the results from it conflict with those reported in other series.

Markers of tumor angiogenesis may discriminate prognostic differences in early stage colorectal cancer. However, lack of standardization of methods and differences in the choice of cut-off levels are relevant problems for the interpretation of results. More retrospective data are necessary before planning prospective investigations with tumor microvessell counts or VEGF expression for identifying high-risk Dukes’ B patients.

Metastasis and invasion

Plasminogen-related molecules and MMPs are considered crucial enzymes for tumor invasion and metastasis. They have been overexpressed in colorectal carcinomas and their prognostic role has been investigated in retrospective series of patients with early stage disease.

Enzyme-linked immunosorbent essay (ELISA) and immunohistochemistry techniques have been employed to determine plasminogen-related molecules in tumor tissues. In the present review 10 studies are quoted on the prognostic role of urokinase-type plasminogen activator (uPA) and its receptor (uPAR), tissue-type plasminogen activator (tPA), plasminogen and the plasminogen inhibitors 1 and 2 (PAI-1, PAI-2) [33–42]. In these studies, one or more of these molecules showed independent prognostic role in early stage disease, but only three investigations were addressed to Dukes’ B and Dukes’ C patients [40–42] (Table 4). These studies found that uPAR [40], tPA [41] and uPA [42] were independent prognostic indicators in patients with stage Dukes’ B colorectal cancer. The largest investigation was reported by Stephens et al. [40], who determined preoperative plasma levels

...
of soluble uPAR in 591 patients. In this study, 219 patients were classified as having Dukes’ B disease and those who had high plasma suPAR levels showed shorter survival than patients who had levels below the median value ($P < 0.0001$).

At least 15 MMPs are known, and types 1, 2 and 9 have been investigated as prognostic markers in colorectal cancer [3]. In nine retrospective studies that included patients with stage Dukes’ B tumors [43–51], there was no specific analysis of the prognostic role of MMPs in the subgroup of patients with node-negative disease. The results of MMPs as prognostic markers are conflicting, and differences in MMPs and techniques used for analysis (immunohistochemistry, mRNA, serum levels) cause relevant problems for the interpretation of results. It is unclear which of the several MMPs may be the best prognostic marker, and it is not clear whether the levels of naturally occurring tissue inhibitors of MMPs may contribute to the prognosis [52].

Plasminogen-related molecules are promising prognostic parameters in early-stage colorectal cancer, and the ease with which they can be analyzed supports further investigation. However, it is necessary to clarify which of the plasminogen-related molecules has the best prognostic power, and to define cut-off levels before planning their validation in prospective studies.

**Thymidylate synthase**

TS is a rate-limiting enzyme in the DNA synthesis pathway and is the main intracellular target of 5-fluorouracil and raltitrexed, two of the most important drugs used in the treatment of colorectal cancer. TS quantification can be performed by immunohistochemistry or RNA levels, and high TS levels have been correlated with poor response rates to fluorouracil-based chemotherapy in advanced gastrointestinal carcinomas [53]. In colorectal cancer, fluorouracil-based chemotherapy is widely used as post-surgical adjuvant treatment, and the prognostic role of TS quantitation has been investigated in patients with early stage disease [8, 54–62].

Since the first report in 1994 by Johnston et al. [54], who found a significant association between TS levels and survival in 294 patients with rectal cancer, further studies have addressed the prognostic role of TS in early stage colorectal cancer. Ten studies that included stage Dukes’ B patients have been identified [8, 54–62], and in the majority of these TS quantitation was found to be an independent prognosticator of postoperative outcome; in particular, patients with TS-positive tumors showed poorer survival than patients with TS-negative tumors [54–62]. Only four studies were performed with prognostic analysis in the subgroup of Dukes’ B patients (Tables 5), and only two of these had a sufficiently large sample to explore the efficacy of fluorouracil-based adjuvant chemotherapy according to TS expression [8, 60]. In 465 patients with Dukes’ B2 (220 patients) and Dukes’ C (245 patients) disease, Allegra et al. [8] failed to demonstrate a consistent and significant association between TS quantification and either disease-free survival or overall survival. In this study, the prognostic role of TS was evaluated according to different TS scores that were based on staining intensity and staining patterns.

### Table 8. Characteristics of studies on the prognostic role of microsatellite instability

<table>
<thead>
<tr>
<th>Ref.</th>
<th>No. pts</th>
<th>Stage</th>
<th>Method</th>
<th>% of MSI-H</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guidoboni [120]</td>
<td>118</td>
<td>B–C</td>
<td>Molecular analysis with 5–10 microsatellite markers</td>
<td>40</td>
<td>MSI-H was independently associated with better survival: $HR = 0.31; 95% CI 0.14–0.68; P = 0.02$. The difference in improved outcome was not significant in Dukes’ B patients only</td>
</tr>
<tr>
<td>Samowitz [121]</td>
<td>1026</td>
<td>A–D</td>
<td>Molecular analysis with BAT-26 and TGFβRII</td>
<td>12</td>
<td>MSI-H was independently associated with better survival: $HR = 0.43; 95% CI 0.28–0.69; P &lt;0.01$. The difference in improved outcome was not significant in Dukes’ B patients only</td>
</tr>
<tr>
<td>Halling [124]</td>
<td>508</td>
<td>B–C</td>
<td>Molecular analysis with 10 microsatellite markers</td>
<td>15</td>
<td>MSI-H was independently associated with improved survival: $HR = 0.51; 95% CI 0.31–0.82; P = 0.006$. The difference in improved outcome was not significant in Dukes’ B patients only</td>
</tr>
<tr>
<td>Curran [107]</td>
<td>159</td>
<td>B</td>
<td>Molecular analysis with 4 microsatellite markers</td>
<td>14</td>
<td>MSI-H was not associated with significant differences in long-term survival</td>
</tr>
<tr>
<td>Gryfe [125]</td>
<td>607a</td>
<td>A–D</td>
<td>Molecular analysis with 5–10 microsatellite markers</td>
<td>17</td>
<td>MSI-H was independently associated with improved survival: $HR = 0.45; 95% CI 0.30–0.68; P&lt;0.001$. The prognostic role of MSI-H was found in all disease stages</td>
</tr>
<tr>
<td>Watanabe [98]</td>
<td>121</td>
<td>B</td>
<td>Molecular analysis with 15 microsatellite markers</td>
<td>21</td>
<td>MSI-H was not associated with significant differences in long-term survival</td>
</tr>
<tr>
<td>Gervaz [108]</td>
<td>88</td>
<td>B</td>
<td>Molecular analysis with 7 microsatellite markers</td>
<td>24</td>
<td>MSI-H was not associated with significant differences in long-term survival</td>
</tr>
</tbody>
</table>

*Analysis of microsatellite instability in patients aged ≤50 years.

aWatanabe et al. [98] performed a separate analysis in stage Dukes’ B and stage Dukes’ C patients. In this study, both groups had received adjuvant chemotherapy.

MSI-H, high-frequency microsatellite instability; TGFβRII, transforming growth factor β-type receptor II; HR, hazard ratio; CI, confidence interval.
Edler et al. [60] found that TS expression was an independent prognostic factor in the whole group of 862 patients and in the 442 Dukes’ B patients who underwent surgery alone. On the contrary, in 420 patients treated with fluorouracil-based adjuvant chemotherapy, TS quantitation lost its prognostic value and patients with low TS levels showed even worse outcome than patients treated with surgery alone. In this study, TS expression was dichotomized in high and low categories according to staining intensity.

On the whole, data from the published literature suggest that TS may be an innovative and promising prognostic biomarker in early stage colorectal cancer. However, the recent and almost unexpected findings reported by Allegra et al. [8] and Edler et al. [60] have no apparent explanation, and deserve further evaluation before planning prospective TS-based prognostic trials.

**p53**

p53 is a tumor suppressor gene located on chromosome 17p13.1, encoding a protein that is involved in cell cycle regulation, DNA replication and apoptosis in response to DNA damage [63]. Accordingly, p53 status has been studied as prognostic factor, and more recently as predictor of response to cancer chemotherapy. Two major techniques are used for p53 analysis: DNA analysis to detect a variety of mutations in the p53 gene [63] and immunohistochemistry to detect abnormal nuclear accumulation of the p53 protein [64]. Immunohistochemically detected p53 overexpression has been generally used as a surrogate marker of p53 mutations, but this assumption may not always be correct. Many genetic changes do not result in p53 overexpression, and positive immunohistochemistry analysis of p53 may occur in the absence of p53 mutations. In colorectal carcinomas, the correlation between p53 gene status and p53 staining has been estimated at ~70% of cases or more [63, 64].

It is worth noting that controversies also exist on the prognostic significance of genetic p53 abnormalities. In a recently published report, a specific p53 mutational site was found to be associated with patients’ prognosis; in particular, patients with p53 mutations affecting a region of the core domain (the L3 zinc-binding domain) had a significantly shorter cancer-related survival [65]. Conversely, a number of p53 mutations in colorectal cancer do not seem to promote disease progression. Some mutations within conserved regions may even counteract negative functional effects of other p53 structural alterations [66].

Despite the above-mentioned difficulties in p53 analysis, this genetic marker has provoked great enthusiasm among researchers, and over 3000 patients have been included in prognostic studies in colorectal cancer between 1990 and 2000 [3, 67].

In a pooled analysis of published studies on the p53 prognostic value in colorectal cancer, neither p53 overexpression nor p53 mutations emerged as a powerful prognostic indicator in patients with colorectal cancer [67]. This analysis was performed after including survival data from 4416 patients from 28 published studies up to June 1999. In recent years, further retrospective studies have attempted to evaluate the prognostic role of p53 abnormalities in early stage colorectal cancer, but again, the results do not suggest that this marker can provide significant prognostic information. Twelve studies that included stage Dukes’ B patients [8, 12, 17, 18, 66, 68–74], and the results of seven studies with prognostic analysis in the node-negative disease [8, 12, 17, 68–71], are summarized in Table 6.

Available data are conflicting, and p53 abnormalities do not always show an independent prognostic role [8, 12, 17, 18, 70, 72]. In the largest investigation of p53 gene mutation analysis in 995 stage Dukes’ B and C patients, Soong et al. [70] failed to demonstrate any prognostic role of p53 mutations. To date, the largest study of p53 overexpression in early stage colorectal cancer has been reported by Allegra et al. [8], who investigated tumors in 465 patients (220 with Dukes’ B2 disease) and determined no significant prognostic role.

More investigations are needed to evaluate the prognostic role of p53 abnormalities in colorectal carcinomas. Immunohistochemistry may represent an easy and accessible technique for determining p53 overexpression, but available data are inconclusive and the definition of standardized methods of analysis and cut-off levels are mandatory. To date, p53 analyses do not seem to be the best candidate for further prognostic evaluation in prospective studies.

**K-ras**

K-ras belongs to the RAS family (K-ras, H-ras, N-ras) of cellular proto-oncogenes and encodes a 21 kDa protein (p21) located in the inner surface of the plasma membrane. This protein controls cell growth and differentiation by transduction of extracellular mitogen signals [75]. The functions of K-ras support its putative prognostic role in colorectal cancer, and several studies have been performed in recent years in this setting. K-ras abnormalities have been studied by molecular genetic assay for determination of point mutations at codons 12, 13, 31 and 61. The point mutations that trigger the oncogenic potential of ras have been detected in more than 80% of cases on codons 12 and 13. In addition, immunohistochemistry has been used to evaluate the ras/p21 protein expression on fixed fresh or paraffin-embedded tissue.

The large collaborative RASCAL studies [76, 77] have collected and reanalyzed survival data of patients worldwide whose tumors have been investigated for ras mutations. The first RASCAL analysis in 2721 patients was published in 1998 [76] and the second, in 4268 patients, was published in 2001 [77]. In the first assessment [76], mutations of K-ras codon 12 or 13 were detected in 37.7% of tumors, and overall survival was reduced by any mutation [hazard ration (HR) = 1.22; 95% confidence interval (CI) 1.07–1.40; \( P = 0.004 \)]. When the authors repeated the multivariate analysis taking into account individual mutations, only glycine to valine on codon 12 (10% of the study population) was found to be an independent factor for increased risk of death \( (P = 0.004) \). The second RASCAL analysis [77] confirmed the significant association of the valine mutation on codon 12 with aggressive biological behavior in colorectal cancer \( (HR = 1.29; 95\% \, CI \, 1.08–1.55; \ P = 0.008) \). The large study sample allowed subgroup analysis in patients with stage Dukes’ B and Dukes’ C disease and this genetic change (8.6% of all patients) lost its prognostic role in the node-negative disease.
After the publication of the latest RASCAL analysis, three additional studies investigated the prognostic role of K-ras mutations in early stage colorectal cancer [78–80] and two found an independent prognostic role of K-ras mutations [78, 79]. Font et al. [79] found poor survival of Dukes’ B patients whose tumors had specific aspartic and serine mutations on codon 12 ($P = 0.03$). Bazan et al. [78] showed that codon 13 K-ras mutations, but not any mutation, were independently related to risk of relapse or death.

Data on the prognostic role of ras/p21 oncoprotein expression in Dukes’ B patients are still limited and unconfirmed [81–83].

According to current data, K-ras may act as prognostic factor in colorectal cancer, but this effect seems related to a limited number of defined mutations, probably in the node-positive disease. K-ras mutations occur in ~30% of colorectal carcinomas, but the prevalence of the potentially more aggressive genotypes seems lower [76, 77]. For these reasons, K-ras mutational status does not seem to be the best candidate for prognostic validation in Dukes’ B patients. In addition, further investigations are needed to clarify the influence of specific mutations on the biological behavior of colorectal carcinomas.

### Deleted in Colorectal Cancer (DCC)

The DCC gene is localized on chromosome 18q and encodes a transmembrane protein with high homology to cell adhesion molecules [84]. Lack of the DCC protein may lead to impaired contacts between cells and contribute to tumor growth and invasion. For these reasons, the DCC gene and its protein product may have a role as natural history prognostic factor. In human colorectal carcinomas, DCC status has been investigated by molecular genetic assays or immunohistochemistry [84, 85].

Fifteen studies including stage Dukes’ B patients investigated the prognostic role of loss of heterozygosity (LOH) at chromosome 18q or absence of DCC expression in early-stage colorectal cancer [2, 79, 86–98]. A specific subgroup prognostic analysis in stage Dukes’ B patients was performed in 10 studies, which are listed in Table 7. Overall, two studies were based on the DCC expression analysis [86, 93] and the remaining eight studies investigated LOH at chromosome 18q with microsatellite markers. All these investigations were retrospective and a consistent and significant prognostic role of the DCC status was found in eight of these studies [79, 86–90, 92, 93].

To date, Shibata et al. [86] have reported the largest prognostic assessment of DCC protein expression in early-stage colorectal cancer. In 132 paraffin-embedded tumor samples of patients with curatively resected stage II/III colorectal cancer, the DCC staining was an ‘all or nothing’ event, and the immunohistochemistry assessment was scored as presence of any cytoplasmic reactivity or negative staining. The 5-year survival rate for patients with DCC-positive tumors was 94.3%, compared with 61.6% for patients with DCC-negative tumors (Table 7). The outcome in patients with DCC-negative stage II tumors was very similar to the outcome in patients with DCC-positive stage III tumors.

On the whole, data on the DCC functional assessment for identifying high-risk patients with early-stage colorectal cancer are encouraging. The prognostic role seems to be confirmed by the subgroup analyses of stage Dukes’ B patients, and the prevalence of DCC abnormalities in colorectal carcinomas is ~40–50% of all cases. These data are in favor of further testing of the DCC status in prospective clinical trials.

### Microsatellite instability

The presence of a defective DNA mismatch repair mechanism results in somatic alterations in the size of simple repeat nucleotide sequences (microsatellites). This phenomenon is known as microsatellite instability (MSI) and is due to silencing of the mismatch repair genes, primarily MLH1 and MSH2 [99]. Analysis of MSI can be performed by molecular assays with microsatellite markers in tumor and corresponding normal DNA [99]. In 1999, the National Cancer Institute sponsored an International Workshop and a validated panel of five microsatellites was proposed for the identification and the assessment of MSI [100]. Tumors may be characterized as high-frequency MSI (MSI-H), if two or more of the five markers show instability, and low-frequency MSI (MSI-L), if only one of the five markers shows instability. MSI-H colorectal tumors seem to share a less aggressive clinical course than stage-matched MSI-L or microsatellite stable (MSS) tumors [100]. In addition, analysis of the specific non-coding mononucleotide BAT-26, a component of the consensus panel, was shown to be highly correlated with generalized dinucleotide repeat instability [101]. Immunohistochemistry for hMLH1 and hMSH2 expression may represent a practical test for identifying DNA mismatch repair-deficient tumors, and it has been used in retrospective prognostic analyses in colorectal carcinomas [102].

MSI was detected in ~90% of tumor samples with the hereditary non-polyposis colorectal cancer syndrome (HNPCC), and patients’ prognosis in this group appeared to be better compared with patients with sporadic colorectal carcinomas [100]. In perspective of future adjuvant chemotherapy trials for high-risk stage Dukes’ B patients, this review focused on the prognostic role of MSI in early-stage, sporadic colorectal carcinomas. In this setting, among 26 selected studies [97, 98, 103–126], 17 retrospective investigations [109–125] showed a significant association between MSI-H or abrogated hMLH1 and hMSH2 expressions and improved prognosis. The majority of studies showed a 5–20% frequency of MSI-H in sporadic tumors, and only a minority of these investigations were performed with a large sample of patients that allowed the prognostic analysis in the subgroup of stage Dukes’ B patients [98, 107, 108, 120, 121, 124, 125] (Table 8). Only one of these studies confirmed a consistent and independent association between MSI-H and improved survival in stage Dukes’ B patients [125].

Gryfe et al. [125] found MSI-H in tumors of 102 out of 587 patients (17%) who were ≤50 years of age at diagnosis. The 5-year survival rate for all patients with MSI-H tumors was significantly better than that of patients with MSS tumors (76% versus 54%, respectively; $P <0.001$). This prognostic role was confirmed in all Dukes’ stages, including 173 patients with Dukes’ B disease. In the largest analysis of MSI in sporadic colorectal carcinomas of 1026 individuals, Samowitz et al. [121] found 12% of unstable
tumors with BAT-26, and this feature was associated with improved 5-year survival. However, the relationship between MSI and prognosis was not confirmed in patients with node-negative disease (328 Dukes’ A patients and 318 Dukes’ B patients).

Available data are insufficient to support a prognostic role of MSI in stage Dukes’ B colorectal cancer patients. In addition, the low frequency of this phenomenon is a major limitation for planning large prospective trials with MSI-based identification of high-risk patients.

Other prognostic biomarkers

Newly discovered molecular markers of prognosis are under investigation in early stage colorectal cancer. Some of them showed potential prognostic significance, but specific survival data in stage Dukes’ B patients are almost lacking, and further studies are needed [127, 128]. An overview of these new biomarkers is reported in this section.

Transforming growth factors (TGF)-α and -β1 are two components of a superfamily of molecules with mitogenic properties and promoting the growth of colon cancer cell lines in vitro. Their expression has been associated with poor survival in early-stage colorectal cancer patients, and in recent investigations TGF-β1 emerged as independent prognostic factor [98, 129].

The c-erbB2 proto-oncogene, also known as HER-2, is a glycoprotein with tyrosine kinase activity that has been largely investigated in breast cancer. Its overexpression in colorectal carcinomas seems to be detectable in 20–40% of tumors, but its prognostic role remains uncertain [130, 131].

Epidermal growth factor receptor (EGFR) is another transmembrane protein with tyrosine kinase activity. The frequency of EGFR overexpression in colorectal carcinomas seems to be higher than that reported for the HER-2 glycoprotein [131], and EGFR inhibitors are under investigation in clinical trials for the treatment of colorectal cancer. However, the role of EGFR as an independent prognostic marker has not been clearly defined yet [132].

The Bcl-2/BAX complex plays a key role in the regulation of apoptosis. The bcl-2 proto-oncogene encodes a protein with anti-apoptotic properties, whereas the BAX protein is a promoter of apoptosis [128]. According to this biological background, bcl-2 overexpression and/or BAX reduced expression may have negative prognostic role in colorectal cancer. Early investigations seem to confirm this hypothesis [17], but data are still limited.

Gene damage and allelic loss in diverse chromosomes seemed to correlate with poorer survival of colorectal cancer patients [132–136]. These data have not been extensively evaluated and additional studies are required.

Conclusions

Several prognostic biomarkers have been discovered over the last decades, and as we move into the 21st century, their introduction in the planning of adjuvant chemotherapy trials may help to improve and prolong lives of patients with surgically resected colorectal cancer. Unfortunately, puzzling data from current retrospective studies, poor homogeneity of inclusion criteria, variability in the techniques of analysis, and differences in the choice of cut-off levels and statistical methods all represent major barriers for interpreting results and widening applications of biomarkers in the overall management of early-stage colorectal cancer. Lack of standardization across studies is a relevant problem, especially for evaluating the results of immunohistochemically detected biomarkers. Whereas molecular markers may be assessed as a relatively simple ‘yes/no’ signal for the presence of gene damage, several parameters may be included in the analysis of immunohistochemistry; number of positive tumor cells, staining intensity and staining patterns. One or more of these features can be considered in the choice of a cut-off level.

There is no clear indication of the level of evidence to be met for validating prognostic biomarkers in prospective studies. Repeated positive results in retrospective studies may suggest the prognostic utility of a molecular marker. Among these promising molecular markers, those, or the one, with sufficient frequency in colorectal carcinomas and with reliable and repeatable methods of assessment could be tested for validation in prospective trials. In this perspective, angiogenesis features, plasminogen-related molecules and loss of DCC function may warrant further investigation. Their frequency in about half of colorectal carcinomas allows the possibility of performing well-designed prospective studies with reasonable sample sizes [137]. In addition, many of these markers can be determined by immunohistochemistry, thereby enabling their analysis in the majority of laboratories worldwide. The analysis of DCC function could be preferable. In fact, it is still not clear which of the several angiogenic features and plasminogen-related molecules do have the best prognostic power. Also, immunohistochemistry for DCC showed unambiguous dichotomization between negative (0–5% stained cells) and positive cases, and consequently has been described an ‘all or nothing’ phenomenon [86, 93].

The simultaneous testing of multiple molecular predictors of survival may supply major information [137], but the necessity of applying a multivariate model of analysis mandate for a large sample size. In a more realistic way, confirmatory studies of prognostic factors should test a single biomarker in a prospective fashion. These investigations can be embedded in trials evaluating new therapies, where the prognostic biomarker is used to identify categories of high- and low-risk patients. The population of patients with Dukes’ B colorectal cancer represents the target population for biomarker-based trials. In these patients, the role of adjuvant chemotherapy is unclear, and the optimal treatment strategy is still matter of debate [138, 139]. In a prospective adjuvant trial, Dukes’ B patients with preserved DCC could not be treated following surgical resection, whereas patients with loss of DCC function could be randomized to observation or chemotherapy.

We believe that hopes for improving the efficacy of post-operative adjuvant chemotherapy in early-stage colorectal cancer should not rely only on new drugs. Biomarker-based clinical trials do not receive the same commercial hype as new chemotherapeutic compounds, but clinicians should consider the opportunity...
of individualized cancer treatments based on the molecular characteristics of the tumor.

References


