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Human breast cancer: frequent p53 allele loss and protein overexpression

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Abstract. A sample of 114 primary breast tumors and corresponding constitutional DNA were tested for loss of heterozygosity (LOH) of the YNZ22 and p53 genes, both located in the 17p13 region. Loss of the p53 allele was found in 28 of 44 primary breast carcinomas (64%). In contrast LOH in only 26 of 61 tumors (43%) was detected with the variable number of tandem repeats (VNTR) probe YNZ22 mapping at 17p13.3 close to the p53 locus at 17p13.1. Among 19 tumors informative for both probes allele loss at 17p13.3 never occurred without p53 involvement. These data suggest, that p53 is the target of 17p13 allelic deletions in human breast cancer. Immunohistochemistry showed overexpression of the p53 protein in 25 of 50 cases (50%) presumably reflecting activating point mutations. Overexpression was not correlated with allele loss but seemed to be closely related to the presence of point mutations in this study. No homozygous deletions or rearrangements of the p53 gene were detected. This would argue for an important role of heterozygous p53 mutations in human breast cancer.

Introduction

Recently allele losses at chromosome 17p13 have been reported in a variety of human cancers including carcinoma of the breast, colon, ovary and lung, and astrocytoma, and osteosarcoma (Mackay et al. 1988; Baker et al. 1989; Nigro et al. 1989; Toguchida et al. 1989; Coles et al. 1990; Devilee et al. 1990; Varley et al. 1991; Prosser et al. 1990). A common target region of 17p deletion, defined by a number of polymorphic markers, was found to be 17p12.1–17p13.3 in colorectal carcinoma and 17p13 in osteosarcoma (Baker et al. 1989; Toguchida et al. 1989). Similar results have been reported in a recent study on breast cancer, which indicated that a target gene may lie in a region extending between 17p13.3 and 17p13.1 (Devilee et al. 1990). It is commonly believed that p53, which maps to 17p13.1 (Baker et al. 1989) is this putative target gene.

In normal cells p53 protein is virtually undetectable by immunohistochemistry owing to the short half-life and the small amount present. Missense mutations are known to stabilize the gene product and may be responsible for p53 overexpression (Lane and Benchimol 1990). A steadily growing body of evidence suggests that p53 point mutations are indeed a frequent feature of human neoplasias (Baker et al. 1989; Nigro et al. 1989; Prosser et al. 1990; see Levine et al. 1991 for review). Therefore elevated p53 protein levels, which have been found by immunohistochemical methods in a number of cancers including breast carcinoma, may reflect a high rate of somatic point mutation (Cattoretti et al. 1988; Rodrigues et al. 1990; Bartek et al. 1990a). Also germ line p53 missense mutations have been identified as the cause of a rare familial pattern of cancers known as Li-Fraumeni syndrome (Malkin et al. 1990).

However, the involvement of a different locus has also been discussed (Coles et al. 1990; Chen et al. 1991). In their investigation on breast cancer Coles et al. (1990) found independent allele loss with the variable number of tandem repeats (VNTR) probe YNZ22 from 17p13.3 and a genomic p53 clone. Therefore they postulated a second target gene at 17p13 distinct from p53.

Data on heterozygous deletion of the p53 locus itself are scarce. In this study we tried to gain more insight into the allele loss pattern at 17p13 and the putative involvement of p53. For this purpose we employed the probe YNZ22 and a p53 cDNA probe. The highest rates of loss of heterozygosity (LOH) at 17p13 in breast cancer have been obtained with YNZ22 (Coles et al. 1990; Devilee et al. 1990). Only a small body of data on the relationship of allele loss and p53 protein expression is available (Varley et al. 1991; Davidoff et al. 1991). In this investigation we report on the frequency of p53 protein overexpression and its relation to p53 allele loss and point mutations as well.

Materials and methods

Nucleic acid isolation and hybridization

Simultaneous DNA and RNA preparation from tumor specimens, preparation of constitutional DNA from the patients' blood, DNA digestion with restriction enzymes, Southern blotting and hybridization were according to standard procedures as described previously (Singh et al. 1991). Before use the RNA was further purified by a guanidinium thio-

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cyanate step. Electrophoresis and Northern blotting were according to Sambrook et al. (1989). RNA prehybridization was performed at 42°C for 2 h in 50% (v/v) formamide, 5 × SSPE, 0.5% SDS, 50 μg/ml yeast tRNA, 50 μg/ml poly(A). For hybridization (at least 16 h at 42°C) 2×10^6 cpm/ml of the p53 probe labeled as for DNA hybridization were added to a fresh sample of prehybridization solution. Procedures for washing and exposure were as for DNA membranes.

DNA probes

The probes pHP53B (American Type Culture Collection, Rockville, Md., USA), and pYNZ22 (courtesy of Dr. Y. Nakamura) were used. For restriction fragment length polymorphism (RFLP) analysis of p53, *Sca*I, *Ban*II (Masharani et al. 1988), and *Bgl*II (de la Calle et al. 1990) were used, and for RFLP analysis of YNZ22, *Taq*I (Nakamura et al. 1987) was used.

Polymerase chain reaction (PCR) and single strand conformation polymorphism (SSCP) analysis

Coding regions of the p53 gene between exons 4 and 9 were amplified by using pairs of specific oligonucleotide primers based on published sequences (Buchman et al. 1988, and P. Chumakov, Moscow, personal communication). For exon 4 the primers were: 5′-ACCCATCTACAG-TCCCCCTTG-3′ and 5′-CAGGGCAACTGACCGTGCAAGT-3′; for exons 5 and 6, 5′-TTCCTCTTCCTGCAGTACTC-3′ and 5′-CAAAC-CAGACCTCAGGCGGCT-3′; for exon 7, 5′GTGTTATCTCCTAG-GTTGGCTCTGAC-3′ and 5′-TGGCAAGTGGCTCCTGACCTGGA-3′; and for exons 8 and 9, 5′-CCTATCCTGAGTAGTGGTAAT-3′ and 5′-TCCCAAGACTTAGTACCTGAAG-3′. Additionally primers 5′-GCCCCAGCTGCTCACCATCGCT-3′ and 5′-CTCACTGATTGCTC-TTAGGTCT-3′ respectively were used paired with the first or second primer used to amplify exons 5 and 6 together in order to amplify exon 5 or 6 alone. The primers were synthesized on a model 391A DNA synthesizer (Applied Biosystems) and were purified by passing through an NAP-column (Pharmacia) before use.

Amplification was performed in a 100-μl volume containing 1 μg genomic DNA, 1.5 μ*M* $MgCl_2$, 70 m*M* TRIS-HCl pH 8, 200 μ*M* each of deoxynucleotide triphosphates (dATP, dCTP, gGTP, dTTP) 200 ng of each primer and 2 units of *Taq* polymerase. A few drops of mineral oil were pippeted on this to prevent condensation. Denaturation was carried out at 93°C for 3 min followed by 40 cycles of denaturation at 92°C for 2 min annealing at 52°C for 2 min, and extension at 70°C for 2 min in a thermocycler (Biomed). For each subsequent cycle the primer extension reaction was increased by 1 s. The reaction products were checked for the size of the amplification product in 2% agarose gels.

For SSCP analysis the amplification was performed in the presence of 2.5 μl of [α-^{32}P]dCTP (3,000 Ci/mmol, 10 μCi/μl, Amersham). The SSCP analysis was done essentially as reported by Orita et al. (1989). Two microliters of the above PCR product were diluted 50-fold in buffer containing 96% formamide, 20 m*M* EDTA, 0.1% SDS, 0.05% each of xylene cyanol and bromophenol blue. Aliquots (50 μl) were heated to 80°C for 3 min and 3 μl samples were applied to 6% or 8% neutral polyacrylamide gels with and without 10% glycerol. Electrophoresis was carried out in 90 m*M* TRIS-borate buffer at 30 W for 3–5 h. The gels were autoradiographed at room temperature as such or after drying.

Direct sequencing of PCR products

Two strategies were employed for this purpose. In the first, 2 μl of PCR product generated from the appropriate exon was reamplified for a further 35 cycles using only a single primer. This asymmetric PCR product was purified on a Centricon 30 column (Amicon), ethanol precipitated, redissolved in water and one-third of it was sequenced using the Sanger technique as detailed below. Alternatively PCR was performed with one of the primers labeled with biotin at the 5′ end. This reaction product was shaken for 5 min with 50 μl of prewashed Dynabeads M280 streptavidin (Dynal Hamburg). The supernatant was removed using a magnetic concentrator (MC) and 100 μl of 0.15*M* NaOH was added and gently mixed. The supernatant was again removed with the help of an MC and the beads washed with 100 μl of TE as above and dissolved in 25 μl of water. Five microlitres of these Dynabeads with ssDNA was used with 1 pmol of relevant primer for the Sanger sequencing reactions as explained below. In some cases the PCR product was separated in 2% low melting temperature agarose gels, the DNA fragment excised and eluted overnight in 0.5 *M* sodium acetate, 1 m*M* EDTA, and ethanol precipitated. It was sequenced directly using 25-nmol sequencing primer after a 2 min preincubation at 42°C using [α-^{32}P]dATP and Sangers dideoxy chain termination method with the Sequenase II kit (United States Biochemicals) according to the vendor's protocol.

Electrophoresis was carried out for 3–5 h in 6% polyacrylamide 7 *M* urea gels and exposed to Kodak X-Omat films at room temperature. In general the same primers as those used for the PCR reactions were used as sequencing primers. In some cases, especially for dynabead protocol, smaller nested primers were synthesized for this purpose.

Immunohistochemistry

Frozen tissue section (5 μm) were air dried and immediately fixed in 1:1 acetone/methanol at –20°C for 10 min. All washing steps were carried out in PBS, pH 7.4. Antibodies used were (1) PAb 1801 and PAb 240 for immunohistochemical detection of p53, (2) TRP-E and c-*neu* (Ab-3) as negative controls (all antibodies from Oncogene Science, NY, USA). PAb 1801 recognizes an epitope lying between amino acids 32 and 79 of the p53 protein (Banks et al. 1986). PAb 240 is an antibody against an epitope between amino acid residues 156 and 335 (Gannon et al. 1990).

After overnight incubation detection was carried out using a avidin-biotin-peroxidase kit (Oncogene Science) according to the manufacturer's protocol with diaminobenzidine and cobalt and nickel chloride as intensifier (Amersham Buchler, Braunschweig, FRG). Hematoxylin was sometimes used lightly to counterstain nuclei.

Clinical and pathological parameters were as routinely obtained in the Department of Gynecology. Above 20 fmol/ml was considered as the cutoff value to assign estrogen or progesterone receptors as positive.

Results

The results of the investigation on allele loss are summarized in Table 1. Typical examples of restriction patterns after hybridization with probes pHP53B and pYNZ22 are presented in Fig. 1. 19 cases were informative for both loci. LOH was observed at both loci in ten cases, and there was no LOH in five cases. In a further four cases LOH was observed only at the 53 locus.

In the total sample of 99 tumors analysed with the p53 probe, only 1 tumor (T127) showed a novel band in addition to the heterozygous constitutional *Sca*I pattern of lower intensity. A partial LOH (loss at least in part of the tumor) was

Table 1. Frequency of allele loss in blood/tumor pairs at the p53 and YNZ22 locus

Locus	Enzyme	Number tested	Informative	Loss of heterozygosity (LOH)	
p53	*Sca*I	99	34	22	
	*Bgl*II	52[a]	12[a]	8[a]	
	*Ban*II	11	0	0	
	Total	99	44	28	64%
YNZ22	*Taq*I	101	61	26	43%
17p13	Total	114	86	44	51%

[a] Includes 2 tumors already informative for *Sca*I

observed in this tumor with the probe pYNZ22 (Fig. 1c). No change in the normal restriction pattern was observed in this tumor using *Ban*II, *Taq*I, *Pst*I and *Hin*dIII. Interestingly no protein overexpression in this tumor was detected. Also no gross changes in the hybridization patterns with the p53 probe were observed using the restriction enzymes *Hin*dIII and *Hin*fI in 33 and 18 blood/tumor pairs respectively.

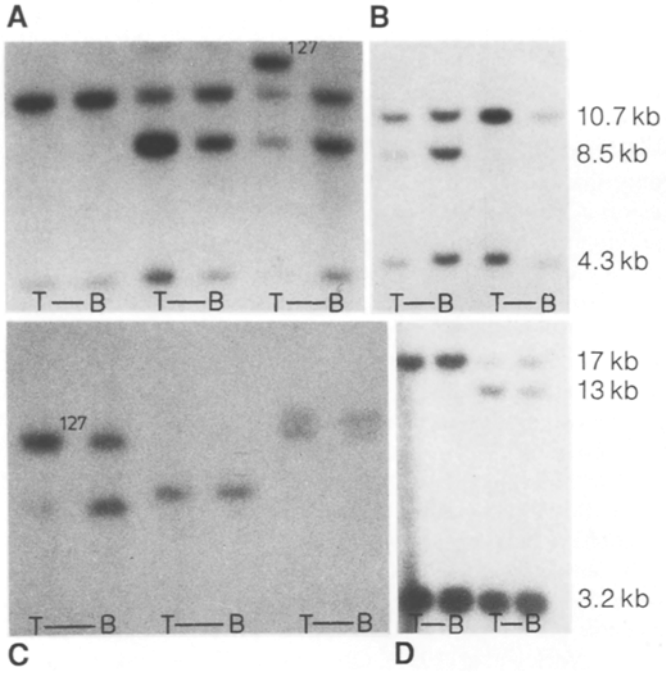

Fig. 1 A–D. Typical examples of Southern hybridization of pairs of tumor *(T)* and blood *(B)* DNA samples with different probes. **A, B** *Sca*I and **D** *Bgl*II restriction digests probed with pHP53B. **C** *Taq*I restriction digest probed with YNZ22. Band loss or marked quantitative decrease was evaluated as LOH

In a subset of 20 tumors tested by Northern analysis, p53 mRNA was always expressed, and no abnormally sized transcript was found.

Positive immunoreactivity with PAb 1801 and/or PAb 240 was detected in 25 of 50 cases (50%). Only nuclear staining of clearly malignant cells was scored as positive. Nine tumors were positive only with PAb 1801, whereas in all cases positive for PAb 240 at least a faint positive staining with PAb 1801 was seen. The number of immunostained cells in a positive tumor ranged from virtually all to only a few. Sometimes the positive cells were more focally distributed. Their staining intensity was also quite variable and not related to cytological features (Fig. 2).

Tumors with LOH at 17p13 were not more frequently p53 positive than those without. From 24 positive tumors 12 showed LOH and 12 did not. Among 23 negative tumors 11 showed LOH.

A subset of 22 tumors screened for the presence of point mutations by the SSCP technique (Fig. 3a) showed in 13 altered mobility of denatured PCR products from some of the tested exons from conserved regions of the p53 gene. On direct sequencing these showed the presence of different types of point mutations (manuscript in preparation). Two examples of such tumors are shown in Fig. 3b, c. In this limited investigation the presence of a point mutation could be correlated with overexpression in all except one tumor tested, which showed a comparatively lower staining intensity.

We examined these results for correlations with a number of parameters usually employed to characterize breast can-

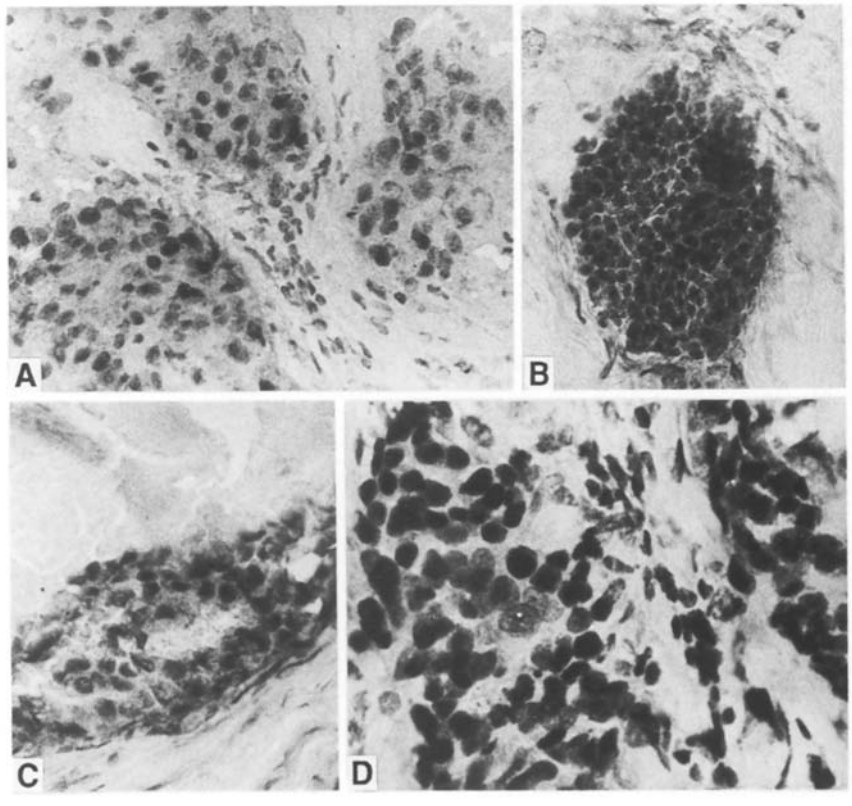

Fig. 2 A–D. Typical examples of immunohistochemical analysis in primary tumor sections. **A** Nuclear staining with PAb 1801 of varying intensity in a proportion of the malignant cells in carcinomatous lobules. **B** Homogeneous PAb 1801 immunoreactivity of all tumor cells in a lobule. **C** PAb 240 immunopositive intraductal carcinoma. **D** Infiltrating neoplastic cells showing nuclear immunoreactivity of variable degree with PAb 240. Sections **A, C,** and **D** are lightly counterstained with hemotoxylin

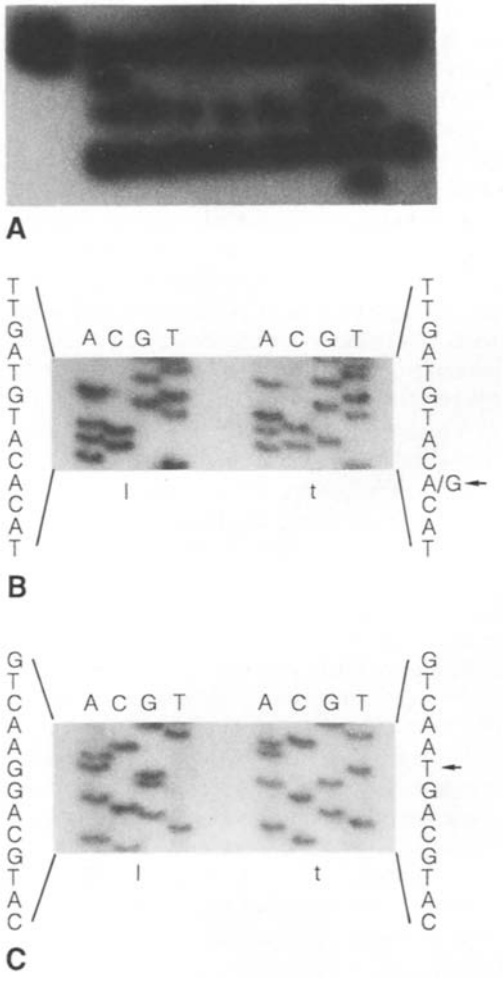

Fig. 3 A–C. Examples of single strand conformation polymorphism (SSCP) analysis and direct sequencing of 32P-labeled polymerased chain reaction (PCR) products: **A** SSCP analysis of exon 7 PCR products. *First lane* depicts an nondenatured (double-strand) normal sample; the *other lanes* show denatured samples from different tumors, some with mobility changes. Direct sequencing of PCR products showing (**B**) a point mutation, A→G (Cys→Arg), in codon 238 and (**C**) a point mutation G→T (Ser→Tyr), in codon 241 of different tumors *(t)* compared with their corresponding blood lymphocyte DNA *(l)*. Non coding sequences are shown

cer: pTNM, steroid hormone receptor status, grading, histology, and age. Only an association between higher nuclear grading and YNZ22 allele loss as well as p53 immunopositivity was found ($P = 0.037$, $P = 0.077$). Preliminary follow-up data show a trend of reduced metastasis free survival and dfs in tumors showing overexpression, but no other significant association of any of the (LOH) mutations investigated in this study and the disease free survival of the patients, some followed up to 45 months, was registered.

Also, no correlation with the amplification of the c-*erb*-B2 oncogene in this set of tumors (data not shown, Singh et al. 1992) was observed.

Discussion

Our results confirm previous reports on frequent loss of heterozygosity at 17p13 in human breast cancer (Mackay et al. 1988; Coles et al. 1990; Devilee et al. 1990; Chen et al. 1990; Varley et al. 1991). In addition we showed that deletions indeed affect the p53 locus.

We found a considerably higher rate of LOH (64%) at the p53 locus than at YNZ22 (43%). Furthermore, no tumor among the subset of 19 tumors informative for both loci showed LOH only at YNZ22, whereas 4 tumors had lost one p53 and retained both YNZ22 alleles.Thus our data support the view that the putative common target for the 17p13 deletions maps at least close to p53 and is probably p53 itself.

Recently Coles et al. (1990) also reported LOH at p53, but only amounting to 27% in a total of 81 informative tumors by using a genomic p53 probe (pBHP53) detecting a *Bam*HI polymorphism. Our finding of a conspiciously higher frequency of LOH (64%) detected with the p53 cDNA probe pHP53B, seems to be due to the different regions monitored by the probes. In their study allele loss at the YNZ22 locus was independent of LOH at p53. A correlation between YNZ22 allele loss and p53 mRNA overexpression led them to propose a gene close to the YNZ22 locus regulating the expression of p53. In contrast, our sample of tumors informative for both loci showed no evidence of YNZ22 allele loss alone. Possibly a number of tumors harboring p53 allele loss might have been missed with pBHP53.

In our series 25 (50%) of tumors were positively labeled when tested for immunoreactivity. Similar frequencies have recently been reported (Cattoretti et al. 1988; Bartek et al. 1990b; Varley et al. 1991). Often both p53 negative and positive cells were present in the same tumor. The distribution pattern resembled that described by Varley et al. (1991).

P53 overexpression is commonly believed to reflect underlying point mutations, especially if assessed with the monoclonal antibody PAb240, shown to be mutation specific in immunoprecipitation experiments (Bartek et al. 1990a, Gannon et al. 1990). In our subset of tested tumors we found a very close correlation of the presence of point mutations with overexpression of p53. There was only one exception in this series. This tumor, however, showed a comparatively lower intensity of expression, which extended from the nucleus partly into the cytoplasm. Davidoff et al. (1991) also found a complete correlation of point mutation and overexpression in breast cancer, whereas Bodner et al. (1991) first reported an imperfect correlation of p53 mutations and immunostaining in lung cancer cell lines, but further analysis in their series revealed that 16 out of 17 high p53 expressors harbored missense mutations. They further concluded that there are low- and high-expression groups of p53 mutants in lung cancer (Bodner et al. 1992). Also the recent study of Maestro et al. (1992) showed a clear association of immunohistochemical nuclear overexpression of p53 with structural and/or transcriptional alterations of the p53 gene in human sequamous cell carcinoma of the larynx. However, not all point mutations have been reported to result in protein overexpression (Rodrigues et al. 1990). Possibly T127 of this study belongs to this group.

Additional mechanisms also seem to influence p53 protein levels. Fibroblasts from patients with Li-Fraumeni syndrome contain amounts of p53 comparable to those of normal tissue, although they harbor point mutations that have previously been shown to encode high protein levels in tumors (Vogelstein 1990). In normal cells p53 expression is strictly regu-

lated during the cell cycle (Bischoff et al. 1990), a mechanism that may not altogether be lost in tumor cells.

The presence of p53 negative tumor cells in positive tumors is not understood. Cell cycle regulation or tumor subclones differing with respect to p53 alleles, and mechanisms influencing the protein level may account for this phenomenon (Bartek et al. 1990a; Varley et al. 1991).

Thus these data indicate that the rate of p53 point mutations in primary breast cancer is at least as high as 50%.

Our observations suggest an important role of p53 loss of function mutations as well as gain of function mutations. Among 47 carcinomas, in which both 17p13 allele loss, presumably reflecting p53 involvement, and protein levels could be assessed, 35 (74%) had lost some p53 wild-type activity through allelic deletions or point mutations. Fifty percent of the tumors harbor activating point mutations resulting in overexpression.

However, in individual tumors the precise interactions of alleles affected by different mutations are not well defined. In contrast to the high rate of allele loss no homozygous p53 deletion was noted in this series of 99 tumors. Furthermore no rearrangements were detected. Chen et al. (1991) also found neither rearrangements nor homozygous p53 deletions in their sample of 50 breast carcinomas. In addition our Northern analysis showed a normally sized transcript in all tumors examined so far. It seems that simple homozygous inactivation is at least not the predominant feature of p53 involvement in breast cancer. All p53 clones used successfully in transformation assays turned out to be mutant (for review see Lane and Benchimol 1990; Levine et al. 1991). Thus it is tempting to speculate that some oncogenic activity conferred on the gene by a point mutation accounts for the tendency to retain this allele grossly intact.

In this study p53 overexpression was not related to LOH at 17p13. Therefore it seems unlikely that point mutations leading to increased protein levels are the reason for p53 hemizygosity in breast cancer. This conclusion is also supported by the recent study of Varley et al. (1991). Thus from our data it is reasonable to assume that heterozygosity for some mutations is sufficient to provide a growth advantage in primary tumors, although cell culture experiments suggest the opposite (Chen et al. 1990; see Levine et al. 1991 for review).

Conflicting reports exist regarding the correlation of p53 mutations and clinical parameters in breast cancer (Cattoretti et al. 1988; Mackey et al. 1988; Varley et al. 1991). In contrast to others we did not find a higher incidence of p53 overexpression in ER-negative tumors. LOH and p53 overexpression were not associated with the stage of the disease; only a correlation with higher grading was found. The preliminary follow-up data point to no association of p53 LOH mutations and poor prognosis.

We hope that further investigations will clarify the situation, particularly in view of the therapeutical implications of the tumor suppressor gene concept.

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