Expression of high p53 levels in colorectal cancer: a favourable prognostic factor

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Summary The expression of p53 protein was examined in a series of 111 colorectal cancer adenocarcinomas with a long follow-up. A quantitative luminometric immunoassay (LIA) was used for the measurement of wild-type and mutant p53 protein in extracts from colorectal tumour cytosols, p53 being detected in 42% of the samples (range 0.0–52ng mg⁻¹). Using an arbitrary cut-off value of 2.7ng mg⁻¹, 25% of the tumours were classified as manifesting high p53 levels. There was no association of p53 expression with patient age, sex, serum preoperative carcinoembryonic antigen (CEA) levels, tumour site and size, nodal status or TNM stage. Significant and independent correlation was found to exist between high p53 levels and prolonged disease-free survival (P = 0.05) at a median follow-up of 60 months. This survival advantage was most apparent among stage III cancer patients. The results from this study would suggest that expression of high p53 levels appear to be useful in selecting a group of colorectal cancer patients with a better prognosis.

Keywords: colorectal cancer; p53; immunoassay; luminometric; prognosis; stage III cancer

The relationship between molecular abnormalities and neoplasm has been extensively reviewed and there is strong evidence that abnormalities of p53 gene represent the most common molecular change in human cancer. Such abnormalities can be detected in a number of ways. Several prior studies have revealed p53 protein expression via immunohistochemistry (IHC) in 42–69% of colorectal cancers (Scott et al, 1991; Auvinen et al, 1994). However, the prognostic value of this protein remains to be defined, probably due to variability of detection and retrieval systems (Wynford-Thomas, 1994). To be clinically useful, prognostic markers should be accessible to analysis with simple and reproducible procedures appropriate for routine use. We have adapted the recently developed luminometric immunoassay (LIA) (Borg et al, 1995), to quantify p53 protein in archival colorectal cytosols.

The purpose of the present study was: (1) to evaluate the relationship between p53 overexpression and clinicopathological data and (2) to assess the value of p53 as a biological marker of prognosis within each TNM class in a series of patients resected for colorectal cancer with a long follow-up.

PATIENTS AND METHODS

Patients

A series of 111 patients underwent surgical resection for primary colorectal adenocarcinoma at the II Department of Surgery, University Hospital ‘San Carlos’, Madrid, between 1990 and 1992. Each patient is regularly followed up at 6-monthly intervals for a minimum of 5 years. Cases in which resections have been performed for metachronous carcinoma, carcinoma arising in familial adenomatous polyposis and ulcerative colitis are excluded. None of the patients had received preoperative radiotherapy or chemotherapy. Since 1992, stage III patients younger than 70 received adjuvant chemotherapy with 5-fluorouracil (5-FU) and leucovorin according to the prevailing protocol (four patients). Clinical staging was done on the basis of the TNM classification. Survival time was calculated from the date of surgery to the date of death or last follow-up, with times censored for patients dying of causes unrelated to colorectal cancer and those surviving. Median follow-up was 5 years.

Tissue specimens

Sections from the colorectal adenocarcinoma and normal mucosa at the proximal/distal resection margins were obtained at surgical resection. These specimens were stored in liquid nitrogen and, prior to being pulverized in the frozen state and homogenized for the preparation of cytosols for protein measurement, cryostat sections were evaluated; all tumour samples used contained more than 80% tumour cells.

Luminometric assay

The LIA is based on a combination of two monoclonal antibodies, Ab 1801 and DO 1, which detect both wild-type and mutant p53 protein in a sandwich-type assay. The Ab 1801, which is immobilized onto a solid phase, is used for catching. Ab DO 1, labelled with a chemiluminescent compound (ABEI), is used for detection. The immunoassay was performed by incubating either 100μl of p53 standard, controls or tumour cytosols, together with 100μl of the ABEI-conjugate in pre-coated tubes. After incubation for 18h at room temperature, unbound reagents were removed by washing...
Table 1 Relationship between p53 protein levels in colorectal tumours and clinicopathological variables

| Variable  | n (%) | p53 ≥ 2.7 ng mg⁻¹ | p53 < 2.7 ng mg⁻¹ | P  |
|-----------|-------|-------------------|------------------|----|
| Age       |       |                   |                  |    |
| Mean 66 years |   |                  |                  |    |
| Mean 68 years |   |                  |                  |    |
| Sex       |       |                   |                  |    |
| Male: 54 (49) | 15 (27) | 39 (72.2) | 0.51 |
| Female: 57 (51) | 12 (21) | 45 (79) | 0.51 |
| I: 14 (12.6) | 3 (21.4) | 11 (78.6) | 0.51 |
| II: 50 (45) | 11 (22) | 39 (78) | 0.51 |
| III: 30 (27) | 10 (33.3) | 20 (66.7) | 0.6 |
| IV: 17 (15.3) | 3 (17.6) | 14 (82.4) | 0.6 |
| T status   |       |                   |                  |    |
| T1–T2: 17 (15) | 5 (29.4) | 12 (70.6) | 0.6 |
| T3–T4: 94 (85) | 22 (23.4) | 72 (76.6) | 0.6 |
| N status   |       |                   |                  |    |
| N0: 65 (58.6) | 15 (23) | 50 (77) | 0.8 |
| N+: 46 (41.4) | 12 (26) | 34 (74) | 0.8 |
| Site       |       |                   |                  |    |
| Right colon: 28 (25) | 7 (25) | 21 (75) | 0.94 |
| Left colon: 36 (33.4) | 8 (22.2) | 28 (77.8) | 0.94 |
| Rectum: 47 (39.6) | 12 (25.5) | 35 (74.5) | 0.94 |
| CEA        |       |                   |                  |    |
| <5 ng ml⁻¹: 42 (42) | 7 (16.7) | 35 (83.3) | 0.16 |
| ≥5 ng ml⁻¹: 58 (58) | 18 (31) | 40 (69) | 0.16 |

Figure 1. Stage III colorectal cancer patients. Disease-free survival curve according to p53 levels

Figure 2. Stage III colorectal cancer patients. Overall survival curve according to p53 levels
the tubes three times with 2 ml 0.9% sodium chloride. The chemiluminescent reaction was initiated by the sequential addition of 300 μl microperoxidase solution, immediately followed by measurement of the chemiluminescent counts in a luminometer. The p53 protein contents of the samples were determined from the standard curve plotting the chemiluminescent response (in relative light units (RLU)) against the standard concentrations of p53 protein. The detection limit was 0.01 ng p53 per ml sample. p53 concentration in the cytosols is expressed as ng mg⁻¹ cytosolic protein, the cytosol protein concentration being in the range 0.5–4 ng ml⁻¹.

**Statistical analyses**

To evaluate the relationship between p53 expression and tumour behaviour, different cut-off values were tested and then 2.7 ng mg⁻¹ was selected as the cut-off value that yielded the best discrimination of patients with good vs poor prognosis. Statistical significance of differences was determined by the χ² or Fisher and the Student’s t-test or analysis of variance (ANOVA). Survival rates were estimated by the Kaplan–Meier method and tested for significance by the Breslow exact test, while multivariate comparisons were conducted using the Cox regression analysis. Stratified analysis was used. Relative risks and their 95% confidence intervals (CI) were calculated with the use of the estimated regression coefficients and their standard errors. A P-value of < 0.05 was considered significant. Analysis was performed using the SPSS 7.5 statistical software.

**RESULTS**

Survival data, available on all cases, showed a 7-year specific overall survival of 49% (median 57 months). During follow-up, 37 patients had a relapse and 54 died from cancer-related deaths. Fifteen patients died from other causes. The LIA revealed p53 overexpression only in 47 neoplastic samples (42.3%), while the adjacent colonic mucosa was constantly negative. The p53 protein contents of the samples were determined from the standard curve plotting the chemiluminescent response (in relative light units (RLU)) against the standard concentrations of p53 protein. The detection limit was 0.01 ng p53 per ml sample. p53 concentration in the cytosols is expressed as ng mg⁻¹ cytosolic protein, the cytosol protein concentration being in the range 0.5–4 ng ml⁻¹.

**Stratified analyses**

Since tumour stage at diagnosis is the most powerful indicator of clinical outcome in colorectal cancer patients, p53 expression prognostic significance was assessed within each TNM class. Only patients with stage III cancer whose tumours expressed high p53 levels had longer disease-free survival times than patients with tumours lacking or with low-expressing LIA-detectable p53 protein (59.3% and 28.6% respectively) at a median follow-up of 60 months (P = 0.03). A longer overall survival (58.3% and 30%) was also observed, although this finding did not reach statistical significance (P = 0.09) (Figures 1 and 2).

**Multivariate analyses**

In multivariate analyses for relapse-free survival, including standard prognostic factors as covariates, p53 level < 2.7 ng mg⁻¹ was an independent factor for predicting the rate of relapse, with an adjusted relative hazard rate of 2.13 (CI 0.89–5.07) (P = 0.05). This association was independent of the TNM stage. However, p53 overexpression did not reach statistical significance for overall survival.

**Table 2 p53 immunohistochemical assays and prognosis in colorectal cancer**

| Reference   | Patients | Follow-up | p53 poor prognostic factor | Multivariate analysis Yes/No and other comments |
|-------------|----------|-----------|----------------------------|-----------------------------------------------|
| Sun 1993    | 293      | 5 years   | Y                          | MVA – yes. Nuclear and cytoplasmic p53 associated with adverse prognosis in I-III stages |
| Yamaguchi 1992 | 100      | 6–48 months | Y                          | MVA – yes. 3-year survival significantly worse for p53-expressing cases |
| Bell 1993   | 100      | 34 months | N                          | No MVA. No relationship of p53 and overall survival |
| Yamaguchi 1993 | 203      | 5 years   | Y                          | No MVA. P53-positive cases associated with adverse survival |
| Auvinen 1994 | 144      | 9 years   | Y                          | No MVA. Overall survival reduced in p53 positive cases |
| Nathanson 1994 | 84       | > 5 years | N                          | MVA – yes. No relationship of p53 and overall survival identified in stage II |
| Zeng 1994   | 107      | 62 months | Y                          | MVA – yes. Study conducted in stage III–IV patients with low CEA levels |
| Mulder 1995 | 109      | > 7 years | N                          | MVA – yes. P53 not an independent marker of prognosis in all stages. |
| Ofner 1995  | 109      | 79 months | N                          | No MVA. p53 expression not an independent marker of prognosis |
| Kressner 1996 | 294      | 4.5 years | N                          | MVA – yes. No relationship of p53 and overall survival identified in all stages |
| Poller 1997 | 250      | 4.3 years | N                          | MVA – yes. No relationship of p53 and overall survival identified in stage I-III tumours |
DISCUSSION

A sensitive and quantitative LIA has been developed for the measurement of wild-type and mutant p53 in extracts from tumour tissue. The p53–LIA has been performed previously on breast tumour cytosols (Borg et al, 1995) where it yielded reliable estimates of p53 expression as compared with IHC analysis. This novel immunoassay, although relatively time-consuming, allows an exact quantification of p53 expression in a way that is superior to IHC. In our series, p53 protein was detected in 42% of the tumour samples; this is well in line with previously reported p53 IHC overexpression in colorectal carcinomas, ranging from 42 to 69% (Scott et al, 1991; Auvinen et al, 1994). We found no correlation between p53 levels, tumour stage, site, serum preoperative CEA levels, bowel wall invasion and nodal status. Similar results have been reported previously (Scott et al, 1991; Starzynska et al, 1992; Bell et al, 1993; Nathanson et al, 1994; Mulder et al, 1995; Poller et al, 1997). At least 30 investigations have dealt with the prognostic significance of p53 aberrations in colorectal adenocarcinoma, providing contrasting results (Viale, 1997). In the vast majority of these studies, IHC was used to document an abnormal accumulation of p53 protein in neoplastic cells. The variables related to the staining protocols and scoring systems are so numerous that it is almost impossible to compare the results of different studies. Table 2 lists some of the most relevant published data on the prognostic value of p53 IHC expression in colorectal cancer; most would suggest that expression of p53 is an adverse prognostic factor. However, of the seven investigations using multivariate analysis, three documented a significant inverse correlation of p53 accumulation with patient survival, whereas four did not.

To clarify the influence of p53 overexpression on long-term survival in colorectal cancer patients, we performed LIA based on a combination of two monoclonal antibodies, Ab1801 and DO 1, which detects both wild-type and mutant p53 protein. No difference in survival was found between p53-positive and -negative tumours, whereas correlation of p53 accumulation with patient survival, whereas four did not.

In summary, it appears from our study that p53 protein overexpression may tell us more about the functional status of the p53 control pathway than the presence of a mutation within the gene. While the exact relationship between p53 gene mutation and protein overexpression in tumours is determined, quantification of p53 protein levels may be a useful parameter to optimize treatment for patients with completely resected colorectal cancer.

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