Comparison of p53 gene mutation and protein overexpression in colorectal carcinomas

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Summary

Immunocytochemistry (ICC) has been used routinely to stain for p53 overexpression in a range of human tumours. The underlying assumption has been that positive staining indicates a mutation in the p53 coding sequence. Recently, however, discordancy has been observed and the accuracy of ICC as a marker of p53 gene mutation has been questioned. In this study of 109 colorectal adenocarcinomas, we compared ICC staining with p53 gene mutations detected by single-strand conformation polymorphism (SSCP) analysis. Concordancy between the two techniques was found in 69% of tumours. ICC-positive SSCP-negative cases accounted for 20% of tumours and ICC-negative SSCP-positive cases for the remaining 11%. These results caution against the assumption that p53 protein overexpression is always associated with a gene mutation. Epigenetic phenomena may account for a significant proportion of ICC-positive tumours.

p53 was originally detected as a 53 kDa nuclear phosphoprotein co-immunoprecipitating with SV40 large T antigen (Lane & Crawford, 1979; Linzer & Levine, 1979). It has since been shown to have a sequence-specific DNA-binding capability which regulates entry into the S phase of the cell cycle at a transcriptional level (Kern et al., 1991). Both cytogenetic (Muleris et al., 1985) and restriction fragment length polymorphism (RFLP) studies (Vogelstein et al., 1989) have shown that loss of the p53 gene is a common event in human cancers. In addition to deletion of the gene, more recent studies have shown that mutation of the p53 coding sequence is also a common occurrence in a wide range of human tumours (Nigro et al., 1989; Hollstein et al., 1991). Alterations of the p53 gene are now known to be among the most frequent genetic changes occurring in human malignancies (Hollstein et al., 1991; Caron de Fromentel & Soussi, 1992). Mutation or deletion of this gene effectively removes the regulatory influence of wild-type p53 protein on cell proliferation and may confer a growth advantage on a neoplastic population. This has led researchers to hypothesise that alterations in the p53 gene play a fundamental role in tumour development and progression.

Mutations occur most often in four of the five phylogenetically conserved regions of the p53 gene (Hollstein et al., 1991; Caron de Fromentel & Soussi, 1992). When mutations occur within these regions they generally result in an altered or 'mutant' p53 protein product. Although wild-type p53 protein, because of its short half-life, is normally present at very low levels in the cell, changes in conformation brought about by mutation apparently stabilise the mutant protein (Levine et al., 1991). The resultant accumulation of mutant protein may be detected by immunocytochemistry (ICC). Overexpression of the p53 protein detected by ICC has now been used in a large number of studies on various tumour types as a marker of p53 gene mutation (Bártok et al., 1991; Porter et al., 1992).

Although some studies have shown a good correlation between ICC staining and the presence of mutations within the p53 gene as detected by molecular analysis (Rodrigues et al., 1990; Davidoff et al., 1991; Marks et al., 1991; Kohler et al., 1992; Maestro et al., 1992; Somers et al., 1992; Thor et al., 1992), others have revealed varying degrees of discordancy (Barton et al., 1991; Allred et al., 1993; Kohler et al., 1993). Indeed, the use of ICC as an indicator of p53 gene mutation has been questioned (Wyndford-Thomas, 1992). We undertook the present study to correlate ICC staining with p53 gene mutation in a large series of human colorectal cancers. Mutations were detected by the single-strand conformation polymorphism (SSCP) technique (Orita et al., 1989) and p53 overexpression by ICC using the monoclonal antibody DO-7.

Materials and methods

Tumour specimens

A series of 109 surgically resected tumours were provided by the general surgeons at Sir Charles Gairdner Hospital. These consisted of 65 Dukes' stage B and 44 Dukes' stage C adenocarcinomas. Specimens were grossly examined and portions of tumour and adjacent normal mucosa were excised, snap frozen immediately in liquid nitrogen and stored at −80°C until required for DNA extraction. The remaining tumour was fixed in neutral buffered formalin and embedded in paraffin for histopathology and ICC. The tissue specimen for SSCP analysis was taken from the centre of the tumour mass, while the tissue section for ICC was cut no more than 1–2 mm away from this site.

Immunocytochemistry

Paraffin sections were cut at 5 μm, mounted on slides, dewaxed for 10 min in xylene, passed through alcohol and washed in water. They were then rinsed with 0.2 M Tris-buffered saline pH 7.6 (TBS) and endogenous peroxidase removed by soaking in 3% hydrogen peroxide for 5 min. Sections were incubated with a blocking solution of 1:5 normal swine serum and TBS for 20 min before application of the primary antibody, monoclonal mouse anti-human p53 protein (Dako p53 protein DO-7) at 1:20 dilution. The slides were incubated at room temperature overnight then washed twice with TBS before a biotinylated second antibody, anti-mouse IgG antibody (1:200) (Histocruz Lab, Melbourne Australia), was applied. Streptavidin–horseradish peroxidase conjugate (Histocruz Lab) was added (1:200) to all slides and incubated for 1 h before washing twice with TBS. Chromogen solution containing 6 mg of dianinobenzoate in 10 ml of TBS 30% hydrogen peroxide was then added and colour allowed to develop for 8 min before being stopped by washing in TBS. Preparations in which the primary antibody was omitted were used as negative controls. All slides were...
evaluated by two observers (P.R. and B.D.) and positive reactions required the presence of brown reaction product within neoplastic cells. Reactions in adjoining normal or adenomatous mucosa were also assessed. Tumours were classified as negative (no cells staining or positive) or equivocal (any number of cells staining). If positive, a subjective assessment of the percentage of tumour cells staining was made.

Preparation of genomic DNA

Genomic DNA was prepared from frozen tissue by grinding the specimen to a powder in liquid nitrogen using a mortar and pestle. The powder was then dissolved in an extraction buffer containing 0.5% SDS, 10 mM Tris–HCl pH 8, 100 mM EDTA, 20 μg ml⁻¹ RNase A and 100 μg ml⁻¹ proteinase K. Samples were digested overnight at 50°C on a rotating wheel apparatus and then extracted with phenol–chloroform–isoamyl alcohol (25:24:1). DNA was precipitated with one-quarter volume of 7.5 M ammonium acetate and three volumes of 100% ice-cold ethanol, washed with 70% ethanol, air dried and redissolved in 10 mM Tris–0.1 mM EDTA to a final concentration of 100 ng μl⁻¹.

Amplification by polymerase chain reaction

Exons 5–8 of the p53 gene were amplified separately using the following oligonucleotides (Biotech International) as primers:

Exon 5: 5'-CTCTTCTGCAGTACTCTCCCT-3', 5'-AGCTGCTCACTACGCTATC-3'
Exon 6: 5'-GATGTCTCTGAGTCTGGC-3', 5'-GAAAACAGAGACCTGCGG-3'
Exon 7: 5'-TGTCCTCCTAAGTGGCTCT-3', 5'-GCTTCCGTACAGTGTTCTC-3'
Exon 8: 5'-TCCGTAGTAGGTGTAATGTCA-3', 5'-GCTTCTGTTACCTCGTTAGT-3'

The standard reaction mixture (25 μl) contained 0.4 μM of each primer, 3 mM magnesium chloride, 67 mM Tris–HCl pH 8.8, 16.6 mM ammonium sulphate, 0.2 mM dNTPs, 0.2 mg ml⁻¹ gelatin, 0.45% Triton X-100, 0.5 μCi of [α-³²P]dCTP and 0.2 units of Taq polymerase. Thermal cycling began with an initial denaturation of 10 min, during which time 100 ng of template DNA was added to the reaction. At least 5 min of denaturation time remained after the addition of template DNA. The initial cycle was completed with 1 min of annealing and 2 min of extension. This was followed by 35 cycles of 30 s denaturation, 1 min annealing and 2 min extension. A final extension of 10 min completed the cycling reactions. Denaturation temperature was 94°C, annealing temperatures were 62°C (exon 5), 60°C (exons 6 and 7) and 55°C (exon 8) and extension temperature was 72°C.

SSCP analysis

A 2 μl volume of PCR product was denatured in 5 μl of formamide loading buffer (95% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) by boiling for 10 min. Denatured samples were loaded onto 12% acrylamide–10% glycerol (99:1 acrylamide–bisacrylamide) non-denaturing gels (800 nm high × 0.4 mm thick), focused at 2,500 V for 5 min and run at 1,900 V for 24–26 h. Gels were then wrapped in Saran and exposed to X-ray film (Fuji) with intensifying screens at −80°C for 18–48 h. Samples displaying mutant bands were reamplified and run a second time to confirm the existence of a mutation. Germ-line DNA from patients displaying a mutation in their tumour DNA were also amplified and run on SSCP gels to confirm the mutation was somatically acquired.

Sequencing of mutant bands

Mutant bands were excised from SSCP gels using the autoradiograph as a positional template. DNA was eluted from the gel slice by immersing in 100 μl of water overnight at room temperature, precipitated with a quarter volume of 7.5 M ammonium acetate and two volumes of 100% isopropanol, washed with 70% ethanol, air dried and resuspended in 20 μl of 10 mM Tris–0.1 mM EDTA. The mutant DNA was sequenced by a modification of the chain-termination method (Sanger et al., 1977) using a Gibco BRL cycle sequencing kit. Reamplified mutant DNA was introduced as template to a set of dideoxy sequencing reactions and subjected to a repetitive series of temperature changes similar to PCR. End-labelled primer was extended by these reactions to dideoxy terminations and the sequence was obtained by separating fragments on a 6% acrylamide–7 M urea denaturing gel.

Results

Detection of p53 gene mutations by SSCP analysis

A total of 109 colorectal carcinomas were screened by SSCP for mutations within exons 5–8 of the p53 gene. Mutant p53 was identified by the presence of one or two extra bands migrating above or below the normal single-stranded product (Figure 1). Occasionally mutant bands were also detected between the single- and double-stranded bands and are probably caused by normal/mutant heterodimers. In all cases, normal p53 banding patterns were also observed. Since previous work by our group has shown that p53 allelic loss occurs in 75% of these tumours (Iacopetta et al., 1994), these normal bands probably arise from either an admixture of normal tissue adjoining the tumour sample and/or tumour heterogeneity. All mutations were confirmed by reamplifying samples and running on separate SSCP gels. Whenever the tumour DNA revealed a p53 mutation, the corresponding germ-line DNA was also analysed by SSCP. No germ-line mutations were found, indicating that the changes were of somatic origin.

Mutations were found in 37% (40/109) of carcinomas (Table I). The distribution of these mutations was 41% (17/41) in exon 5, 12.5% (5/41) in exon 6, 15.5% (6/41) in exon 7 and 32% (13/41) in exon 8. In one tumour (84T) two mutations were found in exon 5 and another in exon 8. Several of the most commonly occurring mutant bands detected by SSCP were sequenced and found to contain mutations at codons 175 and 248 (Table II), corresponding to two of the previously identified 'hotspot' regions (Nigro et al., 1989; Hollstein et al., 1991; Castro et al., 1992). None of the mutations we sequenced was found to be a conservative basepair change. Because the primers used in this study cross the intron–exon boundary, splice donor/acceptor mutations should have been detected by SSCP analysis. However, none of the mutations we sequenced was in this region (Table II).

The sensitivity of the SSCP technique in identifying mutant DNA in the presence of large amounts of normal DNA is dependent on the resolution of the SSCP gel. Overexposure of the gel will eventually reveal all aberrantly migrating bands, providing they are sufficiently well separated from the normal bands. To determine the sensitivity of our SSCP gels, normal and mutant DNA bands from two different tumours were excised, eluted, amplified and rerun to confirm the purity of these DNA templates. They were then diluted to the same concentration and the normal and mutant alleles mixed at varying ratios from 1.5% mutant DNA to 50% mutant DNA. These mixtures were amplified and run on SSCP gels (Figure 2). In cases in which the aberrantly migrating bands were well separated from the normal bands, as little as 1.5% mutant DNA in the original template mix could be detected (Figure 2a). Even when the mutant bands migrated close to normal bands, they could be detected in mixes containing 6–12% mutant DNA template (Figure 2b).

In preliminary experiments, we optimised the sensitivity of our SSCP gels by running PCR fragments from the same tumour samples under widely varying conditions of temperature, buffer concentration, glycerol/acrylamide concentration,
run voltage and run duration. The running conditions we selected resulted in good separation of all aberrantly migrating bands in each of the four exons.

Detection of p53 protein overexpression by immunocytochemistry

The anti-p53 monoclonal antibody DO-7 recognizes a denaturation-resistant epitope at the N-terminus of the human p53 protein and reacts with both wild-type and mutant proteins. We used DO-7 to detect p53 overexpression by ICC staining of paraffin sections. In comparative studies using a range of antibodies, DO-7 has shown to be one of the most sensitive for the detection of p53 overexpression (Campani et al., 1993; Jiko et al., 1993; Lassam et al., 1993; Baas et al., 1994). Overall, 46% (50/109) of colorectal carcinomas in this study exhibited positive nuclear staining (Figure 3).

Table I The relationship between p53 protein overexpression detected by ICC and p53 gene mutation detected by SSCP analysis of 109 colorectal carcinomas

| Tumour | Exon | Codon | Mutation | Amino acid substitution | ICC staining |
|--------|------|-------|----------|-------------------------|--------------|
| 12T    | 5    | 153   | 9 bp insertion | Arg → His             | −            |
| 19T    | 7    | 242   | 4 bp insertion  | Arg → His             | +            |
| 28T    | 5    | 175   | G → A       | Arg → His             | +            |
| 38T    | 5    | 175   | G → A       | Arg → His             | +            |
| 44T    | 7    | 248   | C → T       | Arg → Trp             | +            |
| 47T    | 5    | 175   | G → A       | Arg → His             | −            |
| 54T    | 5    | 175   | G → A       | Arg → His             | +            |
| 82T    | 5    | 175   | C → T       | Arg → Cys             | −            |
| 84Tb   | 5    | 173   | Single bp deletion | Arg → His           | +            |
| 84Tb   | 5    | 186   | Single bp deletion | Arg → His           | +            |
| 89T    | 5    | 175   | G → A       | Arg → His             | +            |
| 90T    | 5    | 152   | C → T       | Pro → Leu             | −            |
| 95T    | 5    | 175   | G → A       | Arg → His             | +            |
| 97T    | 6    | 214   | T → G       | His → Gln             | −            |
| 112T   | 7    | 254   | Single bp insertion | −              | +            |

Two cases also displayed small amounts of reaction product in the cytoplasm of malignant cells. There was considerable heterogeneity of positive ICC reactions throughout the series and within individual tumours. The estimated percentage of positively staining neoplastic cells in given tumours ranged from 1 to 90%. No nuclear or cytoplasmic staining was detected in normal tissue which adjoined 82% (89 109) of the tumour sections.

The concordance of results between SSCP and ICC (i.e. both positive or both negative) was 69% (75 109) (Table I). A further 20% of carcinomas (22 109) stained positively by ICC but failed to display any mutation by SSCP, while the remaining 11% (12 109) displayed mutant bands by SSCP but showed no staining by ICC.

Discussion

Mutations of the p53 gene are the most common genetic alteration, known to occur in a wide range of human cancers (Hollstein et al., 1991). Under normal conditions wild-type p53 protein is rapidly degraded and is therefore present only at very low levels within the cell. The acquisition of a mutant genotype is thought to increase the half-life of the mutant protein and lead to its accumulation within the cell. This accumulated protein is detectable by immunocytochemical techniques and ICC has been proposed as an indirect method of screening tumours for mutation within the p53 gene. Recently however, a number of discordant results have been...
The frequency of p53 gene mutation detected by SSCP analysis in our study was 37% (40/109) in primary colorectal carcinomas, a frequency similar to recent molecular studies on large numbers of colorectal cancers (Kikuchi-Yanoshita et al., 1992; Lothe et al., 1992). Compared with the study by the Japanese group (Kikuchi-Yanoshita et al., 1992) we found 3-fold more mutations in exon 8 and 3-fold fewer in exon 7, possibly reflecting exposure to different dietary or environmental carcinogens. As described in the results (Figure 2), we believe that the high resolution of our SSCP analysis allowed the identification of tumours containing very low levels of mutant p53 allele. For this reason we conclude that approximately 40% of colorectal carcinomas have at least some cells carrying mutations within exons 5–8 of the p53 gene (Table I).

The frequency of p53 protein overexpression detected using ICC on the same series of colorectal carcinoma specimens used for SSCP analysis was 46% (50/109). This is very similar to the range of 42–49% reported in previous ICC studies (Purdie et al., 1991; Scott et al., 1991; Cunningham et al., 1992; Starzynska et al., 1992; Sun et al., 1992; Bell et al., 1993). Because of the variety of methods used (different antibodies, detection systems, tissue fixation, etc.) it is often difficult to make a direct comparison between the results obtained by different groups. However, the DO-7 antibody we used appears to give the same staining profile as two other commonly used p53 antibodies, 1801 (Campiani et al., 1993; Lassam et al., 1993) and CM-1 (Jiko et al., 1993). A comparative study of six different anti-p53 antibodies has shown DO-7 to be the most sensitive and specific for ICC detection of p53 protein in colorectal cancers (Baas et al., 1994).

Although the frequencies of p53 gene mutation and protein overexpression we observed were quite similar (37% and 46% respectively), the concordancy of results from the two techniques (i.e. both positive or both negative) was only 69% (75/109). Recently, Allred et al. (1993) reported 62% concordance between SSCP and ICC detection of p53 mutation in breast cancer. As proposed by these authors and others who have observed discrepancies between p53 gene mutation and protein overexpression (Börresen et al., 1991; Wynford-Thomas, 1992; Oliner et al., 1993; Slingerland et al., 1993), several explanations could account for these findings. Firstly, for the group of SSCP-negative/ICC-positive tumours, (20% of cases) some mutations may have occurred outside exons 5–8. We believe that this is unlikely to account for all cases, however, especially in view of the very low frequency (<5%) of mutations known to occur outside the highly conserved regions of the p53 gene (Hollstein et al., 1991; Caron de Fromentel & Soussi, 1992). Nevertheless, some of the tumours classified as SSCP negative may contain a mutation outside of exons 5–8. Masking of mutant DNA owing to the presence of large amounts of normal tissue or because of heterogeneity within individual tumours may also account for some of the SSCP-negative/ICC-positive cases. However, the SSCP technique has proven to be very sensitive in our hands, and when mutant and normal bands are well separated a mutant DNA content of as little as 1.5% is adequate for detection (Figure 2a). When mutant and normal bands were less well separated a higher mutant DNA content of about 6–12% was required for detection (Figure 2b).

In addition, the SSCP technique may not be 100% sensitive, and therefore some mutations may have escaped detection. Another explanation for SSCP-negative/ICC-positive cases is that p53 protein may accumulate in the absence of gene mutation by forming complexes with other molecules (Lane & Crawford, 1979; Sarnow et al., 1982; Momand et al., 1992). This sequestered protein may be functionally inactive but still detectable by p53 antibodies.

We observed 12 cases (11%) of SSCP-positive/ICC-negative tumours. The most likely explanations for these are, firstly, that the mutations do not lead to protein stabilisation...
and hence accumulation in the cell. Secondly, the mutated gene may code for a stop codon and lead to production of a truncated form of the protein or a halt in protein production altogether. Thirdly, the antibody used. For the ICC staining may not have detected all mutant protein epitopes. We sequenced five of these 12 cases (12T, 47T, 82T, 90T and 97T) and found that four had mutations leading to amino acid substitutions and the fifth was a frameshift mutation which introduced a stop codon downstream (Table II).

Despite the 31% (34/109) discordancy, the overall correlation between SSCP and ICC detection of p53 mutations is still highly significant (P < 0.001). Wider screening for mutations outside of the conserved gene regions and the use of other antibodies in ICC staining are likely to have uncovered other mutations which would have reduced this percentage. Nevertheless, our results suggest that ICC detection of p53 protein overexpression does not always indicate the existence of an underlying gene mutation and vice versa. This observation in colorectal cancer confirms other recent studies in large numbers of lung cancer cell lines (Bodner et al., 1992) and primary breast cancers (Allred et al., 1993). As suggested previously (Bodner et al., 1992), overexpression of p53 protein may depend upon the type of gene mutation.

An obvious question which arises from this work is the comparative biological significance, if any, of p53 gene mutations and protein overexpression in neoplasia. It has been suggested that ICC detects functionally significant mutations (Wyndon-Thomas, 1992). Certainly, in breast cancer, overexpression of p53 appears to be a significant marker of poor prognosis (Isola et al., 1992; Thor et al., 1992; Allred et al., 1992; Silvestrini et al., 1993). In colorectal cancer, however, the association between ICC positivity and patient survival is still controversial (Scott et al., 1991; O'Connell et al., 1992; Remvikos et al., 1992; Starzyńska et al., 1992; Sun et al., 1992; Yamaguchi et al., 1992; Bell et al., 1993). Much less work has been done correlating p53 gene mutation and outcome. Two studies in breast cancer indicate poorer prognosis in the presence of p53 gene mutation (Allred et al., 1993; Thorlacius et al., 1993), but to our knowledge none has been published so far on colorectal carcinoma. Although the post-operative follow-up time of patients whose tumours were examined in the current study is still quite short (average 30 months), our preliminary results show no significant correlation between p53 gene mutation and survival (Dix et al., 1994).

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