p53 mutations and overexpression in locally advanced breast cancers

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Summary Alterations in the p53 gene were analysed in 39 patients with locally advanced breast cancers (LABCs) (stage III–IV) with inflammatory signs in most cases (UIICC stage T4d = 32 patients) by molecular and immunohistochemical (IHC) approaches. All patients were included in the same therapy protocol. Using polymerase chain reaction (PCR) and a single-strand conformational polymorphism migration technique (SSCP), the presence of mutations in exons 2–11, covering the entire coding sequence of the p53 gene, was evaluated. Using the mouse specific anti-human p53 monoclonal antibody (PAB 1801), we also looked for overexpression of the p53 protein in tissue sections. In 16 cases shifted bands were reproducibly identified by PCR-SSCP, and all but one (localised to exon 10) were in exons 5–8, the usual mutational hotspots. Fifteen of these 16 sequences were shown to be associated with larger tumour diameter ($\chi^2 = 7,490, P = 0.0062$) and the presence of clinical metastases (stage IV) ($\chi^2 = 10,113, P = 0.0015$). A non-statistically significant trend of association was observed between p53 mutation, negative oestrogen receptor and lower response rate to therapy. Our results in this group of patients and the heterogeneity of the staining of tumour cells in tissue sections suggest that p53 mutations could be a late event in this non-familial form of breast cancer.

Locally advanced stage III and IV breast cancers (LABCs) have a high likelihood of distant metastasis at diagnosis or more often at recurrence, and are associated with a poor survival even when treated with neoadjuvant chemotherapy (Beahrs et al., 1988). A peculiar feature of LABC is represented by inflammatory breast cancers (IBCs) (stage T4d of UIICC classification) (Beahrs et al., 1988). These represent less than 5% of breast tumours and are characterised by a high frequency of metastases at diagnosis and even shorter survival than other LABCs. These forms are characterised by clinical inflammatory signs in the breast skin and dermal lymphatic emboli. To date, no peculiar biological characteristics for these cancers have been described, although oestrogen receptor (ER) negativity, epidermal growth factor (EGF) receptor positivity and c-erbB2 overexpression have been reported to be more frequent than in other LABCs (Levine et al., 1985; Sherry et al., 1985; Jaiyesimi et al., 1992).

The tumour-suppressor gene p53 is a phosphoprotein that negatively regulates cell proliferation and plays a role in differentiation and apoptosis of cells (Clarke et al., 1993; Lane, 1993; Lowe et al., 1993). Wild-type p53 has been shown to block the cell cycle near the G1/S phase (Diller et al., 1990; Lane, 1992). Mutational inactivation of p53 appears to be the most common genetic abnormality in cancers (Baker et al., 1989, 1990a; Negro et al., 1989; Caron de Fromentel et al., 1991; Hollstein et al., 1991), leading to the loss of growth control. p53 mutations tend to cluster in regions between exons 5 and 8, known to include four highly conserved sequence blocks (Negro et al., 1989; Caron de Fromentel et al., 1991). In colon cancers, the p53 mutation rate is as high as 70% (Baker et al., 1989, 1990a,b), but this event occurs in 20–40% of breast tumours (Prosser et al., 1990; Davidoff et al., 1991a,b; Levine et al., 1991; Mazars et al., 1992; Moll et al., 1992). While in both types of tumours the p53 locus is reduced to homozygosity in 70% of cases (Baker et al., 1989; Davidoff et al., 1991a,b), colon cancers retain the mutant allele in 90% of cases (Baker et al., 1990a).

In contrast, 40% of breast tumours preferentially retain the wild-type allele (Davidoff et al., 1991a). Therefore, mechanisms underlying the negative regulatory effects and the bypass of wild-type protein function are not totally elucidated.

A large series of studies have shown that p53 mutations in breast cancers are associated with poor clinical parameters and bad prognosis. Most of these studies are retrospective and deal with tumour samples from heterogeneous patients treated with various therapies (Prosser et al., 1990; Davidoff et al., 1991a,b; Mazars et al., 1992).

We were mostly interested in studying the mutational pattern of p53 in a homogeneous group of aggressive breast tumours, and relating this pattern to proliferation, response to therapy and metastasis. We studied a group of 39 patients with stage III and IV LABC, 32 of IBC type, diagnosed, followed and treated in the same institution, and included in the same therapy protocol. p53 mutations were sought by both PCR-SSCP and immunostaining using PAB 1801, since p53 mutations are characterised by overexpression of the protein. Mutations were determined by sequence analysis.

We finally attempted to correlate these data with the usual parameters and clinical events that occurred in this cohort of patients.

Materials and methods

Patients

Thirty-nine patients who were classified as having a locally advanced breast cancer (stage III–IV), without prior treatment, were included in the SIM 85 protocol from 1985 to 1990. All these patients had an initial surgical biopsy followed by 4–6 courses of intensive chemotherapy ('4-epi-adriamycin and cyclophosphamide) followed by mastectomy, radiation therapy and 6 months' maintenance chemotherapy using a rotational regimen.

Patients were staged according to the UIICC/AJCC classification (Beahrs et al., 1988). Tumours were classified according to WHO (1982) pathology criteria and Scarff—
Bloom Richardson (SBR) grading system. Two patients were suspected of having Li–Fraumeni syndrome. ER determination was made by ELISA (Abbott).

Human tumours

Breast tumour samples were obtained from patients undergoing surgery. They were immediately quick frozen in liquid nitrogen and stored at −80°C until the extraction of DNA. Tumour typing was done according to the WHO histological typing of breast cancers (1982).

DNA extraction

High molecular weight genomic DNA was extracted from tumour tissues by conventional methods (Maniatis et al., 1982).

Single-strand conformational polymorphism (SSCP) analysis

PCR-SSCP analysis was performed according to the method of Ortt et al. (1989) with slight modifications. The nucleotide sequences of the primers used for exons 5–8 and 10 are listed in Table I, and the sizes of the amplified fragments are described according to the nucleotide numbers of the Chumakov human p53 sequence (Buchan et al., 1988). Briefly, PCR was performed with 100 ng of DNA (1 μl), 4 pmol of each primer, 200 mM deoxynucleotide triphosphate, 10 mM Tris (pH 8.8), 50 mM potassium chloride, 1 mM magnesium chloride, 1 μCi of [α-32P]dCTP (Amersham; specific activity 3,000 Ci mmol−1) and 0.5 U of Taq polymerase. Thirty cycles were performed using an automated DNA Perkin-Elmer-Cetus thermocycler (model 9600) with denaturation at 92°C, annealing at 60°C for exons 2–5 and 8–11, at 65°C for exon 7 and at 55°C for exon 6, and extension at 72°C. One microlitre of the reaction mixture was diluted in 20 μl of 20 mM EDTA/0.1% SDS, and 2 μl of this dilution was mixed with 2 μl of sequencing stop solution. Samples were heated at 95°C for 8 min, immediately cooled at 4°C and 1.5 μl was loaded onto a 6% polyacrylamide gel. Samples were run at 2000 V/m 1 m gel containing 5% or 10% glycerol, and one at 4°C in a gel without glycerol (Spinardi et al., 1991). Gels were dried at 80°C, autoradiography was performed with an intensifying screen for 12 h and the pattern of single-stranded DNA was analysed for shifted bands.

DNA sequencing

DNA from regions showing band shifts upon SSCP analysis was amplified in a separate PCR and subcloned by ligation to pCR™II vector using the TA cloning kit (Invitrogen Corporation) according to the manufacturer’s instruction. The recombinant clones were colour selected and white recombinants were amplified. Double-stranded DNA was then sequenced by the dyeoxy chain-termination method using a Sequenase version 2.0 kit (US Biochemicals, USA) and primering the reaction with forward and reverse primers of the TA cloning kit. Sequences were analysed on a 6% polyacrylamide gel containing 7 M urea. After drying, gels were exposed to Kodak XAR film overnight.

Immunochemistry

Frozen sections (4 μm) were cut, air dried and fixed in acetone for 10 min. p53 was detected with the mouse anti-human p53 monoclonal antibody PAb 1801 (Novocastra, NCL-p53-1801) following the procedure of Davidoff et al. (1991a). PAb 1801 recognises an epitope between amino acids 32 and 79 at the N-terminus of both wild-type and mutant human p53 (Banks et al., 1986). Staining was performed by incubation at 4°C overnight with the primary antibody, followed by incubation by a biotinylated horse antibody to mouse immunoglobulin (Vector Laboratories). The reaction was revealed using diaminobenzidine (DAB), and the sections were counterstained with haematoxylin and mounted. Normal mouse serum was used as a negative control. The breast cancer cell line MDA-MB231 served as positive control.

Results

Characterisation of p53 abnormalities recognised by PCR-SSCP

Using PCR-SSCP, 39 tumour samples from a highly homogeneous series of locally advanced breast cancer patients were screened for abnormalities of the p53 gene (Figure 1). Mutations were recognised as shifted bands on non-denaturing polyacrylamide gels. Shifted bands due to conformational changes produced by mutations allow the wild-type gene to be distinguished from the mutated form of the gene. DNA analysis was performed on every exon of the coding sequence (exons 2–11). In those highly evolutive breast cancers there was one or more mutational pattern. All except one of the 16 variant conformers were found in exons 5–8, which are recognised as classical mutational hotspot regions (Nigro et al., 1989). Exon 5 was the most frequently involved (five cases, 12%). Four mutations were recognised on exon 7, three on exons 6 and 8, and one on exon 10. In nine cases out of 16, four bands were clearly detected by SSCP, verifying that they had retained the wild-type form. This could be caused by only one mutation was per exon. All except either the wild-type or mutated form of p53 or by the presence of both mutated and normal alleles in the same tumour cells. In no case could this be explained by normal tissue, the percentage of which was negligible when observed on tissue sections.

DNA sequence analysis of SSCP variants

We reamplified and sequenced 15 of the 16 samples with variant conformers (Table II). A mutation was confirmed in 14 cases (36%), one sequence being normal after repeat experiments. In 13 of these 14 cases a functional mutation was present, while in one case (patient 32) a deletion (patient 24), one base pair deletion (patient 39) and 12 substitutions, more frequently transitions (eight cases) than transversions (four cases).
Among the 16 band shifts with PCR-SSCP, 13 had mutations leading to mRNA and protein amino acid substitution and were considered as mutants for the clinicopathological correlation. As can be seen in Table III, 82% of this series of patients had an inflammatory form of breast cancer, and 18% had metastasis at diagnosis. Patients with p53 mutations had significantly larger tumours ($\chi^2 = 7.490; P = 0.0062$) and more metastatic forms at diagnosis ($\chi^2 = 10.113; P = 0.0015$).

All but one patient with p53 mutations had ductal cancer. Two other patients with p53 immunostaining positivity but without p53 SSCP abnormality had a lobular breast cancer. Patients with p53 mutations had more frequently, but without statistical significance, SBR grade III tumours, ER-negative tumours, and lymphatic emboli. The response rate to induction chemotherapy was higher in patients without p53 muta-
tions but there was no difference in relapse rates. Surprisingly, three CNS relapses were observed in the patients with p53 mutation.

**Discussion**

Using SSCP we detected p53 shifted bands in 16 (41%) of 39 patients with locally advanced and inflammatory breast tumours. This is a fairly high percentage for breast cancers, in which mutations recognised by this approach are generally lower, even if they greatly vary among studies (17–46%) (Prosser et al., 1990; Davidoff et al., 1991a, b; Osborne et al., 1991; Coles et al., 1992; Mazars et al., 1992; Moll et al., 1992). These data were obtained by studying the whole coding sequence, and SSCP was performed under various conditions known to miss very few, if any, mutations (Spinardi et al., 1991). As a band shift may be associated with nonsense mutation or silent polymorphism (De la Caille Martin et al., 1990; Carbone et al., 1991; Chumakov et al., 1991; McDaniel et al., 1991; Prosser & Condie, 1991), and since a mutation does not necessarily alter protein function, we sequenced the samples with shifted bands and analysed protein expression by immunohistochemistry. Of the 15 samples sequenced, one nonsense mutation was observed and we could not show a mutation in one other sample. These two tumours were in fact not stained by the antibody to p53. Mutations, except for one in exon 10, were found exclusively in exons 5–8, which are regions highly conserved between species and which contain the usual mutational hotspots, as reported by others (Prosser et al., 1990; Davidoff et al., 1991a; Osborne et al., 1991; Coles et al., 1992). In this series, tumour mutations were scattered throughout the DNA-spanning exons 5–8, and we did not find either specific mutations or mutational hotspots as seen in tumours induced by chemical carcinogens or UV irradiation. Eight out of 12 point mutations were G:C to A:T transitions, with one G→A transition occurring at one of the CpG dinucleotides (codon 248) supposed to proceed from deamination of a methylcytosine and known to be a hotspot for spontaneous mutation (Hollstein et al., 1991; Jonveaux et al., 1991). The four other cases were transversions. A high prevalence of transitions favours spontaneous mutations (Caron de Fromentel et al., 1991). However one C→T transition occurred at a CC dimer, which is known to be provoked by UV light damage (Moles et al., 1993).

One of the four transversions was a G→T substitution, known in lung cancer to be caused by benzopyrenes contained in tobacco smoke (Suzuki et al., 1992). One tumour with a shifted band on exon 6 was unavailable for sequence, and although improbable, in this case we cannot exclude a silent polymorphism at codon 213 (Carbone et al., 1991) since the tumour was positively stained with anti-p53 antibody. We cannot speculate if every mutation encountered has the same prognostic value since each mutation alters the p53 conformation differently, leading to different biological properties and tumorigenic potential (Haley et al., 1990; Rayco et al., 1991; Mukhopadhyay et al., 1993). Mutants on exons 5 and 6 encode conformers which bind heatshock proteins (hsp70) and are associated with humoral responses, whereas exons 7 and 8 do not (Callahan et al., 1992; Davidoff et al., 1992a; Schlichtholz et al., 1992).

Owing to its extremely short half-life, normal p53 protein is undetectable by IHC. Point mutations are believed to change its conformation, resulting in prolongation of its half-life (Hinds et al., 1988) and its detection by IHC. To better correlate mutations with protein stability, immunohistochemical studies were performed. Of 16 tumours with a band shift, 12 exhibited overexpression of p53. Of the four samples with band shift associated with lack of immunoreactivity, one had no detectable mutation, one had a nonsense mutation, one a missense mutation and one an intronic insertion. This last type of mutation may generate a short and modified mRNA, leading to a truncated protein. The first two examples raise the issue of technical problems linked

![Figure 2. Examples of immunohistochemical analysis of p53 protein in breast cancers. Tumour tissues were frozen-sectioned, fixed in acetone and stained with monoclonal antibody against p53 (PAb 1801). Counterstaining was performed with haematoxylin. a. Staining of tumour tissue from patients 43 (× 65). b. Staining of tumour tissue from patient 9 (× 325) (both patients had shifted bands on exon 5).](image_url)
to the heterogeneity of the tumours, suggesting that microdissection and PCR in situ would be of greater specificity than classical techniques. Only two tumours without any band shift were heavily stained with PAb 1801. Elledge et al. (1993) found in breast tumours a lower prevalence of mutations of p53 detected by SSCP than with immunohistochemical staining. Other investigators have found either mutations recognised at translation, but negative by immunohistochemistry (Rodrigues et al., 1990; Bennet et al., 1991; Borresen et al., 1991) or overexpression of a stable apparently wild-type protein. This was recently reported in undifferentiated neuroblastoma cell lines (Davidoff et al., 1992b) and in normal epithelial and mesenchymal cells in certain forms of hereditary breast tumours (Barnes et al., 1992). However, there is an abundance of reports of a good correlation between protein detection and the presence of mutation at gene level (Bartek et al., 1990; Borresen et al., 1991; Davidoff et al., 1991a; Varley et al., 1991).

As in other reports, we found marked heterogeneity of staining (Cattoretti et al., 1989; Davidoff et al., 1991a; Thompson et al., 1992; Thor et al., 1992). Three types of staining were observed in our tumours: large isolated foci of tumour cells within a negative population of cancer cells; dispersed cells; and mixed tumorous positive cells. The localisation of staining was usually exclusively nuclear. Two tumours had mixed nuclear/cytoplasmic staining and one had cytoplasmic staining only.

Cytoplasmic staining with nuclear sparing has been described by Moll et al. (1992) in normal lactating breast epithelial cells and in some breast cancers, in which case this type of staining correlated with wild-type p53. This indicates that inactivation of p53 may be obtained through mechanisms involving sequestration of p53 protein in the cytoplasm.

This work was conducted as a pilot study in an infrequent group of patients in Western countries (McGuire et al., 1991). All of them had LABC (stage III–IV), 31 (82%) had an IBC (T4d) as diagnosed after surgical biopsy, and all patients were treated with an identical intensive therapy regimen.

Tumours with p53 mutations were significantly larger ($\chi^2 = 7.490, P = 0.0062$) and more often metastatic at the time of diagnosis ($\chi^2 = 10.113; P = 0.0015$). Six of eight tumours with a diameter greater than 10 cm had a p53 mutation, and all were in patients with metastases. Five of these six patients relapsed after an initial response to therapy, three as a result of CNS disease. Less significantly, tumours with p53 mutations were related to already known (Horak et al., 1992; Poller et al., 1992) poor prognostic characteristics such as absence of oestrogen receptors.

In conclusion, these clinicopathological correlations and the initial response to treatment link p53 mutations with poor prognosis and suggest that within this subset of patients with a severe prognostic form of cancer p53 mutations are related to the more aggressive forms.

A preferential association of p53 mutations with the size of the tumour, the heterogeneity of staining and the presence of metastasis leads us to speculate that p53 mutagenesis could give a selective growth advantage and could be a late event in breast tumour evolution.

We wish to thank Thierry Soussi for fruitful discussions on the p53 gene and on the PCR–SSCP technique and for providing us with some of the primers used. Carolyne Moyret for introduction to sequence analysis of p53, Rima Zoour for fruitful discussions and introduction to TA cloning, and Charles Auffray and Dominique Piattier for discussions.

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