Antibodies against p53 are associated with poor prognosis of colorectal cancer

JGA Houbiers1,2, SH van der Burg1,2, LMG van de Watering1, RAEM Tollenaar2, A Brand1, CJH van de Velde2 and CJM Melief3

1Department of Immunohaematology and Blood Bank and 2Department of Surgery, University Hospital Leiden, PO Box 9600, 2300 RC Leiden, The Netherlands.

Summary Mutation of the p53 gene is a common event in colorectal cancer. This alteration can result in cellular accumulation of p53 and may also induce p53 antibodies. Accumulation of p53 in tumour cells has been associated with poor prognosis of colorectal cancer. We tested preoperative sera from 255 patients with colorectal cancer by enzyme-linked immunosorbent assay (ELISA). A total of 70.2% had reactivity that was higher than the 'low' control serum. Employing a cut-off level of 10% of the 'high' control sample, 25.5% of the patients were positive for p53 antibodies. The presence of p53 antibodies correlated with the following prognostic factors: histological differentiation grade, shape of the tumour, and tumour invasion into blood vessels. Patients with p53 antibodies were shown to have decreased survival and decreased disease-free survival. Specifically for patients with cancer stage A and B1 the presence of p53 antibodies selected a subgroup with poor prognosis.

Keywords: colorectal cancer; p53

Colorectal cancer cells have accumulated several genetic alterations (Fearon and Vogelstein, 1990). The most common of these defects is loss of function of the tumour-suppressor gene p53 (Lane, 1992; Vogelstein and Kinzler, 1992). Although different mechanisms can result in loss of function of p53, allelic loss and mutation of the other gene is most often found (Baker et al., 1989; Levine et al., 1991; Vogelstein and Kinzler, 1992). These point mutations are associated with p53 overexpression caused by the decreased breakdown of the tetrameric form with mutant components (Levine et al., 1991). The normal level of nuclear p53 expression is extremely low, but the aberrant accumulation of p53 in the tumour cell is detectable with p53-specific antibodies (Remvikos et al., 1990; Bartek et al., 1991; Lane, 1992). In colorectal cancer p53 is overexpressed in 50–70% of tumours (Cunningham et al., 1992; Sun et al., 1992; reviewed by Harris and Hollstein, 1993).

Because mutation in the p53 gene and the consequent overexpression of p53 are associated with tumour tissue, both, wild-type and mutant p53 may act as targets of tumour-specific humoral and cellular immune responses. p53-specific antibodies in the sera of patients have been studied in breast cancer (Crawford et al., 1982; Davidoff et al., 1992; Schlichtholz et al., 1992; Mudenda et al., 1994), in lung cancer (Winter et al., 1992; Schlichtholz et al., 1994), in B-cell lymphoma (Caron de Fromenti el et al., 1987) and more recently in various other types of cancer (Lubrano et al., 1993; Lubin et al., 1993; Angelopoulou et al., 1994). In breast cancer, a p53 antibody response was found to be an indicator of poor prognosis (Schlichtholz et al., 1992; Mudenda et al., 1994).

Using an ELISA, we tested the serum samples taken preoperatively from 255 patients that participated in the CRAB clinical trial on the relationship between blood transfusion and colorectal cancer prognosis (Houbiers et al., 1994), for the presence of p53 antibodies. The correlations between the presence of serum antibodies against p53 and prognostic factors of colorectal cancer and patient survival were investigated.

Patients and methods

Patients

Preoperative sera of 255 patients were tested for p53 antibodies. The colorectal cancer patients were enrolled in the CRAB clinical trial by seven different hospitals between 1987 and 1991 (Houbiers et al., 1994). Data on patient history, surgery, tumour pathology, clinical complications and outcome during an average follow-up of 36 months were available. A total of 231 sera were drawn from patients operated upon with curative intent, while 24 sera were from patients with known metastatic disease at the time of surgery.

Assay

The detection of p53 antibodies in patient sera was performed with a commercially available sandwich enzyme-linked immunosorbent assay (ELISA, p53-AK ELISA, cat. no. dia0301E, Dianova, Hamburg, Germany). The assay was performed according to the manufacturer’s instructions and has the following specifications: 1:100 diluted patient serum was added for 60 min at 37°C to microtitre wells coated with recombinant p53. After washing, goat anti-human IgG antibody conjugated with peroxidase was added for 30 min at 37°C; finally the substrate 3, 5, 3, 5-tetramethylbenzidine (TMB) was added for 30 min. The enzymatic process was stopped by adding 2 N hydrogen chloride. Light absorption was measured at 450 nm on a photometer spectrophotometer (Titertek Multiskan).

This ELISA was validated by comparing the ELISA results of sera from cancer patients and non-cancer patients with results obtained from Western blotting with recombinant p53; the results of these sera showed a high concordance (H Zentgraf and PR Galle 1994, Heidelberg, personal communication). A total of 379 patients without tumours were negative in the p53 antibody assay (Müller et al., 1994). The ELISA absorption data of our study were correlated with the manufacturer’s control sera (‘low’ or ‘high’ concentration of p53 antibodies) by the following formula, which was designated the p53 antibody titre index (TI)

\[
p53\text{-TI} = \frac{\text{patient sample absorption}}{\text{absorption of low control}} \times 100\%
\]

This index was calculated for each patient.
The absorption results of sera from our own serum bank, added to control intertest variation showed good concor-
dance between the different assays. Additionally, testing of
the ‘medium’ control serum that was provided by the
manufacturer resulted in p53-Tls in the narrow range from
31% to 35% in all assays. The sera of two patients that were
excluded from the CRAB study because of a benign colon
tumour had a p53-TI of < 10%. The sera of 40 healthy blood
Donors also had a p53-TI of < 10%.

Immunochemistry

A three step indirect immunoperoxidase technique on frozen
4-μm-thick colorectal carcinoma tissue sections was per-
duced as described earlier (Ravenswaay Claassen Van et al.,
1992). The monoclonal antibodies PAb 122 (Gurney et al.,
1980), DO7 (Vojtesek et al., 1992) and the polyclonal
antiserum CM1 (Bartek et al., 1991) were used to detect
cellular accumulation (overexpression) of p53 and appeared
concordant in all sections, except that PAB 122 stained more
weakly and fewer cells were positive per section compared to
the other two antibodies. From different parts of a resected
tumour, on average five pieces of tissue were taken; if one or
more pieces showed overexpression of p53 that tumour was
considered positive for p53 accumulation. The results of
immunohistochemical staining for p53 of colorectal car-
cinomas is further described elsewhere (Houbiers et al., sub-
mitted).

Analysis

Using the Fisher’s test for 2 × 2 tables (Fisher’s exact test),
the presence of p53 antibodies was correlated to the follow-
ing prognostic factors of colorectal cancer: cancer stage
(Dukes’ classification modified according to Astler and Coller
(1954)), histological differentiation, location of the tumour,
tumour infiltration into adjacent organs, tumour size, tumour
shape, tumour invasion of lymph vessels or blood vessels,
sex, and a history of blood transfusion, blood groups (ABO,
rhesus). Correlations between the presence of p53 antibodies
and disease-free survival, overall survival, and risk of cancer
recurrence were analysed using the Kaplan–Meier method
and log-rank tests (for details, Houbiers et al., 1994). Fur-
thermore the association of p53 antibodies with the accumu-
lation of p53 in tumour cells, as detected by immunohis-
tochemistry on autologous tumour samples, was analysed
with the Fisher’s exact test.

Results

Of the 255 tested preoperative sera from patients with colo-
rectal cancer, 70.2% had a p53-antibody titre index (p53-TI)
that was higher than the manufacturers ‘low’ control serum
(i.e. > 0%, Table I). When results of p53-TI ≤ 10% were
regarded as absence of p53 antibodies, 25.5% of the sera
were positive (Table I). The cut-off level of 10% was not
arbitrarily chosen; samples with a lower absorption than the
‘low control’ that was provided by the manufacturer had a
p53-TI between <10% and 0%. Furthermore, the relevance
of the 10% cut-off level was confirmed by the results of ten
sera that were tested in an unmarked order for p53
antibodies using immunoblotting with recombinant p53
(Western blotting). The six sera that had a p53-TI ≤ 10%
were negative by Western blotting (Figure 1); the two sera
that appeared low or intermediate positive (+ or + +) by
Western blotting scored intermediate positive by ELISA; the
other two sera scored triple plus on the semiquantitative
Western blot scale and had a p53-TI > 60% by ELISA.

The tumours of 20 of the 255 patients had been
immunohistochemically stained for HLA antigens and cell-
ular p53 accumulation (overexpression of p53). The associa-
tion between tumours with p53 overexpression and serum
p53 antibodies was significant: 7 out of the 12 patients with
tumours showing p53 overexpression had a p53-TI > 10%,
while only one out of the eight without p53 overexpression
had antibodies (P = 0.05).

Correlation of p53 antibodies with prognostic factors

Table II gives a summary of the correlation of the p53-
antibody status to known prognostic factors for colorectal
cancer. The analyses with the cut-off level for the antibody-
negative status on p53-TI ≤ 10% is shown. A significant
association was found with histological grade, shape of the
tumour, invasion of the tumour into blood vessels and the
Quetelet index (i.e. the quantitative relation to the ideal body
weight). Division of the p53-TI scale into two classes (<0%,
10–33%, >33%) or into four (<10%, 10–33%, 33–80%, >80%) classes resulted in
similar distributions.

The cancer stage was weakly associated with p53 anti-
bodies: comparison of p53 antibody status of patients with
cancer stage A or B to those with stage C resulted in 21% vs
31% p53 antibody positivity (P = 0.09); while the division of
the p53-TI into four classes revealed an association with a
P-value of 0.06.

No association was found between p53 antibody status
and any of the following factors: age, a history of pregnancy,
history of blood transfusion, ABO blood group system,
rhesus blood group system, sex, a history of cholecystectomy,
diabetes, chronic aspecific lung disease, extension of the
tumour into other organs, ulcerative tumour, presence of
metastatic disease, lymph vessel involvement or size of the
tumour.

Follow-up and p53 antibodies

Colorectal cancer prognosis was expressed in terms of overall
survival, disease-free survival and disease-free period at 3
years of follow-up and was analysed on the basis of Kaplan–
Meier curves as previously reported (Houbiers et al.,
1994). Associations between cancer prognosis and p53
antibodies were observed (Table III). We further analysed
the association of p53 antibody status with prognosis within
the subgroups of the major prognostic variable of colorectal
cancer: the cancer stage. Within the subgroup of patients
with cancer stage A (invasion limited to the mucosa or
submucosa) or B1 (tumour progression into, but not
through, the muscle layer of the large bowel), those who had
induced p53 antibodies showed a significantly reduced sur-
vival. Analyses of the subgroups with cancer stage B2 and
cancer stage C revealed no significant results.

Table I p53 antibodies in 255 colorectal cancer patients

<table>
<thead>
<tr>
<th>p53-TI, (patient sample - low control) / (high control - low control)</th>
<th>n</th>
<th>Percentage of patients</th>
<th>Cumulative percentage of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 0</td>
<td>76</td>
<td>29.8</td>
<td>29.8</td>
</tr>
<tr>
<td>1 – 10</td>
<td>114</td>
<td>44.7</td>
<td>74.5</td>
</tr>
<tr>
<td>11 – 20</td>
<td>20</td>
<td>7.8</td>
<td>82.4</td>
</tr>
<tr>
<td>21 – 30</td>
<td>12</td>
<td>4.7</td>
<td>87.1</td>
</tr>
<tr>
<td>31 – 40</td>
<td>8</td>
<td>3.1</td>
<td>90.2</td>
</tr>
<tr>
<td>41 – 50</td>
<td>1</td>
<td>0.4</td>
<td>90.6</td>
</tr>
<tr>
<td>51 – 60</td>
<td>4</td>
<td>1.6</td>
<td>92.2</td>
</tr>
<tr>
<td>61 – 70</td>
<td>7</td>
<td>2.7</td>
<td>94.9</td>
</tr>
<tr>
<td>71 – 80</td>
<td>1</td>
<td>0.4</td>
<td>95.3</td>
</tr>
<tr>
<td>81 – 90</td>
<td>1</td>
<td>0.4</td>
<td>95.7</td>
</tr>
<tr>
<td>91 – 100</td>
<td>0</td>
<td>0</td>
<td>95.7</td>
</tr>
<tr>
<td>100 – 110</td>
<td>4</td>
<td>1.6</td>
<td>97.3</td>
</tr>
<tr>
<td>110 – 120</td>
<td>0</td>
<td>0</td>
<td>97.3</td>
</tr>
<tr>
<td>120 – 130</td>
<td>2</td>
<td>0.8</td>
<td>98.0</td>
</tr>
<tr>
<td>130 – 140</td>
<td>2</td>
<td>0.8</td>
<td>98.8</td>
</tr>
<tr>
<td>140 – 150</td>
<td>0</td>
<td>0</td>
<td>98.8</td>
</tr>
<tr>
<td>150 – 160</td>
<td>1</td>
<td>0.4</td>
<td>99.2</td>
</tr>
<tr>
<td>160 – 170</td>
<td>1</td>
<td>0.4</td>
<td>99.6</td>
</tr>
<tr>
<td>170 – 180</td>
<td>1</td>
<td>0.4</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* p53-TI, (patient sample - low control) / (high control - low control) × 100%.
Serum p53 antibodies and colorectal cancer

JGA Houbiers et al

Figure 1 Western blot analysis was performed on ten patient sera to confirm the results of the ELISA. The recombinant p53 molecules (lane C) have run through the gel until the mark at the right-hand side and were labelled with anti-p53 antibody (p53). The sera with the numbers (No.) 2, 4, 7, 10, 11 and 13 can be considered negative by Western blot (—); in the ELISA they scored similarly negative (p53-TI < 10%). The sera numbered 3 and 57 appeared low or intermediate positive (+ or ++) and scored intermediate positive by ELISA (p53-TI). The other two sera (1 and 5) scored ++ on the semiquantitative Western blot scale and had a p53-TI > 60% by ELISA.

Table II p53 antibodies and prognostic factors of colorectal cancer

<table>
<thead>
<tr>
<th>Factor</th>
<th>p53-TI (%)</th>
<th>+</th>
<th>-</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhesus factor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>12</td>
<td>30</td>
<td></td>
<td>0.63</td>
</tr>
<tr>
<td>Negative</td>
<td>51</td>
<td>153</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A + B1</td>
<td>16</td>
<td>49</td>
<td></td>
<td>0.17</td>
</tr>
<tr>
<td>B2</td>
<td>19</td>
<td>79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>27</td>
<td>59</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>4</td>
<td>15</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>40</td>
<td>136</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>18</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shape of the tumours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stalked or sessile</td>
<td>16</td>
<td>72</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Flat or circular</td>
<td>41</td>
<td>97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invasion of blood vessels</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>53</td>
<td>173</td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quetelet index</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 21</td>
<td>7</td>
<td>40</td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>22-26</td>
<td>32</td>
<td>70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 27</td>
<td>15</td>
<td>19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p53 antibody status is positive (+) if p53-TI > 10%. *P-value of one-sided Fisher’s exact test (2 x 2) or chi-square (2 x 3). Data missing from patients: 1, 9; 2, 6; 3, 20; 4, 29; 5, 2 (blood vessels); 6, 72.

Multivariate analysis

Multivariate survival analysis (i.e. Cox regression analysis) was employed to establish the independent prognostic value of the p53 antibody status. In a predictive model with the variables cancer stage, extension of the tumour, histological grade, and tumour invasion into blood vessels and survival, disease-free survival or disease-free period as outcome variable, the p53-antibody status appeared not to add significantly to the model.

Table III p53 antibody status and colorectal cancer prognosis

<table>
<thead>
<tr>
<th>Division on basis of p53 antibody status (n)</th>
<th>p53-TI (%)</th>
<th>+</th>
<th>-</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients (249)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall survival</td>
<td>61</td>
<td>68</td>
<td></td>
<td>0.35</td>
</tr>
<tr>
<td>Disease-free survival</td>
<td>51</td>
<td>58</td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>Disease-free period</td>
<td>59</td>
<td>63</td>
<td></td>
<td>0.73</td>
</tr>
<tr>
<td>Curative surgery (226)</td>
<td>58</td>
<td>168</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall survival</td>
<td>64</td>
<td>72</td>
<td></td>
<td>0.22</td>
</tr>
<tr>
<td>Disease-free survival</td>
<td>66</td>
<td>64</td>
<td></td>
<td>0.26</td>
</tr>
<tr>
<td>Disease-free period</td>
<td>64</td>
<td>70</td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>Cancer stage A or B1 (64)</td>
<td>16</td>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overall survival</td>
<td>75</td>
<td>88</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Disease-free survival</td>
<td>69</td>
<td>81</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Disease-free period</td>
<td>87</td>
<td>88</td>
<td></td>
<td>0.06</td>
</tr>
</tbody>
</table>

*p53 antibody status is positive (+) if p53-TI > 10%. Percentage of patients alive or disease free at 3 years of follow-up based on Kaplan–Meier curves; P-value of log-rank test. *No evidence of remaining malignant tissue.

Discussion

Using ELISA, we studied the presence of p53 antibodies in preoperatively collected serum of 255 patients with colorectal cancer. A total of 70.2% of the patients had reactivity above the ‘low’ control (Table I); when employing a cut-off of p53-TI ≤ 10%, 25.5% of the patients were positive for p53 antibodies. The cut-off level of 10% was not arbitrarily chosen; sera with ELISA results below the ‘low control’ ranged from p53-TI = 10% to 0% and the six sera with p53-TI = 10% that additionally were tested with immunoblotting were negative for p53 antibodies.

The only study reporting on p53 antibodies in colorectal cancer patients used different assays and detected a positive antibody status in 13 patients (16%) out of 82 tested (Angelopoulou et al., 1994). That report, however, gives no clinical or pathological details on the patient group, nor does it correlate the results with prognosis.

The observed associations between p53 antibody positivity
Serum p53 antibodies and colorectal cancer

JGA Houbiers et al.

References


