Overexpression of p53 mRNA in colorectal cancer and its relationship to p53 gene mutation

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Summary We analysed the frequency of p53 mRNA overexpression in a series of 109 primary colorectal carcinomas and its association with p53 gene mutation, which has been correlated with short survival. Sixty-nine of the 109 cases (63%) demonstrated p53 mRNA overexpression, without any correlation with stage or site of disease. Comparison with p53 gene mutation indicated that, besides cases in which p53 gene mutation and p53 mRNA overexpression were either both present (40 cases) or both absent (36 cases), there were also cases in which p53 mRNA was overexpressed in the absence of any mutation (29 cases) and those with a mutant gene in which the mRNA was not overexpressed (four cases). Moreover, the mutant p53 tumours exhibited an increase of p53 mRNA expression, which was significantly higher in tumours expressing the mutated allele alone than in tumours expressing both wild- and mutated-type alleles. These data (1) show that p53 mRNA overexpression is a frequent event in colorectal tumours and is not predictive of the status of the gene, i.e. whether or not a mutation is present; (2) provide further evidence that p53 protein overexpression does not only result from an increase in the half-life of mutated p53 and suggest that inactivation of the p53 function in colorectal cancers involves at least two distinct mechanisms, including p53 overexpression and/or mutation; and (3) suggest that p53 mRNA overexpression is an early event, since it is not correlated with Dukes’ stage.

Keywords: colorectal cancer; p53; mRNA overexpression; inactivation; mutation

p53 gene mutation with or without allelic loss has been considered to be the commonest alteration found in sporadic non-familial cancer (Vogelstein, 1990). This alteration occurs in a wide variety of tumour types, including solid and haematopoietic tumours (Hollstein et al, 1991). Furthermore, humans who inherit germline p53 mutations are prone to the development of cancer (Marklin et al, 1990; Srivastava et al, 1990).

The wild-type p53 appears to function as a cell-growth suppressor and may play an important role in genomic stability and DNA repair (Zambetti et al, 1993). Wild-type p53 transactivates the WAF1/p21 gene (El-Deiry et al, 1993; Harper et al, 1993), whose protein product prevents exit from the G1-phase by inhibiting cyclin/Cdk complexes and, in parallel, blocks replicative DNA synthesis by binding to proliferating cell nuclear antigen (PCNA) (Dulic et al, 1994; Waga et al, 1994). When DNA is altered, the cell cycle is blocked in G1-phase, allowing time for repair (Kastan et al, 1991, 1992). If the DNA damage is too great, the cell is engaged in the apoptosis pathway and is subsequently deleted from the tissue (Clarke et al, 1993; Lowe et al, 1993). p53, therefore, acts as ‘the guardian of the genome’ (Lane, 1992).

However, the molecular mechanism of wild-type p53 action is not fully understood. The p53 protein has been shown to activate several genes by interacting with specific DNA sequences, such as the promoter of muscle specific creatine kinase (Weintraub et al, 1991) and the third intron of the GADD45 gene, which is induced after gamma irradiation (Papathanasiou et al, 1991). A consensus p53 DNA-binding site has been derived from these studies (Kern et al, 1991; El-Deiry et al, 1992; Funke et al, 1992). Wild-type p53 has also been implicated in the transcriptional inhibition of several genes involved in cell growth: c-myc (Moberg et al, 1992), retinoblastoma susceptibility gene (Shioi et al, 1992), multidrug resistance gene-1 (Chin et al, 1992), proliferating cell nuclear antigen (Subler et al, 1992), c-fos, interleukin6, c-jun and hsc70, a member of the heat shock family (Ginsberg et al, 1991; Santhanam et al, 1991; Agoff et al, 1993). This inhibitory effect might be caused by a direct action on the TATA box machinery (Liu et al, 1993; That et al, 1995). p53 was also found to be able to stimulate its own transcription, but was unable to bind directly the specific sequences, which have been identified in the p53 promoter (Deffie et al, 1993).

In colorectal cancer, p53 mutations and allelic losses on 17p are genomic alterations that occur as late events in tumour progression (Baker et al, 1990; Fearon et al, 1990). p53 gene mutations have been associated with poor prognosis in human breast carcinomas (Thorlacius et al, 1993) and in non-small-cell lung cancer (Horio et al, 1994). In contrast to previous findings (Kern et al, 1989), when tumours were classified according to their histological stage, a multivariate Cox model analysis showed that p53 mutation, rather than 17p allelic losses, was the only independent prognostic factor (Hamelin et al, 1994). Immunohistochemistry (IHC) studies have indicated that mutated p53 is overexpressed in premalignant head and neck lesions (Shin et al, 1994), oesophageal squamous cell carcinomas (Wagata et al, 1993), ovarian cancer (Kupryjanczyk et al, 1993), breast cancer (Faillie et al, 1994) and hepatocellular carcinoma (Wolkman et al, 1994). In colorectal cancer, overexpression of p53 protein has been correlated with poor short-term prognosis (Yamaguchi et al, 1992;...
Most authors attributed p53 protein overexpression to an increase in its half-life owing to the conformational changes induced by mutations (Zambetti et al., 1993), but this mechanism is not relevant in the case of wild-type overexpression. In order to define more clearly p53 gene expression, we analysed in this study p53 mRNA expression in 109 colorectal carcinomas by the use of a semi-quantitative reverse transcription—polymerase chain reaction (RT–PCR) technique and examined its relationship to p53 mutation.

**MATERIALS AND METHODS**

**Materials**

This study was performed on a series of 109 specimens obtained from patients undergoing resection of colorectal adenocarcinoma between January 1992 and November 1994. Tumour and adjacent normal mucosa samples from the same patient were immediately collected for analysis in the pathology department, frozen in liquid nitrogen and stored at −80°C. Before DNA and RNA isolation, the quality of tumours and normal samples in each case was evaluated by examination of cryostat sections stained with haemalum.

Patients with familial adenomatosis or hereditary non-polyposis colorectal cancer with a highly penetrant genetic predisposition to colorectal cancer were excluded from this study. This series included 63 men and 46 women (mean age 64.5 ± 14.5 years). Twenty-six tumours out of the 109 studied were right-sided (caecum, ascending and transverse colon), 49 were left-sided (descending colon and sigmoid) and 34 were located in the rectum.

According to the modified Dukes’ staging system, 15 were Dukes’ stage A (13.8%), 36 were Dukes’ stage B (33%), 34 were Dukes’ stage C (31.2%) and 24 were Dukes’ stage D (22%).

**Clinical stage and tumour site**

Of the 109 tumours studied, 60 (55%) were associated with nodal involvement or distant metastasis. A significant association was observed between tumour staging and location (P=0.01). Tumours of the rectum exhibited a higher proportion of advanced Dukes’ stage (C+D = 71%) than those located in the distal (45%) or proximal colon (46%).

**Preparation of DNA and RNA**

DNA was extracted from each tumour and normal mucosa sample by treatment with sodium dodecyl sulphate (SDS), proteinase K and phenol–chloroform according to Fritsch et al (1989). RNA was isolated, using 50 mg of frozen powdered sample, with RNAzol (Biorad, France), according to the manufacturer’s recommendations. RNA and DNA concentrations were quantified by spectrophotometry and determined to be intact by migration on 1% agarose gels and staining with ethidium bromide.

**Reverse transcription**

This was carried out in a 20-μl reaction volume containing 500 μM dNTP, 10 mM DTT, 0.5 U μl−1 RNASasin (Promega Corporation); 5 μM random hexamers and 10 μg μl−1 reverse transcriptase (Gibco BRL, Bethesda, MD, USA). RNA extracts were heated for 5 min at 70°C and cooled on ice before being added (1 μg) to the reaction mixture.
Table 1 Primer sequences for p53 analysis

| Name               | Site   | Sequences                                      |
|--------------------|--------|------------------------------------------------|
| p53 (RT–PCR)       |        | Nucleotide numbers                             |
| Set 1              | Primer 1 | Sense       | 168–186          | 5′-ACA CGC TTC CCT GGA TTG G-3′ |
|                    | Primer 2 | Antisense   | 616–634          | 5′-GTT CTG GCC CAG TTC GCA A-3′ |
|                    | Primer 3 | Sense       | 548–571          | 5′-GTC TTC TTG CAT TCT GGG ACA GCC-3′ |
|                    | Primer 4 | Antisense   | 964–985          | 5′-CAG TGT GAT GGT GGT GAT G-3′ |
| Set 3              | Primer 5† | Sense       | 887–907          | 5′-GTT GCC TCT GAC TGT ACC CGG-3′ |
|                    | Primer 6 | Antisense   | 1223–1246        | 5′-CAG CTC TCG GAA CAT TTC GAA GCG-3′ |
|                    | Primer 7 | Sense       | 1087–1104        | 5′-GAA AGG GGA GCC TCA CCA-3′ |
|                    | Primer 8 | Antisense   | 1411–1427        | 5′-GCT GTC AGT GGG GAA CAA-3′ |
| p53 (DNA)          |        | Intron number                                   |
| Set 5              | Primer 9 | Sense       | Intron 4         | 5′-TTT AAC TCT GTG TCC TTC CT-3′ |
|                    | Primer 10 | Antisense | Intron 6        | 5′-TTA ACC CCT CTC CCT CCC AGA AGA-3′ |
| Set 6              | Primer 11 | Sense       | Intron 9        | 5′-GTC TCC CCA AGG CGG ACT GG-3′ |
|                    | Primer 12 | Antisense   | Intron 7        | 5′-GA TGT AGG AGG GGA GTG T-3′ |
| Set 7              | Primer 13 | Sense       | Intron 7        | 5′-GTT CCT ACT GCC TCT TGC TT-3′ |
|                    | Primer 14 | Antisense   | Intron 9        | 5′-GCC AAG ACT TAG TAC CTG AA-3′ |
| β-Actin (RT-PCR)   |        | Exon number                                     |
| Set 8              | Primer 15† | Sense       | Exon 3          | 5′-CGT GGA TGC CAC AGG ACT CC-3′ |
|                    | Primer 16 | Antisense   | Exon 4          | 5′-ATC ATG TTT GAG ACC TCC AA-3′ |

†Fluorescent primer when required.

PCR amplification

All primers and probes, used to optimize RT–PCR procedures, were synthesized using a 391 DNA synthesizer (Applied Biosystem, Foster City, CA, USA), followed by high-performance liquid chromatography. The fluorescent-labelled primers, used to measure mRNA levels and to sequence the PCR products, were purchased from Genset Corporation (France). The sequences of the various primers are given in Table 1. Reverse-transcribed cDNA (5 µl, 0.25 µg) was subjected to PCR amplification with two sets of p53 and β-actin (internal standard) primers. PCR reaction was performed in 25 µl of reaction medium containing 10 mM Tris-HCL, pH 8.3, 50 mM magnesium chloride, 0.001% gelatin, 0.05 U Taq DNA polymerase (Beckman, USA) and 0.5 µl of each primer. Each cycle consisted of 15 s denaturing at 94°C, 15 s annealing at 58°C and 30 s extension at 72°C. Negative controls were subjected to reverse transcriptase. Cycling was performed in a Perkin-Elmer 9600 thermocycler (Cetus).

Quantitation of PCR products

The optimal number of cycles of amplification to allow quantitation of p53 and β-actin gene PCR products was determined using primers 5 and 6 for p53 and 15 and 16 for β-actin. The primers 5 and 15 were chosen to produce a fragment within the linear range of the assay. The optimal number of cycles for each reaction was selected by amplifying a series of samples, each set of four (Table 1) in order to amplify overlapping fragments of the total cDNA. After 35 cycles of PCR, the primers and oligonucleotides were recovered from the reaction mixture using a centrifuge 100 microcentrifuge (Amicon, Beverly, MA, USA). Specific p53 amplification products were identified by electrophoresis on 2% agarose gels. The PCR products (100 ng) were subjected to sequencing reactions using the Prism reaction dye terminators kit according to the protocol supplied by the manufacturer (Applied Biosystem). Taq sequencing reactions were conducted in a Perkin-Elmer thermocycler 9600 as follows: 30 s at 96°C, 15 s at 50°C and 4 min at 60°C for 25 cycles. Extended fragments were purified from non-incorporated nucleotides and primers by using spin columns (Boehringer). The reaction mixtures were then dried, resuspended in 5 µl of dionized formamide: 50 mM EDTA, pH 8.0, heated for 2 min at 90°C, transferred to ice and loaded immediately onto a 6% denaturing polyacrylamide gel. Gels were run for 12 h at 30 W constant power on an ABI model 373 A automated DNA sequencer.

p53 genomic DNA sequencing

Samples without p53 mRNA overexpression were sequenced after amplification of genomic DNA. In these cases, the primers used for DNA amplification are summarized in Table 1. A total of three different polymerase chain reactions were used to screen the entire
Table 2 p53 mRNA levels among the 69 tumours showing overexpression

| Number of tumours | p53 mRNA level* |
|-------------------|-----------------|
| Without p53 mutation | 29              | 0.56 ± 0.32 |
| With p53 mutation   | 40              | 0.56 ± 0.31 |
| Presence of wild transcript | 21          | 0.56 ± 0.31 |
| Absence of wild transcript | 19            | 0.86 ± 0.47 |

*Ratio between the amount of p53 to β-actin RT–PCR products at 26 cycles, expressed in arbitrary units as described in Materials and methods, values are the means ± s.d. F2,67 (one-way analysis of variance) = 4.94; P = 0.01.

Table 3 Relationship between p53 gene alterations and clinicopathological features

| Mut− n (%) | Mut+ n (%) | Absence of expression of wild allele among mut + n (%) |
|------------|------------|-------------------------------------------------------|
| Dukes' stage |            |                                                      |
| A          | 10 (15)    | 5 (11)                                                |
| B          | 24 (37)    | 12 (27)                                               |
| C          | 20 (31)    | 14 (32)                                               |
| D          | 11 (17)    | 13 (30)                                               |
| Total      | 65 (100)   | 44 (100)                                              |
| Intestinal site |        |                                                      |
| Proximal colon | 18 (26)  | 8 (18)                                                |
| Distal colon | 27 (44)   | 22 (50)                                               |
| Rectum     | 20 (31)    | 14 (32)                                               |
| Total      | 65 (100)   | 44 (100)                                              |

mut +, with p53 mutation, mut −, without p53 mutation.

coding sequence contained within exons 5–8 and their corresponding splice junctions.

Cell line

The human colorectal cancer cell line, HT29, with a known p53 point mutation at codon number 273, was used as a positive control for sequencing and RT–PCR analysis of p53 mRNA expression.

Statistical analysis

Results are expressed as the mean ± standard deviation. The mean p53 mRNA for each of the subgroups of tumours was compared by one-way analysis of variance. The chi-square (χ²) test of significance was used to analyse the frequency.

RESULTS

p53 mRNA levels

The p53 mRNA content from colorectal carcinoma and adjacent normal mucosa samples was examined by a semi-quantitative RT–PCR procedure, and the results were normalized against the β-actin mRNA content observed in the same samples. In adjacent mucosa, the relative level of p53 mRNA remained very low, i.e. 0.08 ± 0.06 AU (n = 40) and was always lower than 0.20 AU (Figure 2). This value was therefore chosen as the upper limit of the normal p53 mRNA level. In contrast to normal tissue, the p53 mRNA level observed in tumour tissue was distributed over a wide range of values with an upper limit of 1.55 AU. Forty tumours out of 109 (37%) exhibited mRNA values in the normal range, i.e. lower than 0.20 AU. p53 mRNA overexpression, i.e. higher than 0.20 AU, was observed in 69 out of 109 tumour samples (63%) with a mean value of 0.64 AU (s.d. 0.38).

Comparison between p53 mRNA expression and gene mutation

In the 69 tumours that overexpressed p53 mRNA, an absence of mutation was observed in the coding sequence of p53 mRNA in 29 cases (42%). In 21 out of 40 samples in which mutated mRNA p53 was observed, we also demonstrated coexpression of the wild-type allele. This is visible on the sequence spectrum by the presence of two bases at the same position (data not shown). However, it is impossible to sequence p53 cDNA from normal tissue by RT–PCR. The level of p53 mRNA was significantly higher in tumours expressing the mutated-type allele alone (0.86 ± 0.47 AU) than in tumours also expressing the wild-type allele (0.56 ± 0.31 AU) or in tumours with non-mutated p53 (0.56 ± 0.32 AU) (Table 2). In four of the 40 tumours without mRNA overexpression, we found p53 gene mutation associated with wild-type allele expression.

Relationship between p53 gene alterations and clinicopathological features

The frequency of mutation was not statistically different according to the Dukes’ stage (P=0.40) or the location (P=0.5) (Table 3).
### Table 4: List of individual tumours showing p53 mutations in colorectal cancer

| Tumour Site | Expression of wild allele | Codon number | Base change | Amino acid change | p53 mRNA level |
|-------------|---------------------------|--------------|-------------|------------------|----------------|
|             |                           |              |             |                  | Normal mucosa† | Tumours‡ |
| 121         | R                         | C            | +           | 2–3–4            | 1 → 43         | 1 → 129     | Another polypeptide sequence in N terminus | 0.07 | 0.24 |
| 44          | PC                        | C            | +           | 4                | 33 → 48        | 97 → 144    | Another polypeptide sequence between aa 33 and 48 | 0.09 | 0.48 |
| 37          | DC                        | D            | +           | 5                | 143            | GTG → GCG   | V → A       | ND   | 0.48 |
| 140         | DC                        | A            | –           | 5                | 143            | GTG → GAG   | V → E       | 0.17 | 1.54 |
| 12          | R                         | C            | –           | 5                | 161            | GCC → ACC   | A → T       | 0.01 | 1.54 |
| 120         | R                         | D            | +           | 5                | 167            | CAG → CGG   | E → R       | 0.05 | 0.40 |
| 46          | PC                        | B            | +           | 5                | 168            | CAC → CGC   | H → R       | 0.11 | 0.33 |
| 160         | PC                        | B            | –           | 5                | 175            | CGC → CAG   | R → H       | 0.13 | 1.32 |
| 173         | DC                        | B            | –           | 5                | 175            | CGC → GGC   | R → G       | 0.04 | 0.62 |
| 194         | DC                        | C            | +           | 5                | 177–179        | Deletion of deletion of PHH | ND   | 0.62 |
| 122         | R                         | B            | +           | 5                | 179            | CAT → TAT   | H → Y       | 0.05 | 0.45 |
| 11          | DC                        | D            | +           | 6                | 194            | CTT → CCT   | L → P       | ND   | 0.67 |
| 148         | DC                        | B            | +           | 6                | 196            | CGA → TGA   | R → Stop    | 0.06 | 0.98 |
| 193         | DC                        | B            | +           | 6                | 196            | CGA → TGA   | R → Stop    | ND   | 0.35 |
| 108         | DC                        | D            | +           | 6                | 205            | TAT → GAT   | Y → D       | ND   | 0.25 |
| 125         | R                         | B            | –           | 6                | 220            | TAT → TGT   | Y → C       | ND   | 1.22 |
| 104         | R                         | B            | +           | 7                | 245            | GCC → GAC   | G → D       | ND   | 0.94 |
| 157         | R                         | A            | +           | 7                | 245            | GCC → TGC   | G → C       | ND   | 0.77 |
| 13          | PC                        | B            | –           | 7                | 248            | CGG → CAG   | R → Q       | 0.01 | 0.36 |
| 72          | R                         | C            | –           | 7                | 248            | CGG → TGG   | R → W       | ND   | 0.27 |
| 88          | R                         | C            | –           | 7                | 248            | CGG → CAG   | R → Q       | ND   | 0.46 |
| 96          | R                         | B            | +           | 7                | 248            | CGG → CAG   | R → Q       | 0.12 | 0.30 |
| 150         | DC                        | A            | –           | 7                | 248            | CGG → TGG   | R → W       | 0.18 | 1.49 |
| 138         | DC                        | A            | +           | 7                | 248            | CGG → CAG   | R → Q       | 0.10 | 0.10 |
| 97          | DC                        | D            | +           | 7                | 255            | ATC → TTC   | I → F       | 0.00 | 0.10 |
| 147         | PC                        | C            | +           | 7                | 257            | Deletion    | Frameshift changing aa sequence (stop codon 344) | 0.06 | 1.42 |
| 199         | R                         | C            | +           | 7                | 259            | CTG → TG    | Insertion   | 0.21 |
| 155         | R                         | C            | +           | 8                | 263            | GAC → GTAC  | Insertion of 5b: AA [GGTAA] T | ND   | 0.70 |
| 156         | R                         | C            | –           | 8                | 266            | GGA → GTA   | G → V       | ND   | 1.13 |
| 71          | DC                        | D            | –           | 8                | 272            | GTG → ATG   | V → M       | ND   | 0.56 |
| 41          | DC                        | C            | –           | 8                | 272            | GTG → TTG   | V → L       | ND   | 0.31 |
| 35          | DC                        | D            | –           | 8                | 273            | CGT → TGT   | R → C       | ND   | 0.31 |
| 36          | DC                        | D            | –           | 8                | 273            | CGT → TGT   | R → C       | ND   | 0.97 |
| 43          | DC                        | B            | +           | 8                | 273            | CGT → TGT   | R → C       | 0.08 | 0.25 |
| 77          | PC                        | A            | +           | 8                | 273            | CGT → CAT   | R → H       | ND   | 0.77 |
| 143         | DC                        | D            | +           | 8                | 273            | CGT → CAT   | R → H       | ND   | 0.37 |
| 151         | DC                        | D            | –           | 8                | 273            | CGT → TGT   | R → C       | 0.18 | 1.15 |
| 62          | DC                        | D            | –           | 8                | 273            | CGT → TGT   | R → C       | ND   | 0.70 |
| 129         | R                         | B            | –           | 8                | 273            | CGT → TGT   | R → C       | ND   | 0.69 |
| 32          | DC                        | C            | –           | 8                | 274            | GTT → CTT   | V → L       | ND   | 0.38 |
| 164         | PC                        | D            | +           | 8                | 280            | Deletion    | Frameshift changing aa sequence (stop codon 344) | 0.02 | 0.10 |
| 9           | DC                        | C            | +           | 8                | 301            | Deletion    | Frameshift changing aa sequence (stop codon 344) | 0.10 | 0.10 |
| 186         | DC                        | C            | +           | 9                | 306            | CCA → CA    | CGA → TGA   | R → stop   | ND   | 0.78 |

†PC, proximal colon; DC, distal colon; R, rectum. ‡Expression of wild allele: +, wild and mutated-type alleles are expressed together; –, mutated-type allele is expressed alone. §Ratio between the amount of p53 to β-actin RT–PCR products at 26 cycles. ND, not determined. aa, amino acid.

The proportion of mutated tumours not expressing the wild-type allele was roughly the same at each Dukes’ stage. Conversely, this proportion varied according to site: 25% of the tumours did not express the wild-type allele in the proximal colon vs 50% and 43% in distal colon and rectum respectively, but this difference was not statistically significant (P=0.47) (Table 3).

**p53 gene mutation**

Table 4 provides the exact DNA alteration observed in 44 tumours and its location on the gene. The histological grade and p53 mRNA expression in these tumours and their adjacent normal tissues are also shown. Among the 36 substitutions of a single base pair, 28 (77.8%) were G:C ←→ A:T transitions (21 occurring at CpG

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Three were number273 (six CGT with (CGC reading Insertion at acids. pairs nucleotide a large rearrangement (tumour121) ments tumours, the p53 mRNA level was either 0.46 ± 0.22 AU when the wild-type allele was expressed or 0.87 ± 0.35 AU when not expressed. These results are identical to those obtained with respect to all mutated tumours expressing the wild-type allele (or not) (Table 2). Six of the missense mutations affected codon number 248 (four CGG → CAG, R → Q and two CGG → TGG, R → W), two of the missense mutations affected codon number 175 (CGC → CAC, R → H and CGC → GGC, R → G) and the codon number 245 (GGC → GGA, G → D and GGC → TGC, G → C). We also found two mutations at codon number 272 (GTG → ATG, V → M and GTG → TTT, V → L).

Eight mutations created major rearrangements of the mRNA reading frame (Table 4), four of them were insertions or deletions of one basepair. Deletions of cytosine 983 at codon 257 (tumour 147), adenine 1052 at codon 280 (tumour 164) and cytosine 1115 at codon 301 (tumour 9) gave three putative p53 proteins composed of 343 amino acids differing in their C-terminus. Insertion of a thymine after the guanine 989 at codon 259 (tumour 199) gave a putative shorter polypeptide composed of 262 amino acids. In four cases, more marked rearrangements were observed: a large rearrangement (tumour 121) in the N-terminus (from nucleotide 1 to 129), a non-homologous recombination of 43 basepairs after nucleotide 97 (tumour 44), a deletion of nine bases (amino acids 177 to 179, PIH) (tumour 194) and an insertion of five bases after nucleotide 1002 at codon 263 (tumour 155) giving a p53 protein composed of 343 amino acids. These eight rearrangements all concerned advanced Dukes’ stage (seven were Dukes’ stage C and one was Dukes’ stage D).

DISCUSSION

In this series of 109 primary colorectal carcinomas, we observed p53 mRNA overexpression in 63% of the cases. This overexpression occurred without any correlation with the stage or site of the disease. The mean level of p53 mRNA expression is five- to sixfold higher in these tumours than in adjacent normal mucosa. Some tumours showed high p53 mRNA levels, while others showed a slight increase. In the latter case, p53 mRNA may either be weakly expressed in all tumour cells or highly expressed in a few tumour cells owing to tumour heterogeneity. Indeed, we described that the tumour cell staining varied in extent and intensity when p53 protein is detected by IHC (Zeng et al., 1994). Our results clearly demonstrate that p53 regulation may occur at a pretranscriptional step, involving either an increase in p53 gene expression and/or stabilization of its mRNA. Our findings confirm earlier reports, which showed an elevated level of p53 transcripts in 28 (Gothe et al., 1991) and 25 (Lothe et al., 1992) cases, 70% and 66% of tested tumours overexpressed p53 respectively. In these studies, p53 mRNA quantification was performed by Northern blot analysis.

When DNA sequencing was performed, 44 tumours (40%) exhibited p53 mutation. Our data on the prevalence and spectra of p53 mutations in colorectal cancer agree with those obtained from 960 cases compiled by Greenblatt et al (1994). However, the most striking difference between the two studies concerns the number of non-missense type mutations, which accounted for 18% of mutations in our series vs 8% in Greenblatt’s study. Among the 36 substitutions of a single basepair, 28 of them occurred at CpG dinucleotides; the p53 coding region contains 39 CpG dinucleotides, which are potential sites for the methylation of cytosine. Recently, Tornaletti et al (1995) found that the p53 sequences along exons 5–8 were completely methylated at every CpG site, whatever the tissue. Methylation of CpG dinucleotides is thought to be the cause of the genetic changes occurring through spontaneous deamination of 5-methylcytosine (Rideout et al., 1990) and accounted for the majority of endogenous mutations in vertebrates (Sved et al., 1990). In our study, mutated and wild-type alleles were expressed simultaneously in 25 tumours, and the loss of wild-type allele expression was rarely observed in the proximal colon (25%) vs 50% and 43% in the distal colon and the rectum respectively. The rectum is a site in which a high incidence of 17p allelic loss has been observed. These differences were not statistically significant, and the hypothesis that genetic mechanisms leading to cancer differ in the proximal and distal colon needs to be verified (Delattre et al, 1989).

In 40 of the 44 mutated tumours, p53 mRNA levels were significantly higher in tumours expressing the mutated allele alone than in tumours expressing both wild- and mutated-type alleles. It has been described that p53 stimulates its own transcription (Deffie et al., 1993), and that some p53 mutant forms presented wild-type transactivation activity (Levine et al., 1991). In addition, p53 can induce transcription from an internal promoter located within the mdm2 gene (Juven et al., 1993), whose product, MDM2 oncprotein, has been identified as a negative regulator of the p53 gene. Further, MDM2, by binding to p53, inhibits its ability to activate transcription and may, therefore, be part of a negative feedback loop serving to terminate signals involving the transient activation of wild-type p53 (Oliner et al., 1992; 1993). So, when a p53 gene mutation occurs, the absence of a negative feedback might be responsible for p53 mRNA overexpression. The suppressor function of wild-type p53 may also be compromised in cells containing a mutant allele of p53, since the formation of wild-type – mutant p53 inactive complexes occurs (Milner et al., 1991). Thus, mutant forms abrogate the ability of wild-type p53 to transactivate appropriate target genes in vitro and in vivo (Farmer et al., 1992; Kern et al., 1992), and the relative quantity of mutated to wild-type p53 mRNA could determine the transformed phenotype, and the result could be partial or complete loss of wild-type function. p53 mRNA overexpression can account for an elevated content of p53 protein in the absence of p53 gene mutation. An increase in wild-type p53 has been observed in colorectal adenomas and has been suggested to constitute an early event in the process of adenoma formation and carcinogenesis (Tominaga et al., 1993; Boccuzzi, 1995); our present data showing no correlation between p53 mRNA and tumour stage agree with these results. Other results suggest that, in colorectal cancer, IHC detection of p53 protein does not always indicate the existence of an underlying p53 gene mutation (Dix et al., 1994). This abnormal expression of wild-type p53 protein was also found in normal cells of a patient from a family with a history of cancer (Barnes et al., 1992) and, more recently, a new case of Li–Fraumeni was reported in which no mutation in the coding sequence of the p53 gene was detected (Birch et al., 1994). In all cases, stabilization of the p53 protein depends on factors other than p53 gene mutation, such as (1) binding to other molecules of cellular (mdm2 gene product) or viral origin blocking p53 in an inactive conformation; and/or (2) its
sequestration in a cellular compartment in which it cannot exert its functions. Recent data have demonstrated wild-type p53 protein accumulation in the cytoplasm of astrocytomas (Lang et al, 1994), melanomas (Castresana et al, 1993) and testis cancer (Peng et al, 1993), and that nuclear exclusion of p53 might also be one way of inactivating p53 in breast cancer (Moll et al, 1992). Nuclear exclusion of wild-type p53 is suggested in a study showing its cytoplasmic accumulation in colorectal cancers (Bosari et al, 1995) with a higher prevalence in advanced tumours (Sun et al, 1992). Another study has shown an increase in p53 protein according to the stage of carcinomas from the rectum (Starzynska et al, 1992). In our study, the level of p53 mRNA did not correlate with either the Dukes’ stage or the tumour site, in accordance with previous data showing no correlation in colorectal cancers between p53 overexpression detected by IHC and clinicopathological data (Yamaguchi et al, 1992, ; Bosari et al, 1994). This lack of correlation between p53 overexpression (either mRNA or protein) and tumour stage suggests that it is an early event in these tumours, perhaps beginning with adeno ma formation (Tominaga et al, 1993).

p53 mRNA overexpression with or without p53 mutation suggests two distinct mechanisms of inactivation leading to the development of cancer, and that the p53 status might have important implications for cancer therapy. The tumour-suppressing function of p53 preserves genome integrity and the p53 protein is required for apoptosis in response to radiation-induced DNA damage, a mechanism serving to eliminate potentially oncogenic cells (Lee et al, 1993). The relation between p53 mutations and the therapeutic response has been verified in vivo in athymic nude mice injected with embryonic transformed fibroblasts differing in their p53 status. Tumours expressing the wild-type p53 gene contained a high proportion of apoptotic cells and typically regressed after gamma radiation or doxorubicin treatment. In contrast, wild-type p53-deficient tumours continued to grow and contained few apoptotic cells (Lowe et al, 1994). These data show that much benefit could be gained from identifying cancers without p53 mutations, which are likely to respond more favourably to drug therapy than those with mutated p53. Conversely, patients whose tumours harbour p53 mutations might be spared from the toxicity associated with chemotherapy agents and would be good candidates for novel therapeutic approaches. Recently, Fujiwara et al (1994) reported the in vivo retroviral transduction of wild-type p53 in human lung cancer cells in an orthotopic nude mouse model with endogenous mutated p53. They demonstrated that cancer cell growth can be eliminated or greatly reduced by this in vivo gene therapy beginning 3 days after tumour cell inoculation. So, ‘cancer therapy meets p53 status’ (Kinzler et al, 1994).

Analysis of the level of p53 mRNA in colorectal cancer by quantitative RT–PCR provides a rapid and sensitive method for discriminating between tumours overexpressing p53 mRNA with or without p53 gene mutation. This should be useful for future anti-tumour research and for the design of therapeutic agents specific to the inactivation process. The observation period in our study was too short to clarify the relationship between p53 mRNA overexpression and clinical prognosis of patients with a colorectal carcinoma; we are following these patients.

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**ABBREVIATIONS**

Abbreviations IHC, immunohistochemistry; RT, reverse transcription; PCR, polymerase chain reaction; dNPTs, deoxynucleotide triphosphates; DTT, dithiothreitol; AU, arbitrary units; SD, standard deviation.
p53 mRNA overexpression in colorectal cancer

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