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p53 Mutations Occur in Aggressive Breast Cancer[1]

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ABSTRACT

Using a polymerase chain reaction-single strand conformation polymorphism approach we analyzed 96 human primary breast tumors for the presence of mutations in exons 2, 5, 6, 7, 8, and 9 of the *p53* gene. These exons have been shown to comprise highly conserved sequences and the portion including exons 5 through 9 is believed to be the target for over 90% of the acquired mutations in human cancer. Eighteen tumors of the 96 (18.7%) tested showed reproducibly a variant band indicative of a mutation. Most (15 tumors) of the mutations were single nucleotide substitutions and G:C to A:T transitions were prevalent (6 tumors), G:C to T:A transversions came next (4 tumors), and guanines were always on the nontranscribed strand. Concomitant loss of the wild type allele and mutation of the other copy was observed in only 3 of 18 mutated cases; this is consistent with the heterogeneous cellular composition of breast tumors. Furthermore *p53* mutations were correlated to estrogen and/or progesterone receptor negative tumors, thus indicating their relationships to aggressive breast cancer. No association could be observed with DNA amplification events in these tumors.

INTRODUCTION

There is increasing evidence linking development and progression of cancer to an accumulation of mutations at the genomic level. These mutations are thought to result either in the activation of protooncogenes or in the loss of tumor suppressor gene potential. Thus their accumulation will eventually drive cancer cells further into anaplasia. Genomic alterations currently identified in human breast cancer include DNA amplification at five possible chromosomal locations (1–4) and loss of genetic material affecting 11 chromosomes (1–13). Moreover the inactivation of the *RB* gene has been reported in 19% of sporadic breast cancers (14). Most of these aberrations have either been associated with a negative outcome of the disease or found to define particular subgroups of tumors. More recently, mutations inactivating the tumor suppressing potential of the p53 gene have also been reported in sporadic breast cancer (15–22).

Originally thought of as an oncogene, it was only recently discovered that the wild type version of the p53 gene in fact possessed tumor suppressing potential (23, 24). A mutation affecting the coding sequence of the p53 gene consequently leads to the loss of its negative growth control properties or even to its oncogenic activation (25). Presently available results indicate that these inactivating mutations tend to cluster in a portion of the gene spanning codons 132 to 281 (corresponding to exons 5, 6, 7, 8, and 9) known to include four highly conserved sequence blocks (26). At present p53 inactivation proves to be the most common genetic alteration detected in human malignancies, since it has been found in almost every tumor type analyzed to date (for review see Ref. 27). With the exception of certain forms of hepatocellular carcinoma, where codon 249 is exclusively hit (28, 29), mutations in most tumor types do not seem to conform to a specific pattern, since they range from nucleotide substitutions to deletions of variable length and occur at any place in the above defined portion of the gene (27).

Given these facts and since we possessed a large breast tumor collection which we had previously studied for DNA amplifications and allelic imbalances, we undertook a systematic search for p53 mutations in all the exons including conserved sequence blocks (such as exons 2, 5, 6, 7, 8, and 9) in a subset of 96 breast cancer DNAs. Most solid cancer biopsies contain varying proportions of cancer and normal tissue. Furthermore breast tumors are known to have a very heterogeneous cellular composition and mutations, if present, are always at risk to be diluted out by sequences contributed by normal cells. Hence, the SSCP[4] technique on radiolabeled PCR fragments (30), which allows separation of the mutated molecule prior to sequencing, seemed a good approach to screen for mutated sequences in this type of biological material.

Our objectives were to characterize the mutations affecting the p53 gene and determine their incidence in this breast tumor panel, the final aim being to correlate them to the clinicopathological status of the tumors as well as to DNA amplification and allele loss data.

MATERIALS AND METHODS

Human Tumors and Biopsies. Breast tumor biopsies were collected within the last 5 years at the Paul Lamarque Val/d'Aurelle Cancer Center in Montpellier. Tumors were snap frozen in liquid nitrogen at most 30 min after surgical removal and stored at −80°C until extraction. Tumor typing was done according to the WHO Histological Typing of Breast Tumors (31). Steroid receptors were assayed as described (32).

DNA Extraction, Analysis, and Recombinant Probes Used. Genomic DNAs were extracted and the analysis for DNA amplification has been described (33–35). LOH was assessed using two polymorphic markers mapping to chromosome 17p pYNZ22 and pHF 12–2 (generously provided by Dr Y. Nakamura, Howard Hughes Institute at Salt Lake City). Conclusion could be reached only for patients showing a heterozygous pattern for at least one of the markers and where both tumor and normal lymphocytes DNAs could be analyzed.

PCR, SSCP, and Direct Sequencing. PCR conditions SSCP, direct DNA sequencing and autoradiography conditions were as described (36, 37). Twelve couples of primers, selected from the sequence according to Buchman and coworkers (38), were used. Six couples corresponding to sequences chosen within the introns and which are as follows:

Exon 2: 5'tgcagcagctagactgccttcc, 3'caatggatccactcacagtttcc

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[4] The abbreviations used are: SSCP, single strand conformation polymorphism; PCR, polymerase chain reaction; LOH, loss of heterozygosity; EGFr, epidermal growth factor receptor.

Exon 5: 5'gccgtgttccagttgctttatc, 3'gtagatggccatggcgcggaccg
Exon 5': 5'gtggattccacacccccgcccgg, 3'tcagtgaggaatcagaggcc
Exon 6: 5'ctggagagacgacagggctg, 3'gccactcagaaccaccctta
Exon 7: 5'cctcatcttgggcctgtgtt, 3'tcagcggcaagcagaggctg
Exon 8: 5'aggacctgatttccttactg, 3'ctcgcttagtgctccctgggggc
Exon 9: 5'gcctcagattcacttttatcacc, 3'ctttccacttgataagaggtc

six couples corresponding to more internal sequences which are as described (36).

Analysis of Tissue Sections. First 5-μm-thick sections were cut into paraffin embedded tissues for histological reading. These were fixed in a hematoxylin-eosin solution. Then a 20-μm-thick section was collected and placed into 1 ml of xylene (or octane). Paraffin was eliminated in this way by three extractions. Xylene was eliminated by ethanol and the pellet was dried. Then, 300 μl of 20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 10 mM EDTA, 0.6% sodium dodecyl sulfate, and 75 μg/ml proteinase K were added and the mixture was incubated with gentle agitation overnight at 37°C. Proteinase K was subsequently denatured by a 10-min heat treatment at 95°C. The solution was allowed to cool and 150 μl of 5 M NaCl were added, briefly vortexed, and centrifuged for 10 min in a microfuge. The supernatant was taken diluted with 0.5 volume of sterile water and DNA was precipitated by addition of 2 volumes of ethanol. DNA was resuspended in 30 μl of Tris-EDTA and an aliquot was used directly in the PCR reaction.

RESULTS

Detection and Characterization of *p53* Mutations. A total of 96 breast cancer DNAs were screened by a combination of the PCR and SSCP methods for mutations in the coding sequence of the *p53* gene. Radiolabeled PCR fragments are run on a nondenaturing polyacryacrylamide gel and mutant sequences appear as shifted bands (Fig. 1A, *arrows*). The variant conformers were isolated and reamplified, and their nucleotide sequence

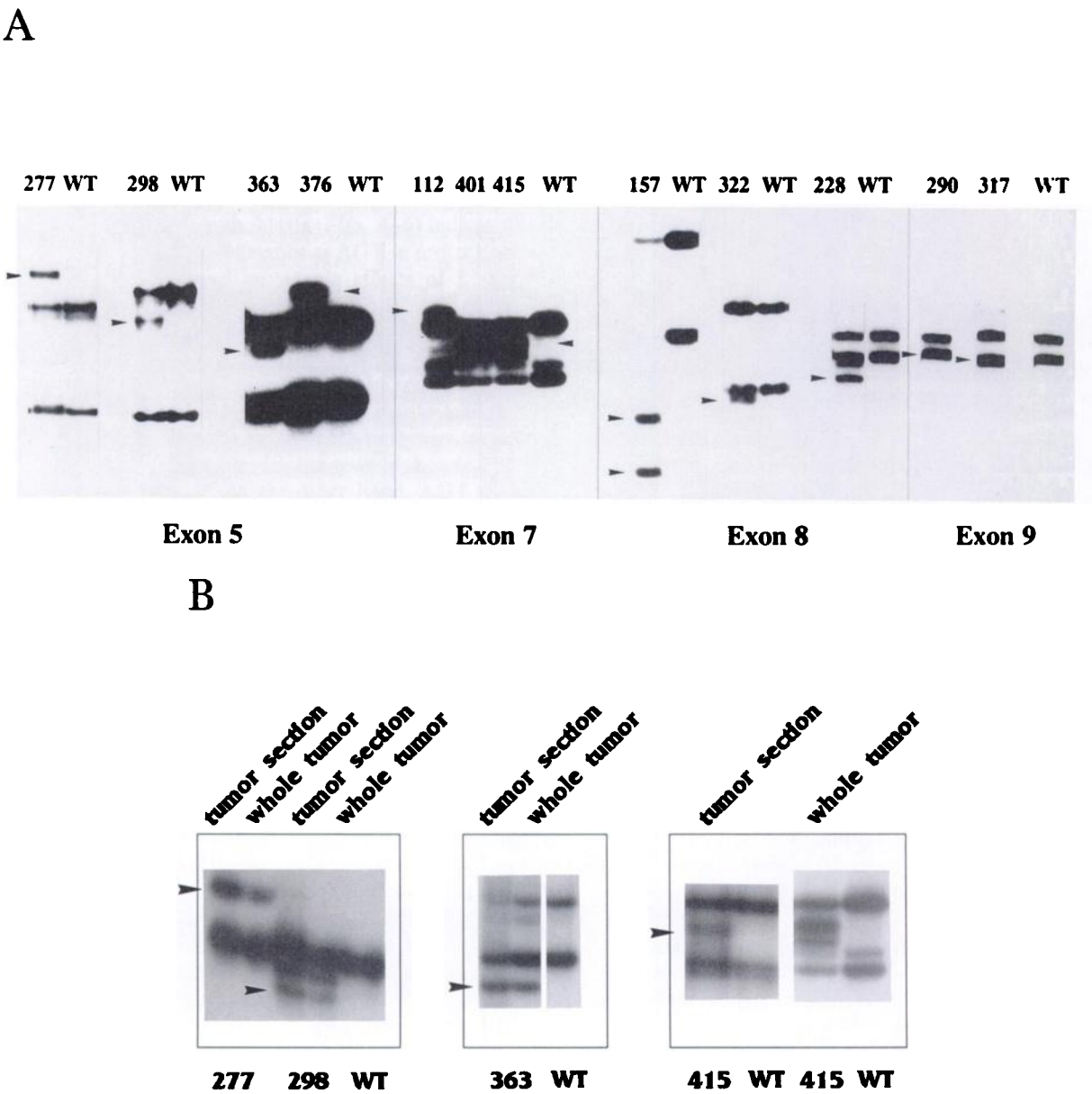

Fig. 1. Detection of *p53* mutations by SSCP analysis. Radiolabeled PCR products were heat denatured and quickly loaded onto a nondenaturing polyacrylamide gel. Single-stranded molecules take specific conformations which will define their electrophoretic pattern. Variant conformers characterize mutant sequences (arrowheads). A, examples of the different mutations observed presented by exons. Numbers on top of each lane, tumor samples. B, SSCP analysis of DNA extracted from paraffin embedded blocks. A 5-μm section fixed and stained in hematoxylin-eosin was first taken for histological analysis and a 20-μm section was then processed for DNA extraction and SSCP analysis. Numbers on bottom of each lane, tumor samples. Tissue sections and whole tumor SSCP are compared for four breast carcinomas (277, 298, 363, and 415) and show in all cases the same pattern.

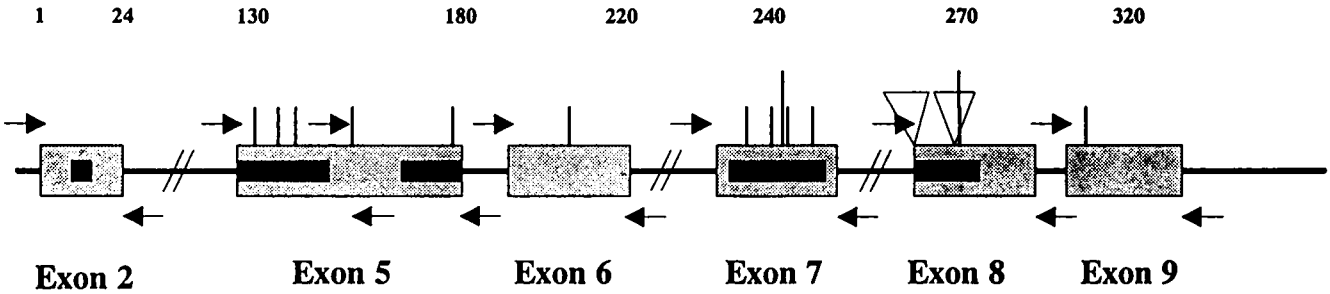

Fig. 2. Map of the p53 mutations detected in breast carcinomas. The genomic map of the region of the p53 gene were screened for mutations has been schematized as follows: □, exons; lines, introns; ■, blocks of conserved amino acid sequences; arrows above and below, site of the PCR primers. Vertical lines, site of mutations found at each particular location. ▽, the two microdeletions.

Table 1 *Description of the p53 mutations found in breast cancer*

Tumor sample	Exon	Codon	Nucleotide substitution	Amino acid substitution	LOH[a]
241	5	176	TGC to TTC: G-T	Cys to Phe	No
277[b]	5	135	TGC to TGG: G-C	Cys to Trp	No
	5	139	AAG to AAT: G-T	Lys to Asn	No
298	5	132	AAG to AAT: G-T	Lys to Stop	No
363	5	157	GTC to TTC: G-T	Val to Phe	No
376	5	165	CAG to CTG: A-T	Gln to Leu	No
361	6	196	CGA to TGA: C-T	Arg to Stop	No
112	7	249	AGG to AGC: G-C	Arg to Ser	No
325	7	232	ATC to AGC: T-G	Ile to Ser	No
401	7	248	CGG to TGG: C-T	Arg to Trp	No
415	7	248	CGG to TGG: C-T	Arg to Trp	No
456	7	255	ATC to AAC: T-A	Ile to Asn	No
590	7	241	TCC to GCC: T-G	Ser to Ala	No
157	8	Deletion from codon 279 to 287			
192	8	272	GTG to ATG: G-A	Val to Met	No
228	8	Deletion from codon 265 to intron 8			Possible
322	8	272	GTG to ATG: G-A	Val to Met	No
290	9	306	CGA to TGA: C-T	Arg to Stop	Yes
317	9	Sequence not determined			Yes

[a] LOH was detected here only by SSCP.
[b] Tumor 277 presented two mutations on the same allele.

was determined in order to identify the mutation. This shift in electrophoretic mobility allowed us in some instances to identify mutations heavily diluted by wild type sequences. Since breast tumors are known to have a very heterogeneous cellular content, SSCP appears to be well suited to this context. Sequences analyzed included exons 2, 5, 6, 7, 8, and 9 (Fig. 2).

We identified 18 (18.7%) breast tumor DNAs presenting a mutated p53 allele (Table 1). All of the mutations, except one (tumor 317), were identified by direct dideoxy sequencing of the mutated fragment isolated from the SSCP gel and reamplifiifed by PCR. Interestingly one sample (tumor 277) presented two sequential mutations on codons 132 and 139 affecting the same allele (Table 1). All the mutations were found in exons 5 through 9, with exons 5, 7, and 8 being the most frequently involved (Table 1; Fig. 2). Although exon 2 contains the first domain of conserved sequences defined by the study of Soussi et al. (26) no mutation could be found there. The majority (15 of 17) of the mutations were single nucleotide substitutions leading to a nonconservative amino acid change. The two remaining tumors (samples 157 and 228) both exhibited internal deletions of 23 and 11 base pairs, respectively, in exon 8. Although some regions, including codons 132 through 139, codons 248–249 as well as codon 272, seemed frequently involved, no indication of mutational hot spots could be evidenced in our breast tumor series. Almost every possible nucleotide substitution has been found amoung our 15 single base change mutants (6 G:C to A:T, 4 G:C to T:A, 2 G:C to C:G, 2 A:T to C:G, and 2 A:T to T:A). G:C to A:T transitions were predominant (40%) and one-half of these occurred at one of the CpG sites present in this portion of the p53 coding sequence. G:C to T:A transversions came next and interestingly all occurred in the G to T orientation, the guanine being on the nontranscribed strand.

Due to the conformational polymorphism, produced by the presence of a mutation, SSCP allows determination of whether a tumor has retained or lost the wild type p53 allele. Only 3 tumors of the 18 presenting a mutation on 1 allele had lost the wild type counterpart (Table 1; Fig. 1A; tumors 157, 290, and 317). In some of the tumors, retaining the wild type allele, the signal ratio between the variant and the wild type band was largely in favor of the latter, clearly indicating that the mutation originated from a minor component of the tumor (for example see samples 376 and 112 in Fig. 1). Often, however, a 1:1 ratio between the mutated and the normal alleles was observed leading us to suspect some tumors to be heterozygous for the p53 mutation (Fig. 1A, samples 401 and 415). Since the analysis had been performed on DNA extracted from a frozen biopsy we could not discount the fact that the wild type sequences were contributed by normal cells present in the tumor tissue. To address this question we performed a PCR/SSCP analysis on DNA extracted from 20-μm-thick tissue sections cut into paraffin embedded blocks from the archival tissue bank. The actual proportion of tumor cells composing the section was estimated upon histological reading on a serial section for 4 tumors (277, 298, 363, and 415) presenting both the wild type and the mutated conformers (Fig. 1B). The tissue sections had a diameter of approximatly 4 mm and tumor and stromal cells were counted within four microscopic fields using the 16 × 10 focal for each of them. This assessment was done on a morphological

basis. The estimated proportion of tumor cells corresponded to the mean of the numbers counted in each of the 4 fields and all 4 sections displayed 85 to 90% of tumor cells. Interestingly all four samples displayed, upon SSCP analysis, a similar pattern in bulk DNA and DNA extracted from tumor sections; *i.e.*, both the wild type and the mutated allele could be evidenced (Fig. 1B).

Relations with Clinicopathological Parameters. The presence of a mutated p53 allele was computed against parameters commonly used in the clinical management of breast cancer (Table 2). We included in the statistical analysis only the 17 tumors the mutation of which had been characterized by sequencing. Mutations in the p53 gene were prevalent in steroid receptor negative tumors: χ^2 analysis revealed statistically significant correlations with estrogen receptor negative ($P = 0.044$) and progesterone receptors negative ($P = 0.012$) tumors (Table 2). Moreover the incidence of p53 mutations in tumors from patients older than 50 years was 3 times higher than that found in younger patients suggesting a preferential trend according to the age of the patient (Table 2). Noticeably no association or preferential trend could be evidenced with histopathological grade or nodal invasion.

Relations with DNA Amplification and Allele Losses on the Tip of Chromosome 17p. Most of the tumor DNAs (from 79 to 86 of 96 total) presently analyzed for mutations in the p53 coding sequence had also been tested for DNA amplifications at five loci [BEK (FGFR2), ERBB2, FLG (FGFR1), MYC, and sequences on chromosome 11q13 including INT2, HST, and BCL1]. Moreover a subset of 44 tumors gave informative data concerning LOHs on the tip of chromosome 17p. These LOHs were assessed by pooling the results obtained with polymorphic probes D17S5 (pYNZ22) and D17S1 (pACY184). Table 3 shows the distribution of the p53 mutations according to each of the events listed above, as well as the result of the correlation analysis. Although in some instances mutations did not seem evenly distributed, none of the tumors amplified for the BEK gene presented a mutant p53 allele and interestingly p53 mutations were almost twice as frequent in tumors with an amplification at the ERBB2 locus compared to tumors showing a normal copy number for this gene, no statistically significant association was observed (Table 3). Furthermore it is interesting to note that p53 mutations were no more associated with LOHs at chromosome 17p than they were with DNA amplification. These data thus indicate that p53 mutations occur independently from other genetic alterations found in breast cancer and particularly from deletions detected on the same chromosomal arm carrying the p53 gene.

Table 2 *Correlations of p53 mutations with clinicopathological parameters*

Histopathological grading was done according to the WHO Histological Typing of Breast Tumors (31) and hormonal receptors were assayed as described (29). All tumors with assays above 20 fmol/mg of protein were scored positive for hormonal receptors. ER, estrogen receptors; PR, progesterone receptors; NS, not significant. The χ^2 test for dependence relationships with continuity correction was computed using the Epi Info 5.00 software from the Centers for Disease Control Epidemiology Office, Atlanta, GA.

Clinical parameters	*p53* mutations		
	n		%
S&B grade			
1	0/5		0.0
2	9/46		19.5
3	7/30		23.3
P		NS	
Nodal status			
0	6/47		12.7
1 to 3	4/19		21.0
>3	4/23		17.4
P		NS	
ER+	6/55		11.0
ER−	11/37		29.7
P		0.044	
PR+	4/49		8.1
PR−	13/42		30.9
P		0.012	
Age			
>50 yr	9/51		21.6
<50 yr	2/28		7.1
P		NS	
Total	17/96		17.7

Table 3 *Correlations of p53 mutations with current DNA amplification data*

Most of the tumor DNAs have previously been studied for gene amplification (29, 32). Amplification was considered positive when the signal ratio target probe/control was repetitively >2.

Genomic alteration	*p53* mutations		*P*
	n	%	
Amplification			
BEK			
Yes	0/12	0.0	
No	15/74	20.3	NS
ERBB2			
Yes	6/21	28.0	
No	9/58	15.5	NS
FLG			
Yes	3/14	21.4	
No	12/72	16.6	NS
MYC			
Yes	5/20	25.0	
No	10/59	17.0	NS
11q13			
Yes	3/18	16.6	
No	12/62	19.3	NS
LOH			
Yes	3/12	25.0	
No	4/32	12.5	NS

DISCUSSION

Using a combination of PCR and SSCP methods we detected 18 (18.7%) breast tumors bearing a mutated p53 allele of 96 samples screened. No gross structural rearrangement could be detected by Southern blotting (data not shown) and most (15 of 17) of the identified mutations were nucleotide substitutions, the remaining 2 being internal deletions. Mutations scatter along the portion spanning exons 5 through 9 with no apparent hot spot and no mutation could be found in exon 2, although this exon also contains a block of conserved sequences (26). Of the 15 single nucleotide changes 6 (40%) appear to be G:C to A:T transitions among which 3 occurred at 1 of the CpG sites present in the p53 coding sequence (27), thus suggesting a possible deamination of a methylcytosine. G:C to T:A transversions ranked second (4 of 15) and interestingly there seems to be a strand bias since in all of them the guanine was on the nontranscribed strand. A general conclusion on mutation patterns in breast cancer may, however, need larger numbers of characterized mutants.

Incidences reported for other tumor types vary from a high of 70 (possibly 100%) in small cell lung carcinoma (38) to rare occurrences in thyroid carcinoma (39). Our finding would thus place breast cancer in the lower half for incidence of p53 mutations since 30 to 40% seems a reasonnable average when all

tumor types are combined. Several studies have reported p53 mutations in primary breast cancers and observed frequencies vary from 13 to 46% (17–22). At present the reasons for these variations remain unclear. These may stem from the different experimental approaches used, such as direct sequencing on PCR amplified fragments, chemical cleavage, constant denaturant gel electrophoresis, or SSCP. It is noticeable that in the two studies using SSCP respective incidences were 17 and 46% (21, 22). Alternatively these differences may stem from differences in tumor sampling. In any case our numbers may in fact represent a minimal figure (a) because we cannot rule out that some mutations might be missed by SSCP, although we have made extensive tests and brought some changes to the original technique (36), and (b) because we did not analyze the entire coding sequence of the gene. It is, however, noticeable that even the highest frequency of mutations in the p53 coding sequence is about one-half of the 50 to 62% of breast tumors staining positively with anti-p53 antibodies in some immunocytochemical studies (40–42). However, variability in staining have been reported: different ranges of positivity have been observed when results obtained with two antibodies are compared (18, 20); and a proportion of the cancers present focal staining corresponding to clusters of stained cells disseminated within negative tumor cells (18, 41–43). Homogeneous staining was, indeed, observed in 22 to 34% of the cases (18, 41). It is, at present, difficult to estimate accurately the correspondence between positive immunostaining and the presence of a mutation at the genetic level. It may prove of interest that in a limited set of tumors showing strong and homogeneous immunocytochemical staining all DNAs bore mutated p53 sequences (18). It could be that mutations present in tumors with focal immunocytochemical positivity are missed by molecular techniques, because of a high dilution factor by wild type sequences.

The p53 gene has been assigned to the short arm of chromosome 17, band 17p13, in a region where LOHs are observed at a high incidence in a number of different human cancers. This made the p53 gene a prime candidate for a target tumor suppressor in the area. However, although mutations were more frequent in the group of tumors showing a LOH at 17p, we observed a lack of correlation between the occurrence of LOHs at the distal end of chromosome 17p (detected with polymorphic probes pYNZ22 and pHF12-2) and the presence of mutations in the p53 gene. These data, indicating that both events may occur independently, are in agreement with results presented by several other groups (18–20, 22). This could thus suggest the presence of at least another tumor suppressor gene, in addition to p53, on the short arm of chromosome 17. Transfection experiments as well as the inherited nature of p53 mutations in Li-Fraumeni syndrome argue strongly in favor of the recessive nature of the p53 mutation (44, 45). Thus, to be phenotypically expressed a mutated p53 allele requires the loss of its wild type counterpart. Using SSCP we could discriminate the wild type from the mutated allele and hence could verify that 3, possibly 4, (Table 1) of the 18 tumors with a mutation in the p53 gene presented only the mutated conformer. The remaining 15 exhibited both the mutated and the wild type forms. We and others made similar observations indicating that a mutation on one allele of the p53 gene did not necessarily imply the loss of the wild type copy in breast (18, 19), lung (46), and ovarian cancer (36). This could be the result of the cellular heterogeneity of human solid tumors, which are known to be composed of varying proportions of normal and tumor cells and where cancer cells bearing a mutated p53 gene may lie next to other cancer cells with a normal p53 locus.

Correlation studies with clinicopathological parameters showed that p53 mutations were significantly associated to steroid receptor negative tumors, whereas they remained independent of either nodal involvement or tumor grading. The loss of estrogen and/or progesterone receptors is, in breast cancer, indicative of evolved and undifferentiated tumors. Estrogen and progesterone receptor negativity is universally considered a bad prognostic indicator (47). Thus the association with the absence of steroid receptor suggests that p53 mutations are related to aggressive breast cancer. This is in concordance with data presented by Horak et al. (42) which show a correlation between positive staining with anti-p53 antibodies and overexpression of EGFr in a series of 111 primary breast cancers. Indeed, high levels of EGFr expression have been related to a negative outcome of the disease and although these authors did not find a statistically significant correlation between p53 staining and absence of estrogen receptor they show that EGFr overexpression is correlated to estrogen receptor negative tumors. Moreover we were interested in looking for possible overlaps with DNA amplifications in order to test for potential sets of genetic alterations in breast cancer. The association of p53 mutations with estrogen receptor negative and/or progesterone recepter negative tumors similar to that observed for amplification of the ERBB2 gene (33) was suggestive of such a possibility. Our data show that, although a positive trend can be seen with respect to the amplifications of MYC and ERBB2, no statistically significant association could be observed. In conclusion p53 mutations occur independently of DNA amplifications and are related to evolved breast cancer. It should be of interest to test for their occurrence in premalignant or early breast cancer stages.

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