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Molecular and immunohistochemical analysis of p53 mutations in scrapings and tissue from preinvasive and invasive breast cancer

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Abstract Mutations of the p53 gene appear to be one of the most common abnormalities in human cancer. Although many studies have been published about p53 alterations in breast cancer, data on molecular biological detection of p53 mutations in in situ lesions are still rare, and the implications for breast cancerogenesis are unclear. Tissue samples from 83 patients with different stages of breast cancer and from 13 patients with benign breast lesions were screened for p53 gene mutations by polymerase chain reaction (PCR) followed by temperature-gradient gel electrophoresis (TGGE). p53 protein accumulation was analysed by immunohistochemistry (IHC). Samples were gained from fresh-frozen tissue, scrapings, or paraffin embedded tissue. Additionally, 23 pairs of primary tumours and corresponding lymph nodes were examined. p53 gene aberrations were found in 55.7% of the infiltrating carcinomas, in 31.5% of the ductal carcinomas in situ (DCIS) and in one atypical ductal hyperplasia. A positive correlation was seen with high-grade tumours and with comedo. There was no statistically significant relationship with respect to age, menopausal status, tumour size, hormone receptor status or lymphatic invasion. Concordance between TGGE and IHC was seen in only 63% of the cases analysed. However, with regard to p53 mutation screening by TGGE, a high significance (P = 0.0008) was seen between standard tissue extraction and our scrape preparation technique. Among 8 pairs of primary tumours and their corresponding lymph node metastases, only 3 harbored identical p53 mutations in the same exon, while in 5 cases with mutant p53 in the primaries, no mutation was seen in the lymph node. Our data indicate that p53 mutations are frequent in breast tumours associated with unfavorable prognosis, including high-grade or the comedo histotype. There is evidence that p53 gene alterations occur early in breast cancerogenesis, as mutations were detected not only in in situ carcinomas but also in atypical ductal hyperplasia.

Key words: Protein p53 · Breast cancer · Immunohistochemistry · p53 mutation · Temperature-gradient gel electrophoresis (TGGE)

This work is dedicated to Prof. Dr. H.-E. Stegner on the occasion of his 65th birthday

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Introduction

Alterations of the p53 gene are probably the most common genetic abnormalities in human malignancies, including breast cancer [18]. There is evidence of an important role for p53 protein in gene transcription, DNA repair and induction of apoptosis [24, 25]. Wild-type p53 binds in a sequence-specific manner to double-stranded DNA [22]. Several genes, including mdm2 or p21, are known to be regulated by p53. p53 levels rise in response to DNA damage and lead to p21-mediated G_1 arrest in the cell cycle [13, 21]. This transient interruption of replication gives time to repair DNA prior to duplication or may lead to programmed cell death [38]. Mutations of the p53 gene lead to conformational change of the protein and often loss of DNA binding activity. In some cases mutant protein acts in a dominant-negative fashion, forming inactive hetero-oligomers with wild-type p53 [28]. The majority of point mutations (93%) occur between amino acid residues 120 and 290 in the DNA binding domain corresponding to the exons 5–8 of the p53 gene [7].

From the viewpoint of the clinical course, the phenotype and the genotype, breast cancer is a heterogeneous disease. The incidence of p53 overexpression in breast cancer ranges from 13% to 62% [5, 10] and appears to be generally higher than the incidence detected by mole-

cular techniques. Previous data indicate that p53 mutation could be an early event in mammary tumour evolution, as p53 overexpression has also been reported in some in situ carcinomas [35].

In this study we looked for p53 accumulation in breast samples by immunohistochemistry. Alterations in exon 5–8 of the p53 gene were detected by polymerase chain reaction (PCR) and temperature gradient gel electrophoresis (TGGE). Samples were gained from fresh frozen tissue, scrapings and paraffin-embedded tissue. Attention was paid to the value of the special scrape preparation technique in preinvasive and invasive lesions.

Our aim was to study the role of p53 in breast cancer evolution by determining the p53 mutation rate in benign breast samples, in ductal carcinoma in situ (DCIS), particularly in the comedo subtype, and in invasive carcinomas. We also examined whether or not p53 mutations are conserved during breast cancer progression and lymph node metastasis.

Materials and methods

Tissue samples were obtained from 80 patients who had undergone surgery at the Department of Gynaecology, University of Hamburg for either in situ carcinoma (n = 19) or invasive breast cancer (n = 61). For invasive cancer, 23 pairs of primary tumours (18 with mutations proven by TGGE) and corresponding axillary lymph nodes were examined. The patients ranged in age from 24 to 90 years, with a mean age of 48. Forty-four patients were premenopausal. In addition, 13 samples of nonmalignant breast lesions and 3 samples obtained from patients with intramammary recurrence were analysed. The tissue samples were fresh frozen in liquid nitrogen immediately after excision and stored at –80°C until DNA analysis, while in some cases a special scrape preparation technique was applied. p53 analyses in lymph nodes were carried out on paraffin-embedded tissue. Histological classification done by light microscopy revealed 32 ductal, 17 comedo, 8 lobular, 1 mucinous, 1 medullary, and 1 anaplastic carcinomas, and 1 carcinoma with a mixed histological pattern (Table 1). Among the in situ carcinomas were 15 comedo and 4 noncomedo DCIS. Most of the invasive tumours and all of the in situ carcinomas were examined by gross-mount sections to estimate the size and the extent of the in situ component.

In scrape preparations, the surface of a freshly cut tumour was carefully scraped off with a scalpel blade. Cell clusters obtained by this procedure were suspended in 2 ml of sodium chloride solution and stored at –80°C; 400 µl of this cell suspension was then centrifuged for 5 min. The pellet was washed three times with 200 µl PBS and finally dissolved in 100 µl PBS containing 20 µg proteinase K, 5% Tween 20 and 5% Nonidet P-40. The solution was incubated for 1 h at 55°C, followed by 10 min at 94°C. The samples were used for PCR were 10 µl in volume.

DNA was extracted from fresh-frozen tissue using cesium chloride, guanidinium isothiocyanate and proteinase K, followed by phenol-chloroform extraction and ethanol precipitation as described by Chomcynski and Sacchi [8]. For the analysis of lymph nodes, 8-µm sections were cut from paraffin-embedded tissue with sterile blades. In the case of metastasis, tumour tissue was microdissected from the sections and treated as described previously [23].

For TGGE, exons 5, 6, 7 and 8 of the p53 gene were separately amplified by PCR as described by Scholz et al. [32]. The sequences of the PCR primers were obtained from Dr.V.C. Sheffield, Iowa City, IA [6], with the exception of the anti-sense primer of exon 8, which was designed by our group. With the object of to detecting single-base alterations more efficiently, one primer in each group contained a GC-rich clamp [33] (Table 2). PCR products were extracted with phenol/chloroform/isoamylalcohol followed by ethanol precipitation. Sediments were dissolved in ethanol, lyophilized and dissolved in 30 µl buffer containing 20 mM MOPS, 1 mM EDTA, pH 8.0. TGGE was performed as described previously [20, 32].

p53 immunostaining was performed using mouse monoclonal p53 antibody Ab-6 (Oncogene Science, Dianova, Hamburg, Germany), which identifies a denaturation-resistant epitope located near the amino terminus of the p53 protein between amino acids 37 and 45. Deparaffinized slides were washed in phosphate-buffered saline (PBS) and pre-incubated in 0.5% blocking serum for 20 min to reduce nonspecific binding. The monoclonal p53 antibody Ab-6 was diluted 1:150 in 1% BSA (Sigma) and used for overnight incubation at 4°C. A biotinylated sheep anti-mouse antibody (Amersham-Buchler, Braunschweig, Germany; diluted 1:100) was applied to the sections as secondary antibody for 1 h at 37°C, followed by streptavidine-alkaline phosphatase conjugate

Table 1 Histological classification and p53 analysis by TGGE and immunohistochemistry (*TGGE* temperature-gradient gel electrophoresis)

Histological diagnosis	TGGE						IHC	
	Analysed cases	Positive cases	Tissue extraction				Analysed cases	Positive cases
			Exon 5	Exon 6	Exon 7	Exon 8		
Normal breast and benign lesions								
Fibroadenoma	5	0					4	0
Lipoma	1	0					1	0
Fibrocystic disease	7	1			1		7	0
In situ carcinoma								
Comedo	15	5		2	3	2	15	6
Noncomedo	4	1		1			4	1
Invasive carcinoma								
Ductal	32	12	1	5	6	1	31	14
Comedo	17	12	3	2	4	5	17	8
Lobular	8	2			2	1	6	1
Mucinous	1	0					1	0
Medullary	1	1		1			1	0
Other	2	1			1	1	2	1
In breast recurrence	3	2	2		1		3	1

Table 2 Primers used for PCR

Exon	Sense	Anti-sense
5	5′-(GC 40) TTCCTCTTCCTACAGTACTC-3′	5′-CTGGGCAACCAGCCCTGTCGT-3′
6	5′-(GC 40) ACGACAGGGCTGGTTGCCCA-3′	5′-AGTTGCAAACCAGACCTCAG-3′
7	5′-(GC 40) TCTCCTAGGTTGGCTCTGACTG-3′	5′-GCAAGTGGCTCCTGACCTGGA-3′
8	5′-CCTATCCTGAGTAGTGGTAATC-3′	5′-(GC 40) CCGCTTCTTGTCCTGCTTGCTT-3′

(GC 40) GC-rich clamp: CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG

(Gibco-BRL, Eggenstein, Germany; diluted 1:1000). Colour development was performed with NBT/BCIP (NBT:0.3 mg /ml; BCIP: 0.2 mg/ml) for 15 min in the dark without counterstaining. Only nuclear staining in the tumour tissue was scored: no positive cell nuclei –, up to 25% positive nuclei +, 26–50% positive nuclei + +, 51–100% positive nuclei + + +.

All statistical analyses were performed by Statistica 4.5 software StatSoft (Tulsa, Okla, USA). Chi-square analysis was used to determine statistical associations. Mean values for patient age and tumour size were compared with the aid of a *t*-test.

Results

Structural aberrations of the p53 gene, as indicated by altered mobilities of the amplified DNA fragments (Fig. 1), were detected by TGGE in 29 (55.7%) of the invasive breast carcinomas and in 6 (31.5%) of the ductal carcinomas in situ (DCIS). In 33 of the 35 cases with mutant p53, germline mutations or polymorphisms could be ruled out either by comparison with normal tissue from the same patient showing wild-type p53 or by the typical TGGE pattern with predominance of the wild-type or mutant homoduplex band. The 2 remaining cases harboured exon 6 mutations, probably natural polymorphisms in codon 213, because of the typical TGGE patterns identical to those in cases with polymorphisms demonstrated by direct sequencing [27]. These 2 cases were not included in the evaluation, as the mutations were found in normal tissue.

In the invasive carcinomas, 12 mutations were found in exon 7, 8 in exon 8, 7 in exon 6, and 4 in exon 5. In the DCIS group, TGGE revealed 3 mutations in exon 6, 3 in exon 7, and 2 in exon 8. Three tumours showed a double mutation (exon 5/8, exon 6/7, exon 7/8), and in 1 case of DCIS there was even a triple mutation in exons 6/7/8. Of 3 patients with intramammary recurrences, 2 had mutations; 1 double mutation (exons 5/7), and 1 mutation in exon 5.

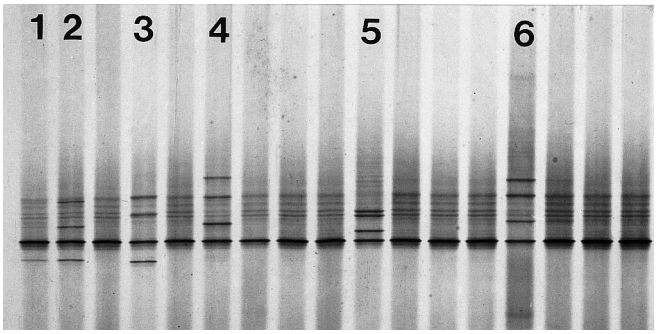

Fig. 1 TGGE (temperature-gradient gel electrophoresis) analysis of exon 7. p53 Mutations in invasive breast cancer (*1,2,3,5,6*) and in an atypical ductal hyperplasia (*4*)

Table 3 Correlation between TGGE from scrape analysis and tissue extraction

Scrape analysis	TGGE	
	Positive	Negative
Positive	6	0
Negative	2	10

χ^2=11.25, *P*=0.0008

It is remarkable that in 1 of our 13 cases of benign breast disorders we detected a mutation in exon 7. Several gross mount sections were prepared, but the histological state was found not to exceed atypical ductal hyperplasia when examined independently by two pathologists. The distribution according to histological diagnosis is shown in Table 1. Of the 18 pairs of primary carcinomas (with mutant p53) and corresponding lymph nodes, 16 were available for evaluation, as DNA could not be extracted from 2 lymph nodes. Eight nodes showed metastases. In 3 of them, exon 7 mutations were detected. These mutations matched with the mutations found in the primary tumours. In all 3 cases IHC was also positive as well.

Scrape preparations were made in 30 cases (20 invasive carcinomas, 6 DCIS and 4 benign tissues). In 18 cases, DNA extracted from frozen tissue had also been analysed. All cases showing mutations in scrape preparations were also positive after tissue extraction, but 2 mutations detected in tissue (both DCIS) could not be found in scrape preparations. Nevertheless, there was a significant correlation between the two methods (P = 0.0008). Also, the location of the mutations found corresponded to the tissue extraction findings (Table 3).

TGGE profiles indicating a structural aberration of the p53 gene were correlated with common clinicopathological parameters (Table 4). There seems to be a relationship with histological tumour type, as mutations were more frequent in comedo carcinomas (70.6%) and less common in other ductal (34.4%) or lobular (25.0%) carcinomas (P = 0.01). A strong correlation was found with high grade (P = 0.01). Statistically significant correlations were not observed with any further variable but mutant p53 tended to be more frequent in premenopausal patients and in angioinvasion.

Table 4 Association between clinicopathological variables, p53 mutations and p53 overexpression

Variable	TGGE				IHC			
	No. of cases	No. of p53 mutations	χ^2	*P*-value	No. of cases	Positive cases	χ^2	*P*-value
Invasive carcinoma								
Histological type								
Comedo	17	12 (70.5%)			17	8 (47.0%)		
Noncomedo	44	16 (36.4%)	5.78	0.01	43	18 (41.8%)	0.13	0.71
Lymph node metastasis								
N^+	35	18 (51.4%)			34	17 (50.0%)		
N^-	22	10 (45.5%)	0.19	0.66	22	8 (36.4%)	1.00	0.31
Grading								
G I	7	3 (42.8%)			7	1 (14.3%)		
G II	21	5 (23.8%)	I+II vsIII		20	9 (45.0%)	I+II vsIII	
G III	33	20 (60.6%)	6.25	0.01	33	16 (48.5%)	0.29	0.58
Hormone receptors								
ER +	40[a]	17 (42.5%)			39	15 (38.5%)		
ER –	20	10 (50.0%)	0.30	0.58	20	10 (50.0%)	0.71	0.39
PgR +	41[b]	17 (41.5%)			40	16 (40.0%)		
PgR –	18	10 (55.5%)	1.00	0.31	18	9 (50.0%)	0.50	0.47
Menopausal status								
Pre –	34	19 (55.8%)			33	15 (45.5%)		
Post –	27	9 (33.3%)	3.08	0.07	27	11 (40.7%)	0.13	0.71
Lymphatic invasion								
Positive	20	12 (60.0%)			20	11 (55.0%)		
Negative	41	16 (39.0%)	2.38	0.12	40	15 (37.5%)	1.66	0.19
[a] ER unknown for 1 patient								
[b] PgR unknown for 2 patients								
In situ carcinoma								
Histological type								
Comedo	15	15 (33.3%)			15	6 (40.0%)		
Noncomedo	4	1(25.0%)	0.10	0.75	4	1 (25.0%)	0.31	0.58
Hormone receptors								
ER +	14	5 (35.7%)			14	5 (35.7%)		
ER –	5	1 (20.0%)	0.42	0.51	5	2 (40.0%)	0.03	0.86
PgR +	10[c]	4 (40.0%)			10	3 (30.0%)		
PgR –	8	2 (25.0%)	0.45	0.50	8	4 (50.0%)	0.75	0.38
Menopausal status								
Pre –	12	6 (50.0%)			12	5 (41.6%)		
Post –	7	0 (0%)	5.11	0.02	7	2 (28.6%)	0.32	0.56

[c] PgR status unknown for one patient

Of the 18 women with ductal carcinoma in situ (DCIS), 6 had mutations of p53, all of them premenopausal ($P = 0.02$). Five of them had comedo-type lesions, and the remaining tumour was a 60-mm lesion of cribriform type. Repeated gross-mount sections revealed small areas of invasion, representing no more than 3% of the total lesion.

Sixty primary breast carcinomas, 19 DCIS, and 13 benign breast diseases were analysed for p53 protein accumulation by immunohistochemistry (Table 4, Figs. 2, 3). None of the benign breast lesions stained positive for p53. Among the 19 DCIS, 7 (36.8%) showed an accumulation of p53 protein. Staining patterns were weak in 5 cases, while moderate or strong immunostaining was found in 2 cases of DCIS harbouring p53 mutations. However, in 3 further cases associated with p53 mutations, staining was completely absent. p53 overexpression was observed more frequently in comedo-type lesions and in premenopausal women.

Twenty-six (43.3%) of the invasive breast carcinomas accumulated nuclear p53 protein. However, strong immunostaining was rare (4 cases), and only 14 invasive carcinomas with p53 protein accumulation harboured gene mutations. As in DCIS, p53 overexpression was found more frequently in comedo-type lesions, and additionally in cases with lymph node metastasis and negative hormone receptors. A significant correlation could be seen between positive staining and increasing tumour size ($P = 0.02$). p53 overexpression was associated with mutant p53 neither in DCIS ($P = 0.4$) nor in invasive car-

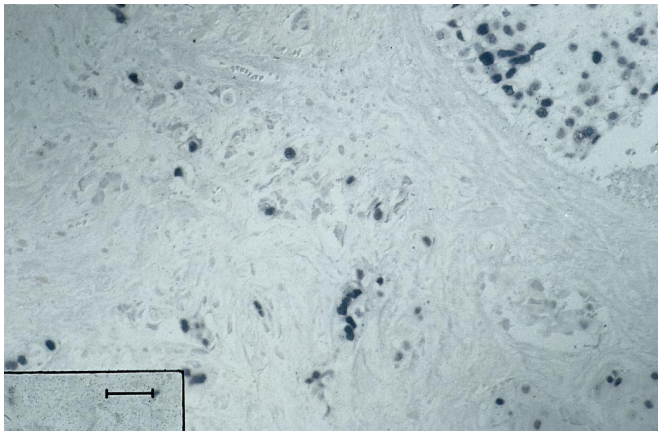

Fig. 2 Immunohistochemical detection of p53 expression in ductal carcinoma in situ and invasive tumour. Original magnification×250

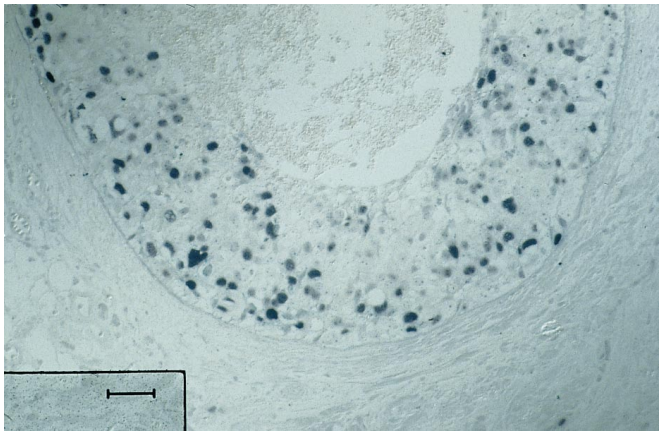

Fig. 3 Higher magnification of p53 staining in ductal carcinoma in situ. Original magnification ×400

cinomas (P = 0.3). In 11 infiltrating tumours with an extensive in situ component, staining patterns were compared in both types of lesions on the same section. Six of these were found to be positive (Fig. 2), and 3 to be negative in both components. In the remaining 2 cases, only the infiltrating lesion stained positive.

Of the 23 lymph nodes analysed, 12 were metastatic. All nonmetastatic nodes stained negative, whereas 5 of the metastatic lesions were positive. In 3 of these positive cases, TGGE revealed p53 mutations in exon 7. The staining patterns of the primary neoplasms corresponded with the metastatic lesions in 8 cases. There was a shift from positive to negative in 4 cases.

Discussion

We have examined different stages of breast cancer and benign breast lesions for p53 protein expression and genetic alterations, using TGGE to screen for unknown mutations in amplified DNA fragments corresponding to exons 5–8 of the p53 gene. Like the denaturing gradient gel electrophoresis (DGGE) [15], TGGE is based on the melting properties of DNA, using temperature instead of a chemical denaturating gradient. The TGGE is rapid and convenient, and appears to be more efficient and easier to interpret than the more commonly used single strand conformational polymorphism analysis (SSCP) shown previously [32]. In a series of 94 ovarian tumours, we demonstrated the reliability of the TGGE by direct sequencing [20].

In order to gain tumour cells rapidly, we developed a simple method for sampling and preparation for genomic analysis. The scrape preparation can be applied to very small samples and needs only short proteolytic treatment instead of time-consuming extraction procedures. Multiple tumour cell clusters can easily be obtained by scraping the cut surface of a fresh tissue carefully with a small scalpel blade. A high correlation to the standard extraction procedure was found.

We analyzed a variety of breast tissues, both benign and malignant, by TGGE and compared them by immunohistochemical (IHC) methods. All benign lesions stained negative for p53. Interestingly, 1 patient with an atypical ductal hyperplasia harboured an exon 7 mutation, but also without detectable p53 protein expression. A point mutation with positive p53 staining has been described in normal tissue by Eeles et al. [12], but that case was a constitutional mutation in a woman who developed multiple tumours. In our case, no mutation was detected in tissue obtained from outside of the lesion, and the patient has developed no tumour so far. Nevertheless, Dupont and Page [11] have demonstrated that patients with atypical hyperplasia have a significantly increased risk of developing breast cancer. The fact that this high-risk lesion can be associated with p53 gene mutation gives further credence to the theory that p53 gene alteration might be an early or even an initiating event in breast cancer cancerogenesis. p53 mutations were detected in 28 of 61 invasive carcinomas (45.9%), a higher incidence than that reported by others [2, 4, 31, 34, 35]. This may be explained by a bias to high-risk criteria, such as low age, positive lymph nodes or aggressive tumour type.

A strong relationship was seen between p53 gene alterations and comedo-type invasive carcinoma, an histotype generally associated with an unfavorable prognosis. p53 mutations were significantly correlated with low differentiation and were more frequent in cases of angioinvasion and in premenopausal women. The p53 expression as detected by immunohistochemistry was related to larger tumour size and negative hormone receptor status. Barbareschi et al. [3] reported similar findings in a larger study on 178 node-negative patients and also showed a significant association with lower age and higher tumour grade, but this did not translate to clinical outcome.

The p53 overexpression in our study did not correlate with p53 mutations found by TGGE, however. Similar findings have been described by others [1, 19, 26, 34] and by us in a recent study on cervical and vulvar cancer, using the same methods [27]. The reasons why mutations

are not associated with p53 expression are manifold: confirmed mutations with negative IHC staining can occur due to nonsense mutations, frameshift errors, silent and some splice mutations; other possible explanations are modifications of epitopes or the effect of different methods of fixation on the stability of the p53 protein [14].

It seems that p53 immunostaining does not inevitably reflect genetic alteration. p53 as a part of the DNA damage response can be up-regulated or stabilized by various factors, such as ultraviolet light, viral products or the cellular environment itself [17, 36]. There is increasing evidence to show that immunostaining properties depend on conformational changes or interactions of p53 with other cellular proteins, since staining patterns change during different points of the cell cycle [16] and are independent of mRNA expression. Comparison of TGGE and IHC results (Table 4) indicates that the association of p53 status and other variables is much more clear cut if gene alteration is studied by molecular biology rather than by IHC alone.

Many data have been published on the distribution of p53 mutations in invasive carcinomas [2, 4, 31, 34, 35], but studies on DCIS are still limited. Bartek et al. [4] found pronounced overexpression of p53 in 2 out of 9 cases of pure DCIS. Strong staining for p53 was primarily seen in comedo-type DCIS [14, 35, 37]. In the largest study on 143 cases of DCIS, detectable p53 expression was confined almost exclusively to large-cell DCIS, a subgroup that includes comedo-type DCIS [30]. In a recently published study, O´Malley et al. [29] showed positive immunostaining in 4 out of 12 comedo DCIS, whereas all the noncomedo DCIS (n = 27) stained negative. SSCP and direct sequencing revealed only 1 mutation among the 4 positive lesions. It was therefore concluded that p53 mutation has to be considered as a rare event even in comedo DCIS. Among the 15 cases of comedo DCIS in our study, 6 (40%) were immunopositive and 5 (33%) harboured a mutant p53 gene, but only in 3 cases did the data correspond to IHC, indicating that mutations are easily missed when not confirmed at the molecular level. In contrast to O´Malley, we believe that p53 mutation is frequent at least in comedo-type DCIS. Moreover, our data demonstrate a high degree of correspondence between in situ and invasive components within the same lesion.

We consider that it is easier to distinguish between high-grade DCIS and low-grade DCIS, than between low-grade DCIS and atypical ductal hyperplasia. The latter might be variants of the same group of lesions, but the small number of cases makes us cautious in interpretation.

Finally, we investigated whether mutations are conserved in metastastic lesions. Our p53 staining of the paired primary carcinomas and lymph nodes are not in accordance with the results of Davidoff et al. [9], who found a total correspondence in a set of 22 pairs analysed by immunohistochemistry. In contrast to Davidoff et al., we examined all metastatic lesions at the molecular level, revealing 3 mutations in exon 7, which corresponded to the findings in the primary tumours. At least in these cases, the p53 mutations are conserved during metastatic spread, but it must be remembered that in 5 metastatic lesions with positive primaries no mutations were found at all. This might be due to dilution of the DNA by normal lymphocytes or stromal cells, as in some cases very small amounts of cancer cells were present in the lymph nodes. A more convenient explanation, however, is genotypic heterogeneity among the cancer cells. The fact that no new mutation was found at metastatic sites lends credence to the hypothesis that p53 occurs well before metastatic spread.

In conclusion, our findings indicate that p53 mutations are mainly seen in tumours with an unfavorable prognosis, such as comedo-type or high-grade tumours. p53 Gene alterations were detectable in all stages of breast cancerogenesis, including atypical ductal hyperplasia.

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