Locally advanced rectal cancer
Reerink, Onne

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Chapter 3

Molecular prognostic factors in locally irresectable rectal cancer treated preoperatively by chemo-radiotherapy.


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Abstract

Background: The aim of this study was to determine the relation between survival and value of molecular markers in the primary tumour in a group of patients with locally irresectable rectal cancer, treated with preoperative chemo-radiotherapy.

Methods and Materials: Immunohistochemistry for p53, p21, bcl-2 and Ki-67 was performed on pre-treatment biopsy specimens of 34 patients with locally irresectable rectal cancer. Preoperative treatment consisted of pelvic irradiation of 45-56 Gy, combined with 5FU and leucovorin (350/20 mg/m² x 5 d; in week 1 and 5 during radiotherapy). The median follow-up was 38 months (range 25-75 months). Endpoints were pathological T-stage and survival after surgery. Eleven patients received intra-operative radiotherapy and 14 patients postoperative adjuvant therapy.

Results: Expression of p21 correlated significantly with survival (p=0.005). Survival and p21 expression also correlated significantly, when adjusted for tumour response (p=0.005, RR=4.8 (1.6-14.7)). No relation was found between p53, bcl-2 or Ki-67 and tumour response or survival. Multi-variate analysis between the different molecular markers showed no significant relation.

Conclusions: Expression of p21 predicts a worse survival in locally irresectable rectal cancer treated with preoperative chemo-radiotherapy. No relationship was found between tumour response in chemo-radiotherapy and p53, bcl-2 or Ki-67.
Introduction
Colorectal cancer is a major public health problem in the Western world and ranks as the third leading cause of death in both males and females. In 1997, 8600 new colorectal cancer patients were registered in the Netherlands, of whom 25% had rectal cancer (1). In early stages a surgical resection is the only curative treatment. Following potentially curative resection however, local recurrence rates vary between 5 and 40% (2-5). Moreover the majority of patients present at an advanced stage. At the time of diagnosis 38% of patients will have regional spread of disease and 25% will already have distant spread (6). (Neo-)adjuvant therapies like pelvic irradiation and chemotherapy, either alone or in combination, have an additional role in these subsets of patients. At present, conventional clinico-pathological parameters cannot entirely identify aggressive tumours that would benefit from (neo-)adjuvant therapy. As for other human malignancies, the development of rectal adenocarcinoma is associated with a series of inherited and/or acquired gene abnormalities that dis regulate cell growth and cell death. These genes or their protein products can be measured in tumour tissue. The aim of this study was to determine the relation between survival after chemo-radiotherapy and the value of molecular markers, in the primary tumour, in a group of patients with locally irresectable rectal cancer treated with preoperative chemo-radiotherapy.

Materials and methods
Patients
Thirty-four patients with locally irresectable rectal cancer treated in the University Hospital Groningen from 1994 to 1998 were studied. Assessment of tumour stage was performed by digital rectal examination, computed tomographic scan and in thirty-three cases by means of a staging laparotomy. All patients received neoadjuvant therapy consisting of preoperative radiotherapy at doses between 45 and 56 Gy administered to the pelvis as described in an earlier paper (7), accompanied by 5-FU and leucovorin (350/20mg/m² x 5d; in week 1 and 5 during radiotherapy). After 4 to 6 weeks patients were subjected to radical surgery with a curative intent. During this study period patients were entered in prevailing protocols, thus intra-operative radiotherapy with a dose of 10 Gy was performed on 11 patients and postoperatively a weekly dose of 5-FU and leucovorin (450/20 mg/m²) for a period of 12 weeks was given at 14 patients. Survival of patients was measured from start of neo-adjuvant therapy.
Immunohistochemical staining

Specimens were fixed in formalin, paraffin embedded and cut into 3 µm thick sections, which were applied to 2-aminopropyltriethoxysilane-coated slides and stretched on a heated plate (30 min at 60°C). Slides were dried overnight in a stove at 37°C. After deparaffinisation of slides, 200 µl blocking solution (2% blocking reagent (Boehringer, Mannheim, Germany) in maleate buffer 0.15 M NaCl, pH 6.0) was added to each slide for antigen retrieval. Slides were heated twice for 10 min at 115°C with 5 min cooling in between and subsequently washed with phosphate buffered saline (8.750 g NaCl, 1.370 g Na2HPO4, 0.215 g KH2PO4 in 1 L H2O, pH 7.3 (PBS)). Endogenous peroxidase activity was blocked with 0.3% H2O2 in PBS for 30 min.

Different monoclonal antibodies were diluted in 1% bovine serum albumin (BSA) in PBS. For p53-staining, slides were incubated for 1 hour at room temperature with a 1:400 dilution of BP53-12-1 (Biogenex, San Ramon, CA), detecting both wild and mutant type p53. For p21-staining a 1:50 dilution of p21-WAF (Ab-1) (Calbiochem, Oncogene Research Products, Cambridge, UK) and for Ki-67-staining a 1:400 dilution of MIB-1 (Immunotech, Marseille, France) were used with 1 hour incubation at room temperature. For bcl-2 staining, slides were incubated overnight at 4°C with a 1:400 dilution of anti-bcl-2 antibody (Dako, Glostrup, Denmark). After washing with PBS, slides were successively incubated with a 1:50 dilution of peroxidase conjugated rabbit-anti-mouse antibody (RaMper, Dako) and a 1:50 dilution of peroxidase conjugated goat-anti-rabbit antibody (GaRper, Dako) in 1% BSA/PBS and 1% human serum for 30 min each. Peroxidase activity was visualised by incubation with 25 mg diaminobenzidine dissolved in 50 mg imidazol in 50 mL PBS and 50 µL H2O2 30%. Counterstaining of the nuclei was performed using Mayer’s haematoxylin (Sigma, St. Louis, MO) for 2 min. For p53, a breast carcinoma specimen was taken as a positive control (2+) and for p21, a normal colon specimen was used as positive control (1+). For bcl-2 staining, incubation with an IgG1 antibody (Dako) and subsequently RaMper and GaRper served as negative control (0+) and bcl-2 staining of infiltrative lymphocytes was used as a positive internal control (3+).

Semiquantitative determination of p53, p21 and bcl-2 expression and Ki-67 index

Evaluation of intensity and extension of staining was performed light microscopically by three blinded observers. Staining intensity was graded qualitatively as -, not detectable; +, weak; ++, moderate, ++++, strong. The intensity was referred relative to corresponding positive controls.
Ki-67 index was defined as the total number of Ki-67 positive cells per total number of nuclei counted. The results of the immunohistochemistry of p53 and Ki-67 performed on the biopsies were scored positive when a strong staining intensity was found. The overall staining intensity of bcl-2 was less intense than that of other scored markers, for that reason the percentage of stained cells was multiplied times the staining intensity, (weak=1, moderate=2, strong=3). An intensity of 50% or higher was considered positive. The expression of p21 was considered positive when 25% or more nuclear staining was found. The percentage of positive cell staining was categorised as follows in table 1.

*Statistical analysis*

Associations between p53, p21 bcl-2 and Ki-67 staining and tumour response were determined by the chi-squared test. The logrank test was used for survival analysis regarding staining of p53, p21, bcl-2 and ki-67 respectively, with or without adjustment for other parameters.
### Table 1. Distribution of percentages cell staining.

<table>
<thead>
<tr>
<th>Staining</th>
<th>Non (-)</th>
<th>Weak (+)</th>
<th>Moderate (++)</th>
<th>Strong (+++)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>&lt; 5 %</td>
<td>5-25 %</td>
<td>25-50 %</td>
<td>&gt;50 %</td>
</tr>
<tr>
<td>p21</td>
<td>&lt; 10 %</td>
<td>10-25 %</td>
<td>25-50 %</td>
<td>50-100 %</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>&lt; 50 %</td>
<td>50-100 %</td>
<td>100-200 %</td>
<td>200-300 %</td>
</tr>
<tr>
<td>Ki-67</td>
<td>&lt; 25 %</td>
<td>25-50 %</td>
<td>50-75 %</td>
<td>75-100 %</td>
</tr>
</tbody>
</table>

### Table 2. Distribution staining intensity of molecular markers.

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>P53</td>
<td>16</td>
<td>18</td>
<td>34</td>
</tr>
<tr>
<td>P21</td>
<td>12</td>
<td>22</td>
<td>34</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>14</td>
<td>20</td>
<td>34</td>
</tr>
<tr>
<td>Ki-67</td>
<td>29</td>
<td>5</td>
<td>34</td>
</tr>
</tbody>
</table>
Results
The effects of the neo-adjuvant treatment on the downstaging of the rectal tumour in the study have been described before (7). Briefly, 7 of the 34 patients remained irresectable after neo-adjuvant treatment. The median survival of these patients was 12 months (range 1.5 - 49 months). Ten had a postoperative staging of T2 or less in which the overall median survival was not reached in 4.5 years. Seventeen patients had a pT3 or higher with a median survival of 76 months (3.5 - 79 months). Patients receiving IORT (n=11) showed no significant difference in survival compared to the group treated without IORT. No impact was also found from postoperative adjuvant chemotherapy (n=14). As expected an evident relation between lymphnode involvement and survival was found in the resectable group (p=0.009, RR=9.3 (1.7-49.6)).

The distribution of positive or negative scoring of the molecular markers is shown in table 2. Further analysis of the relationship between the staining intensity and the clinical parameters survival, resectability or downstaging was performed. In this analysis two groups were formed. The first group are the “good” responders, pT0-2. The second group the “bad” responders, pT3-4 and the irresectable.

p53, bcl-2 and Ki-67 expression
Univariate analysis in the expression of p53, bcl-2 and Ki-67 showed no significant difference in survival in the post-treatment irresectable group (n=7) and the resectable group (n=27). There was no relationship between antigen expression and the histological response to preoperative treatment. There were no associations in expression of p53, p21, bcl-2 or Ki-67.

P21 expression
P21 expression correlated with survival in the whole group (p=0.013, RR=4.3 (1.4-13.6)). All irresectable patients died. In the resectable group a positive expression of p21 still correlated significantly with a worse survival (p=0.009, RR=13.3 (1.9-92.2) (Fig. 1). In this group, survival and p21 expression remained correlated, when adjusted for tumour response (p=0.004, RR=15.5 (2.4-99.5)).

Further analysis in the resectable group of p21 and survival adjusted for IORT or adjuvant therapy showed that p21 still was an independent marker (p=0.013, RR=12.2 (1.7-88.4) & p=0.011, RR=12.7 (1.8-89.9) respectively. P21 and lymph node involvement both were
independent predictors for a worse prognosis (p=0.032, RR=9.9 (1.2-79.9) and p=0.025, RR=7.5 (1.3-43.8) respectively). This significant relation remained when the tumours with less then 10% staining were compared to those with a staining intensity stronger than 25%, p=0.027 RR=3.56 (1.07-11.8).

**Figure 1.** Correlation of staining intensity of p21 and survival in the group which became resectable after neo-adjuvant therapy (p=0.002).
Discussion

In the treatment of locally irresectable rectal cancer some sort of (neo)adjuvant therapy is often applied, usually in the form of a combination of irradiation and chemotherapy, sometimes irradiation alone is used. The results of such schedules can be appreciated at the levels of tumour response, resectability, local relapse and survival. At all levels the number of patients failing treatment is considerable, emphasising the need for treatment alternatives. At present however prediction of treatment outcome is not possible with any standard criterion. The most likely parameters to offer predictive value are cellular proteins that represent pathways engaged in cellular survival or death after anti-tumour therapy and parameters for tumour (re)growth. In this respect the most often studied markers are p53, p21, bcl-2 and Ki-67. Genes in this path have products which play a crucial role in apoptosis, cell proliferation and tumour progression. The proliferative activity of a given lesion is commonly evaluated by MIB-1, a monoclonal antibody to Ki-67 proliferation antigen, or by counting mitotic figures on histologic sections. In a study of Jansson, Ki-67 expression in 255 human colorectal cancers showed no relation to clinicopathology and prognosis (8). Adell showed in a recent study that Ki-67 stained tumour cells can predict a treatment failure after preoperative radiotherapy of rectal cancer (9).

The tumour suppressor gene p53, localised on chromosome 17, is responsible for the production of a protein that targets among many others the p21 gene. Mutations often lead to excess protein that is unable to function in the appropriate pathway. However mutations not leading to abnormal proteins are not detected by immune histochemistry, as applied in this study. Therefor the incidence of p53 mutation may be underestimated.

Bcl-2 was the first human gene known to encode for an inhibitor of apoptosis. When bcl-2 is expressed at high levels in cells, it forms complexes with bax (a bcl-2 like protein); preventing bax homodimerisation and inhibiting cell death. Schwandner and Leahy, found in two different studies on bcl-2 expression in colorectal cancer respectively no relation to recurrence and better long term prognosis (10,11).

In the literature results of the application of these markers are conflicting. A low level of mutated p53 protein either alone or in combination with other parameters was found to be a favourable factor for tumour response (12-14) as well as for local relapse (15) and more favourable histology (16). In a study also incorporating clinical outcome (relapse) the combination of p53 and bcl-2 markers was of value in patients with colon or rectal cancer (17). However, other studies do not confirm these results, neither at the local response level (18-20) nor at clinical levels of relapse and survival (21).
Therefore these markers are probably insufficient as predictors for prognosis in colo-rectal cancer, in any case they seem of limited value compared with breast cancer (22), lung cancer (23) and ovarian cancer (24). It is conceivable that other intervention factors such as surgical technique have a much more important and variable effect on treatment outcome.

In our study no clinical value in predicting tumour response or survival was found for p53, bcl-2 or Ki-67. We found however a distinct relation between p21 expression and clinical outcome, in patients with p21 positive tumours did worse. This result held true after adjusting for T stage, IORT and adjuvant therapy. Previous experimental and some clinical evidence support these findings. In the context of resistance to irradiation, in a number of cell lines an elevated level of p21 protein is found. Characteristically the level of p21 protein remains high in this situation, and there is no induction of the protein by the irradiation, as is often seen in the p53 induced p21 response to irradiation. Rather than following the cell cycle block to its apoptotic climax, cells survive, giving rise to the phenotype of radiation resistance (25-28). Antisense application in this situation restores sensitivity (28) while p21 mutation also interferes with resistance (27). In these studies the effect of radiotherapy may have influenced survival. However in our patient group we do not find a relation between p21 and the effect of radiotherapy as judged by the T status. Therefore the inverse effect on survival may be caused by mechanisms other than radioresistance. This is supported by observations in patients with colorectal cancer treated surgically (29), patients with prostate cancer treated surgically (30) and patients with breast cancer (31). As in many other respects p21 functions as a negative regulator of growth, progression and metastasis (32,33), further analysis of this intriguing observation seems warranted.
Reference list


