p53 Mutations and Expression in Breast Carcinoma *in Situ*

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The p53 tumor suppressor gene is altered in approximately half of human cancers. Although p53 mutations are common in invasive breast carcinoma, few have been identified in breast carcinoma *in situ* (intraductal breast carcinomas). Most studies of p53 in breast carcinoma *in situ* are immunohistochemical studies of p53 staining in paraffin-embedded tissue sections. Few studies have isolated the tumor cells and subjected them to DNA sequence analysis. The current study was undertaken to characterize p53 in a cohort of breast carcinoma *in situ* cases, both with and without invasive disease. Fifty-eight frozen breast biopsy samples were used for these investigations. Twenty-seven cases had only ductal carcinoma *in situ* (CIS) and 31 cases had evidence of both invasive and *in situ* carcinoma. DNA sequence alterations in exons 2 through 11 of p53 were screened by the single-strand conformational polymorphism technique. Exons with altered mobility were sequenced. Among breast CIS cases without invasive disease, 22% had p53 mutations and 7% had DNA sequence alterations of unknown significance. Analysis of breast CIS with concurrent invasive disease demonstrated p53 mutations in 19% of cases and one (3%) DNA alteration of unknown significance. Each carcinoma having a p53 mutation in the breast CIS component had the identical mutation in the invasive component of the same tumor indicating a clonal relationship between the two tumor components. p53 protein overexpression was identified in 22% of pure intraductal breast carcinomas and in 35% of breast CIS with invasive disease. Comparison of immunostaining and DNA sequence alterations showed a significant association between overexpression and mutations ($P = 0.0037$) in cases of CIS without invasion, and similarly between overexpression and mutations in cases of CIS with invasion ($P = 0.007$). p53 mutations and p53 overexpression were relatively common in intraductal breast carcinomas but were not observed in adjacent normal breast lobules or ducts in 9 cases available for DNA analysis. The frequency of p53 alterations when comparing breast CIS with and without an invasive component indicated that p53 muta-

tions usually occur before invasion during the progression of breast cancer, as is observed for a number of other adult solid tumors. (Am J Pathol 2000, 156:183–191)

The molecular genetic profile of p53 alterations has been well characterized in invasive breast cancer. In contrast, precursors to invasive disease, such as ductal carcinoma *in situ* (ductal CIS), are far less understood. Most studies of p53 in ductal CIS report only immunohistochemical analysis of p53 expression using paraffin embedded tissues.^{4–16} Very few studies have used molecular biology screening techniques.^{17–22} Of these, only three studies screened cohorts of ductal CIS for p53 sequence alterations using single-strand conformational polymorphism (SSCP) analysis or constant density gel electrophoresis (CDGE) and confirmed all potential SSCP/CDGE altered mobility patterns using DNA sequence analysis.^{17–19} However, these studies screened only exons 4–9,^{17,19} or exons 5, 7, and 8¹⁸ for alterations. In total, 13 of 82 cases had altered mobility (16%) and 8 (10%) were confirmed by sequencing. To gain a greater understanding of the entire mutational spectrum of ductal CIS, a study of all coding exons of p53 was undertaken. To our knowledge, this present study is the first to completely characterize all coding exons of p53 for alterations in breast carcinoma *in situ*. Using 58 flash-frozen breast CIS cases, the p53 gene was first screened for altered mobility patterns in exons 2 through 11 using SSCP analysis and then the presence of mutations was confirmed by DNA sequence analysis. p53 expression was analyzed by immunohistochemistry also using frozen breast CIS specimens. SSCP analysis and sequencing of exons 2 through 11 revealed novel mutations of p53. The findings further indicated that p53 is mutated in breast CIS as frequently as in invasive breast carcinoma and that the invasive carcinomas are clonally derived from breast CIS as indicated by mainte-

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nance of the same p53 sequence in intraductal and invasive disease from the same case.

Materials and Methods

Tissue

The use of human tissue in this study was reviewed and approved by the University of Southern California Institutional Research Committee. Fifty-eight frozen breast biopsy samples with CIS, stored at -186°C under liquid nitrogen and accessioned sequentially between 1988 and 1996 by the USC Breast Tumor and Tissue Bank, were used for this investigation. Twenty-seven cases had only carcinoma *in situ* and 31 cases had evidence of both invasive and *in situ* carcinoma. Among the cases with only CIS, 13 (48%) breast CIS were classified as comedocarcinoma, 4 (15%) as cribriform ductal CIS, 3 (11%) as micropapillary ductal CIS, 3 (11%) as intraductal papillary carcinoma, and 4 (15%) as solid ductal CIS. In the cases with invasive disease and CIS, 10 (32%) breast CIS were classified as comedocarcinoma, 8 (26%) as cribriform ductal CIS, 3 (10%) as micropapillary ductal CIS, 1 (3%) as papillary ductal CIS, and 9 (29%) as solid ductal CIS. Frozen tissue sections stained with hematoxylin and eosin (H&E) were used to confirm the histological composition of the specimens. Those cases having microinvasion were classified as CIS with invasive disease. Microinvasion, defined as microscopic invasion of breast stroma beyond the ductal-lobular unit <1 mm in maximal diameter as well as invasive breast carcinoma, was identified in histological frozen sections by a board-certified pathologist (M. F. P.). Only one case in this series had microinvasion (C425). Nuclear grading of the CIS was classified from 1 to 3, with 2 cases classified as nuclear grade 1 (low grade), 32 as nuclear grade 2 (intermediate grade) and 24 as nuclear grade 3 (high grade).

Microdissection and DNA Isolation

The different tissue components of the breast specimens were separated according to histomorphological phenotypes by mechanical microdissection with the assistance of a microscope. Ten to twenty frozen serial sections, 10 μm thick, were cut and fixed in 95% ethanol. The initial section was stained with H&E and subsequent sections for microdissection were stained with ethyl green. Microdissection was performed to separate benign breast epithelium, breast CIS and invasive carcinoma. Care was taken to minimize contamination of epithelial cells with stromal cells. For example, only the central portion of the CIS from the ductal lumen was excised from the ductal epithelium. Any possible area of potential contamination was discarded. After microdissection, the DNA of each component was extracted as previously described.²³ In three cases, formalin-fixed, paraffin-embedded tissue was used in addition to frozen tissue. These cases were sectioned and subjected to microdissection and DNA extraction as described previously.²³

p53 Primer Design

Polymerase chain reaction (PCR) was used to amplify exons 2–11. Ten different sets of 19- to 25-mer oligonucleotide primers were designed using the genomic sequence of p53 (GenBank accession numbers U94788 and X54156).²⁴ Primers were designed to span each exon of the p53 open reading frame and sufficient bases of the intronic sequence to ensure the splice donor and splice acceptor sites were included for analysis.²⁴

SSCP and DNA Sequence Analysis

The SSCP technique was initially used as a screen for DNA sequence alterations in p53 exons 2 through 11 as described elsewhere.²³ Exons with altered mobility were analyzed for DNA sequence changes as described²³ using the CircumVent Thermal Cycle Sequencing Kit (New England Biolabs, Beverly, MA) or the ThermoSequenase Kit (Amersham, Arlington Heights, IL), according to the manufacturer's instructions. DNA sequencing was performed twice, from separate PCR reactions, to confirm that the mutation found was not an artifact of PCR.

The term "DNA mutations" is used below to refer to changes in the coding sequence giving rise to a change in amino acid sequence. In contrast, the term "DNA sequence alterations of unknown significance" is used below to refer to base changes outside of the coding region (exon), such as base changes in the intron sequence or in the 3' untranslated region. Finally, the term "DNA polymorphisms" is used to refer to base changes that are either silent and do not code for different amino acids, or base changes which code for amino acid substitutions that have been reported to maintain normal p53 function.

p53 Immunohistochemistry

p53 protein was identified in tissue using the peroxidase anti-peroxidase immunohistochemical technique. Frozen sections 4 μm thick were incubated with anti-human p53 mouse monoclonal antibody (DO-7 or Ab240, Dako Corp., Carpinteria, CA or Zymed Laboratories, South San Francisco, CA, respectively) as previously described.²⁴ The percentage of positively immunostained tumor cell nuclei was determined from the number of nuclei containing immunoreaction product divided by the total number of nuclei, both immunostained and unstained. A minimum of 100 tumor cells were scored. Those breast tissues with p53 immunostaining in at least 10% of the cell nuclei were considered to have p53 overexpression, while those with less than 10% p53 immunostained nuclei were considered to be within the normal range of p53 expression.²⁴

DNA Ploidy Analysis

DNA ploidy analysis was performed on all samples as previously described.²⁵

Figure 1. SSCP analysis of exon 8 of p53 in ductal CIS. Lanes marked wt (wild-type) show typical two-band conformation, representing the two complementary strands of DNA. Lane marked MT (mutant) shows the typical four-band conformation, with two bands migrating at the same rate as normal DNA and two migrating more slowly.

Statistical Analyses

The association between p53 overexpression and p53 mutations was evaluated using Fisher's exact test.

Results

p53 Mutations

SSCP screening identified altered mobility patterns in 25 of 58 cases for at least one of the exons evaluated (Figure 1). Sequencing confirmed the presence of mutations in 12 cases (Figure 2 and Tables 1 and 2), the presence of DNA sequence alterations of unknown significance in three cases (Tables 1 and 2), and the presence of DNA polymorphisms in 8 cases (Table 3).

Figure 2. p53 Immunohistochemistry and DNA sequencing on a single tumor. One representative case with sufficient invasive and *in situ* carcinoma as well as benign ductal epithelium was microdissected and DNA was extracted separately. Each separate morphology is represented in the top 3 photographs labeled "H&E" showing, from left to right, normal breast epithelium (normal breast), breast ductal carcinoma *in situ* (breast CIS), and invasive breast carcinoma (invasive cancer). Original magnifications, $\times 560$. Immunostaining for p53 using the mouse monoclonal antibody DO7 is depicted in the second row of photographs (p53 IHC). p53-overexpressing nuclei are identified by the brown diaminobenzidine immunoprecipitate only in breast CIS and invasive disease. DNA sequencing (third row of photographs) shows part of the analysis of a 16-bp deletion in exon 9 of p53 found only in the breast CIS and invasive disease. On the left of each sequencing gel is the normal sequence, and in the sequencing panels of the CIS and invasive disease the mutant sequence is noted next to the normal sequence, after the backslash.

Table 1. p53 Alterations in Breast Carcinoma *in Situ* without Invasion

Number	Histology	Grade	p53 immunohistochemistry		DNA sequence analysis		
			nl epithelium	CIS	Exon	Codon	Type
C804	comedo	2	14% hyp	0%	none	no mutation	—
C128	papillary	2	20% hyp	0%	none	no mutation	—
C723	solid	2	NA	23%	none	no mutation	—
C2885*	comedo	3	0%	0%	4	112delG	nonsense
C953	comedo	3	0%	59%	7	Arg248Gln	missense
C1022	cribriform	2	NA	34%	7	Arg248Gln	missense
C693*	papillary	3	NA	0%	7	Arg249Gly	missense
C1026	comedo	2	NA	0%	7	Arg249Gly	missense
C1046	micropap	3	0%	10%	9	Gly325Stop	nonsense
C96*	comedo	3	0%	47%	11	post stop [†]	AUS
C2545	comedo	3	NA	40%	11	post stop [†]	AUS

IHC, immunohistochemistry; NA, not available; AUS, alteration of unknown significance; hyp, hyperplasia; IDC, invasive ductal carcinoma; nl, normal.

*Cases with sufficient benign ductal epithelium for DNA analysis.

[†]This AUS is located at nucleotide 18717, Genbank accession no. X54154.

Table 2. p53 Alterations in Breast Carcinoma *in Situ* with Invasion

Number	Histology	p53 immunohistochemistry		Breast CIS				Invasive breast carcinoma			
		Nuclear grade	nl epithelium	CIS	DNA sequence analysis			Histology	p53 IHC	Exon	Codon
					Exon	Codon	Type				
C2330	comedo	3	0%	20%	none	no mutation	—	IDC	24%	—	—
C2798	comedo	3	NA	25%	none	no mutation	—	IDC	30%	—	—
C2829	comedo	3	NA	20%	none	no mutation	—	IDC	30%	—	—
C3124	comedo	2	NA	60%	none	no mutation	—	IDC	70%	—	—
C1518	cribriform	2	NA	40%	none	no mutation	—	IDC	45%	—	—
C470	micropap	2	0%	14%	3rd intron [†]	cctctga→ccAtcCtTga	AUS	IDC	NA	—	—
C2929	solid	2	0%	0%	5	Gln165Stop	nonsense	IDC	0%	5	165delG
C1989*	comedo	3	0%	24%	6	Leu194Ile	missense	IDC	22%	6	Leu194Ile
C2325	comedo	2–3	0%	53%	6	Tyr205Ser	missense	IDC	50%	6	Tyr205Ser
C1752	cribriform	2	NA	65%	7	Arg248Trp	missense	IDC	61%	7	Arg248Trp
C425*	micropap	3	0%	45%	8	Ala276Pro	missense	NA	NA	NA	NA
C2666*	comedo	3	0%	90%	9	del: codons 323–328	nonsense	IDC	90%	9	del: codons 323–328

IHC, immunohistochemistry; NA, not available; AUS, alteration of unknown significance; hyp, hyperplasia; IDC, invasive ductal carcinoma; nl, normal.

*Cases with sufficient benign ductal epithelium for DNA analysis.

[†]This AUS sequence starts at nucleotide 11990 in the p53 genomic sequence (Genbank # X54154).

Analysis of Mutations in Breast CIS Lacking Invasive Disease

In 10 of 27 (37%) breast CIS cases with no concurrent invasive carcinoma there were six (22%) mutations (Table 1), two (7.5%) alterations of unknown significance (Table 1) and two (7.5%) polymorphisms (Table 3). Three of 6 mutations, and both alterations of unknown significance, had comedocarcinoma histology. One mutation had papillary histology, one had micropapillary histology, and one had cribriform histology. One polymorphism had papillary histology and one had solid histology (Table 3). The DNA sequence alterations were distributed between exons 4 and 11 (Figure 3). One deletion of a G in codon 112 was predicted to result in a shift in the open reading frame and the introduction of a stop at codon 122 in exon 4. Four mutations, two Arg248Gln and two Arg249Gly, were found in exon 7. One Ala276Pro mutation was identified in exon 8 and one Gly325Stop mutation was identified in exon 9. A C→T polymorphism (CCG→CCA) was found in the 4th exon at codon 36, a C→G polymorphism (CCC→CGC) at codon 72 and two identical T→A changes, at nucleotide 18717 in the p53 genomic se-

quence, were noted in exon 11 after the termination codon (Table 1).

DNA from benign epithelium from four cases was evaluated for DNA sequence alterations. No sample of benign epithelium had a p53 mutation when assessed by SSCP. This was confirmed by DNA sequence analysis in two cases with a p53 mutation in the DNA from the carcinoma. However, one case with a DNA sequence alteration of unknown significance, a T→A base change (at nucleotide 18717) after the termination site in exon 11, did contain the same sequence alteration in adjacent benign tissue as would be expected for a DNA polymorphism.

Analysis of Mutations in Breast CIS and Invasive Disease

Among the 31 cases of breast CIS with invasive disease, 6 (19%) had mutations, 1 had a DNA sequence alteration of unknown significance, and 6 had DNA polymorphisms. Three of the mutated cases had comedocarcinoma histology, one had a micropapillary histology, one had a

Table 3. p53 Polymorphisms in Breast Carcinoma *in Situ*

Number	Histology	Presence of invasion	DNA sequence analysis			
			Exon	Codon	Sequence	Amino acid
C3540	papillary	no	4	36	CCG→CCA	Ser→Ser
C2398	solid	no	4	72	CCC→CGC	Pro→Arg
C1895	cribriform	yes	4	72	CCC→CGC	Pro→Arg
C2286	solid	yes	4	72	CCC→CGC	Pro→Arg
C1518	cribriform	yes	6	213	CGA→CGC	Arg→Arg
C2340	solid	yes	6	213	CGA→CGC	Arg→Arg
C2775	comedo	yes	6	213	CGA→CGC	Arg→Arg
C2821	cribriform	yes	6	213	CGA→CGC	Arg→Arg

solid histology, and one had a cribriform histology. The alteration of unknown significance had a micropapillary histology. The 6 polymorphisms consisted of 1 case with comedocarcinoma, 2 cases with solid histology, and 3 cases with cribriform histology (Table 2). The DNA alterations occurred between the third intron and the ninth exon.

The six breast CIS cases with p53 mutations had sequence alterations distributed from exon 5 through exon 9 (Table 2 and Figure 3). No mutations were identified in exons 2, 3, 4, 10, or 11. One case had a Gln165Stop mutation in exon 5. Two mutations were in exon 6, coding for a Leu194Ile and a Tyr205Ser change. Two mutations occurred at the DNA binding codons: an Arg248Trp mu-

tation was found in exon 7 and an Ala276Pro change in exon 8. Another case had 16 bases deleted in exon 9, a loss of codons 323 to 328. One alteration of unknown significance occurred in the third intron, a 3 base insertion beginning at base 11992. Among these cases, there were a total of 6 with polymorphisms: 2 in codon 72 of the 4th exon, a C→G transversion (Pro72Arg), and 4 in codon 213 of the sixth exon, an A→C transversion (Arg213Arg). Comparisons of the mutations in the CIS and invasive components in all 5 cases with mutations and sufficient tissue available revealed the same mutation in both components. A sixth case with a DNA mutation did not have sufficient invasive material for analysis.

Figure 3. p53 mutations in ductal CIS compared to the molecular structure of p53. **Top:** The graph is a comparison of mutations found in this study and in previous studies. The horizontal axis shows the evolutionarily conserved regions in gray (labeled "conserved regions"), as described in Harris et al.^{26,30} Below this bar is the exon structure of p53, showing part of exon 4, exons 5 to 9, and part of exon 10. Above the horizontal bars, mutations from the present study are noted in two colors: the red columns denote cases of breast CIS with invasion; mutations in these cases were found in both CIS and invasive components. Mutations shown in green are from cases of breast CIS without invasion. Below the horizontal axis bars, the mutations from previous studies are shown. **Bottom:** The mutation spectrum in the present study compared to the molecular structure of the p53 protein. Regions of p53 that interact with DNA are shown in yellow; regions that serve as support for the DNA interacting domains are shown in green.

In cases lacking p53 mutation in the breast CIS component, no mutations were detected in the invasive component. As in the cases with only CIS, no mutations of p53 were found in the benign epithelium of 3 cases with p53 mutations in both CIS and invasive disease (Table 2).

p53 Protein Expression

In the entire cohort, 17 of the 58 cases (29%) showed p53 overexpression by immunohistochemical staining in the carcinoma. Two cases had p53 overexpression only in benign hyperplastic tissue (Table 1). p53 protein was either exclusively or predominantly nuclear (Figure 2). Cytoplasmic staining without nuclear staining was not identified. In frozen tissue sections immunostaining is observed in nuclei of a low percentage of normal, proliferatively active tissues. Therefore, we have used 10% immunostained tumor cell nuclei as a value for separation of normal expression from overexpression.²⁴ The percentage of cells with nuclear staining for p53 varied from 2 to 90%. Among those cases showing any staining only two cases had less than 10% of tumor cell nuclei positively immunostained. One of these had a p53 polymorphism and the other had wild-type p53.

p53 overexpression was noted in 6 of 27 (22%) cases of breast CIS without invasion (Table 1). The percent of nuclei immunostained ranged from 10% to 47% and the average staining was 32.5%. Among the 6 cases of CIS with p53 overexpression, 3 were classified as comedocarcinoma, one as solid, one as cribriform and one as micropapillary CIS. Six cases had sufficient benign epithelium for interpretation of immunostaining. Four cases with benign ductal epithelium lacked p53 immunostaining, while two cases exhibited p53 immunostaining in areas of benign ductal hyperplasia. Neither case with benign hyperplasia had a p53 mutation in the CIS portion. The amount of hyperplastic breast tissue available from these cases was insufficient for DNA sequence analysis (Table 1). Eleven (35%) of 31 cases of CIS with invasive disease had p53 overexpression (Table 2). Seven cases with CIS components had comedocarcinoma histology, two had micropapillary histology, and two had cribriform histology. The percent of nuclei immunostained ranged from 14% to 90% and the average staining among these samples was 41.5%. Six cases with p53 overexpression in the CIS had no expression in the normal breast epithelium. Only one case (C425) with both p53 immunostaining and p53 mutation had sufficient benign DNA for sequencing. The benign tissue from this case did not have a mutation in 3 separate areas sampled.

Comparison of p53 Mutations with Protein Overexpression

Overall, 11 of the 58 cases (19%) had both p53 overexpression and either mutations or sequence alterations of undetermined significance, 37 had neither mutations nor overexpression, 6 had overexpression without mutations and 4 had a p53 mutation but lacked overexpression. Overexpression of p53 was highly correlated ($P <$

Table 4. Comparison of p53 Overexpression with p53 Mutations in 58 Cases

Mutation	p53 Overexpression		Total
	Positive	Negative	
Any	11*	4	15
None	6	37	43
	17	41	58

$P < 0.0001$.

*Three alterations of unknown significance were included in this group.

0.0001, Fisher's exact test) with the presence or absence of mutations/sequence alterations (Table 4).

Among the 27 breast CIS cases without invasive disease, 5 had both p53 mutations or alterations of unknown significance and overexpression, 18 had neither mutations nor overexpression, 3 had p53 mutations but lacked overexpression, and 1 had overexpression but lacked p53 mutations. Among the 31 breast CIS cases with invasive carcinoma, 6 cases had both p53 mutations or DNA sequence alterations of unknown significance and overexpression, 19 had neither p53 mutations nor overexpression, 5 had p53 overexpression but lacked a p53 mutation, and 1 had a p53 mutation but lacked p53 overexpression. Comparison of immunostaining and DNA sequence alterations revealed a significant association between overexpression and p53 alteration ($P = 0.0037$) in cases of CIS without invasion, and similarly between overexpression and DNA alterations in cases of CIS with invasion ($P = 0.007$).

Discussion

Breast CIS, or intraductal breast carcinoma, is the earliest morphologically recognizable form of breast cancer. Breast CIS is confined to the lumen of breast ducts and lobules without penetration of the basement membrane and, therefore, without invasion of the breast stroma. However, cytological characteristics of intraductal tumor cells are essentially indistinguishable from the cytological characteristics of invasive breast carcinomas and intraductal carcinomas are considered to represent an early stage in a continuum of breast neoplasia. Circumstantial evidence supports this view. Breast CIS is associated with an increased risk of subsequent development of invasive breast carcinoma. Overexpression of HER-2/*neu* (c-*erb* B-2) and other oncoproteins, including p53 tumor suppressor protein product, known to be frequently overexpressed in invasive breast cancer, are also frequently overexpressed in breast CIS. Although overexpression of oncoproteins have been observed in breast CIS, these are immunohistochemical staining studies of archival, paraffin-embedded tissues, which do not provide direct evidence of genetic alterations.

Little direct evidence of genetic alterations in CIS is available in the literature. Only three studies have examined cohorts of CIS using SSCP as a screening tool followed by DNA sequence analysis to confirm apparent mutations.¹⁷⁻¹⁹ Two of these studies screened exons 4 to

9,^{17,19} and one screened only exons 5, 7, and 8.¹⁸ Three other studies screened with either immunohistochemistry^{20,21} or SSCP of exons 4–9.²² However, one immunohistochemistry study confirmed only 2 mutations in 8 cases of p53 overexpression,²¹ and the other immunohistochemical study was able to confirm only 1 mutation among 4 immunohistochemically positive tissues.²⁰ The last report did not attempt to confirm the presence of mutations after a positive SSCP screening.²² Taken together, these screening studies revealed 28 possible mutations; however, only 9 p53 gene mutations were confirmed.^{17–21} These p53 gene mutations include 7 missense mutations: Arg202His,¹⁹ His214Arg,¹⁷ Ser215Gly,¹⁹ Met237Ile,¹⁹ Gly248Asp,²¹ and two Arg273His mutations.^{18,20} Two frame shift mutations were noted and both were predicted to result in premature termination codons and truncated p53 protein.^{17,19} The frequencies of confirmed p53 mutations in the breast CIS cohorts screened were 3%,^{18,20} 4%,²¹ 11%,¹⁹ and 12%.¹⁷

The present work shows a frequency of p53 mutations in breast CIS lacking invasive carcinoma (22%) much higher than has been previously described^{17–21} and similar to the frequency of p53 mutations in breast CIS with coincident invasive carcinoma (19%). This rate is similar to the 22% rate found in a single large study of p53 mutations in invasive breast cancer.²⁶ The higher percentage of mutations in this study as compared to the previous work is probably related to the more detailed molecular analysis performed in the current cohort. For instance, this is the only study of breast CIS to screen all coding exons of p53. Twenty percent of DNA alterations were located outside of exons 4 to 9 in this study, and 40% of all p53 DNA alterations would have been undetected if only exons 5 to 8 had been screened. This study shows that to assess the p53 gene completely for alterations, all exons should be screened.

There is no evidence of increased p53 overexpression during progression from CIS to invasive disease within the same tumor (Table 2). Furthermore, although the frequency of p53 overexpression in breast CIS lacking invasion (22%) was lower than the frequency of p53 overexpression in breast CIS cases having invasion (35%), this difference was not significant.

Those breast tumors with both a CIS component and an invasive component with wild-type p53 in the CIS component by DNA sequence analysis also had wild-type p53 in the invasive component (data not shown). When p53 mutations were identified in a CIS component by DNA sequence analysis, the identical mutation was identified in the invasive component of the tumor, suggesting a clonal relationship between CIS and invasive disease (Table 2 and Figure 2). In those cases of carcinoma with p53 mutations and sufficient benign ductal tissue for DNA analysis, no evidence of p53 mutations was found in the benign ductal epithelium. A recent report showed a similar lack of p53 mutations in benign ductal epithelium compared to CIS.²⁷ These results suggest that p53 mutations occur before invasion in the progression of the disease, primarily in intraductal breast carcinomas.

There were 8 polymorphisms found in this cohort (Table 3). Arg72Pro was the only polymorphism associated with a change in the amino acid sequence. The codon 72 alteration has recently been implicated in the pathogenesis of cervical cancer by one group,²⁸ but this result was recently questioned.²⁹ The effect of this polymorphism in breast cancer is unclear. The Arg213 alteration does not code for an amino acid change. This codon is the most frequently altered non-missense codon³⁰ and is reported to be a polymorphic site.³¹ Noncoding DNA sequence alterations have been reported at high frequency in sporadic breast tumors³² and certain silent mutations at the wobble nucleotide in the fibroblast growth factor receptor 2 gene appear to have phenotypic effects,³³ possibly through the opening of a cryptic splice site. It is not clear whether similar alterations may have similar effects in p53.

The p53 mutations reported in this study can be divided into three groups. Mutations were found in sites which interact directly with DNA (Arg248Gln, Arg248Trp, Ala276Pro),³⁴ in sites that preserve p53 structural integrity (112delG, Gly165Stop, Leu194Ile, Tyr205Ser, Arg249Gly),³⁵ and in sites outside the core domain (Gly325Stop, del323–328, and alterations of unknown significance).^{36,37} Among the 12 mutations, five interfere directly with p53/DNA binding, six interfere with internal stabilization of p53, and one codes for a termination site in the 3' oligomerization domain.

The alterations of unknown significance are not easily classified. One such alteration effected p53 outside of the core domain but, nonetheless, exhibited p53 overexpression. This C-to-A alteration in the third intron does not appear to effect any cryptic splice sites. However, this alteration was within the putative lariat branch point.³⁸ Loss of this site could impair the proper splicing of this intron. The observed overexpression of p53 in this case (14% of nuclei immunostained for p53 protein) suggests that this alteration may be significant.

The T→A alteration of bp 18717 in p53 (Table 1) appears to be a polymorphic site because it exists in the normal epithelium in one case. However, evidence suggests that this alteration is relevant to p53 function. First, the two cases in this study, as well as 4 of 5 ovarian carcinomas (Reles A, Wen W-H, Press M, manuscript in preparation) exhibiting this alteration, overexpress p53 protein. This indicates a possible mechanism of p53 overexpression that is not related to mutations in the coding sequence. It has been noted in the human α -globin mRNA that alterations of the 3' untranslated region can determine RNA localization, polyadenylation, translation initiation, and RNA stability.³⁹ The stability of α -globin mRNA is modulated by the binding of a ribonucleoprotein complex to specific polycytosine sites in the 3' untranslated region of the gene.⁴⁰ The sequence of this site in α -globin is 5'-CCTCCCTCCCC-3'. In p53, the site of the bp 18717 T → A alteration is 5'-CCTCCCT/ACCCC-3', which is an exact match for the consensus binding site of α -globin mRNA. Certain substitutions in the consensus binding site destabilize the α -globin mRNA; substitutions at the T→A site, however, were not tested.⁴¹ It appears possible that a polycytosine binding protein may also influence the stability of p53 mRNA. Alterations

such as the T→A substitution in these consensus sequences could possibly increase translation of p53 protein and lead to the observed p53 overexpression.

In conclusion, this study demonstrated genetic alterations of p53 as well as overexpression of the p53 protein. A number of conclusions can be reached based on this data. First, although the results of prior studies suggested that there was an increased percentage of p53 mutations in invasive disease as compared to CIS, our results did not support this view, suggesting that most p53 mutations in breast occur before invasion of the breast stroma. In combination with the lack of p53 mutations in benign epithelium in cases with p53 mutations in carcinoma components, it appears that p53 may be altered during formation of the CIS lesion in the pathogenesis of breast cancer. Second, identical mutations in both CIS and invasive components of the same tumor support a precursor-product relationship between CIS and invasive disease consistent with a clonal relationship between CIS and invasive disease. Finally, p53 mutations occur in most histological types of intraductal breast cancer. These results suggest that p53 alterations are important in the pathogenesis of early breast neoplasia.

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