Jpn. J. Cancer Res. 85, 825–830, August 1994

Nuclear Accumulation of p53 Protein Correlates with Mutations in the p53 Gene on Archival Paraffin-embedded Tissues of Human Breast Cancer

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Fifty invasive ductal carcinomas of the breast were analyzed by immunohistochemistry, polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) and direct sequencing after microdissection of conventional formalin-fixed, paraffin-embedded tissues. A highly significant association between the presence of p53 gene mutation and nuclear accumulation of p53 protein was found ($P<0.0001$). Of 13 tumors that demonstrated p53 gene mutations, 11 (84.6%) showed nuclear accumulation of p53 protein. However, of 37 tumors in which gene mutations were not detected, only 5 (13.5%) showed nuclear accumulation of p53 protein. There was a statistically significant association between the nuclear accumulation of p53 protein and a higher histological grade ($P<0.001$) or mitotic index ($P<0.01$). In addition, gene mutations had a statistically significant association with a higher histological grade ($P<0.05$) or mitotic index ($P<0.0001$). Therefore, p53 abnormalities might be associated with an aggressive phenotype in breast cancer. We conclude that the immunohistochemical detection of nuclear p53 protein accumulation is highly associated with p53 gene mutations in archival paraffin-embedded tissues, and that this method is useful for rapid screening of p53 abnormalities.

Key words: Breast cancer — p53 — Gene mutation — Immunohistochemistry

Recently, many abnormalities of p53 in human breast cancer have been demonstrated by immunohistochemistry[1–4] or molecular biological means.[5–8] A correlation between the immunohistochemical detection of p53 protein accumulation and gene mutations has also been reported.[9–12] However, the association between immunohistochemical findings and DNA mutations of p53 is not clear-cut[12] and concordant ratios between these methods have not yet been precisely established. In addition, almost all studies of p53 gene mutations were performed using DNA extracted from fresh-frozen tissues. Therefore it could be that mutations present in heterogeneous tumors (such as breast cancer) with focal immunohistochemical positivity are undetectable by molecular means, because of high dilution by wild-type sequences.[6] The current investigation was designed to evaluate the precise correlation between the immunohistochemical detection of p53 protein accumulation and gene mutations analyzed by polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) and direct sequencing of the same archival paraffin-embedded tissues after microdissection, which enables precise sampling. In addition, we investigated the relationship between p53 abnormalities and the clinicopathological status.

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MATERIALS AND METHODS

Patients and tissues Tissue samples of primary breast cancer and axillary lymph nodes were obtained from 50 Japanese female patients who underwent mastectomies at the Sagara hospitals (Kagoshima) between November 1990 and November 1992. All specimens were histologically diagnosed as invasive ductal carcinoma (IDC) according to the WHO Histological Typing of Breast Tumors.[13] The mean age at the time of surgery was 52.2 years (range: 34–81 years). The mitotic index was defined as the total number of mitoses in ten high power fields (400× magnification) as previously described.[14] Histological grade was determined using a slight modification of the methods of Bloom and Richardson.[15]

Microdissection and DNA extraction Pieces of fresh tumor were stored at −80°C for estrogen receptor (ER) assay. All other samples of IDC and lymph nodes were fixed in 10% neutrally buffered formalin and embedded in paraffin. Paraffin-embedded blocks of neoplastic tissues were serially sectioned at 4–15 μm and microdissected. The first sections were stained with hematoxylin-eosin (H-E), the second for DNA analysis and the third for H-E and immunohistochemical staining. For DNA extraction, sections were cut from the block and the circumscribed tumor areas were separated from adjacent noncancerous tissues by direct scraping of the slides under the light microscope. These microdissections resulted in

neoplastic cell enrichment of about 80% or greater. The area of specimens was typically about 16 mm^2. DNA was extracted according to the method of Lyons *et al.*[16] with slight modifications. DNA was also extracted from lymph nodes of matched patients without metastasis as normal controls.

PCR-SSCP analysis Oligonucleotide primers were synthesized using a 392 DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). A set of PCR primers flanking the intron/exon junctions 4, 5, 6, 7 or 8 were used according to the genomic sequence data by Buchman *et al.*[17] The nucleotide sequences of the primers were as follows:

exon 4: 5′-ATCTACAGTCCCCCTTGCCG-3′ (sense)
5′-GCAACTGACCGTGCAAGTCA-3′ (antisense)
exon 5: 5′-TTCCTCTTCCTGCAGTACTC-3′ (sense)
5′-CAGCTGCTCACCATCGCTAT-3′ (antisense)
exon 6: 5′-TTGCTCTTAGGTCTGGCCCC-3′ (sense)
5′-CAGACCTCAGGCGGCTCATA-3′ (antisense)
exon 7: 5′-GTGTTATCTCCTAGGTTGGC-3′ (sense)
5′-CAAGTGGCTCCTGACCTGGA-3′ (antisense)
exon 8: 5′-AGTGGTAATCTACTGGGACGG-3′ (sense)
5′-ACCTCGCTTAGTGCTCCCTG-3′ (antisense)

For each patient, the five primer pairs described above were used in individual PCRs to amplify p53-coding sequences in genomic DNA from tumor and axillary lymph node specimens as templates. PCR was performed in a thermal cycler (Astek, Fukuoka). The PCR reaction mixture (total 5 μl) contained genomic DNA (50 ng), 1 m*M* each deoxynucleotide triphosphate (dNTP), 1 μM ^{32}P-end labeled primer, 6.7 m*M* $MgCl_2$, 67 m*M* Tris-HCl pH 8.8 and *Taq* polymerase (0.1 units, Perkin Elmer Cetus Corp., Norwalk, CT) for exons 4, 5, 7 and 8. For exon 6, the following conditions were used except for the DNA, primer and *Taq* polymerase: dNTP (200 μM), $MgCl_2$ (1.5 m*M*), Tris-HCl pH 8.3 (10 m*M*), KCl (50 m*M*) and 0.001% gelatin. Thirty cycles consisting of 60 s at 94°C, 45–60 s at 60°C, 58°C, 62°C, 59°C and 60°C for exons 4, 5, 6, 7 and 8, respectively, and 2 min at 72°C were performed. The PCR products were diluted 50 to 200 fold with formamide dye mixture (95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). The diluted mixture was denatured at 80°C for 5 min, then 1 μl was applied to a 6% polyacrylamide gel (acrylamide:methylene-bisacrylamide, 49:1) containing 45 m*M* Tris-borate (pH 8.3), 4 m*M* EDTA, with or without 10% glycerol. Electrophoresis was performed at 10 W for 12–15 h with cooling by a fan at room temperature. The gel was dried on filter paper and exposed to an X-ray film for 6–14 h at −80°C with an intensifying screen. PCR-SSCP was performed at least twice to ensure that the results were reproducible in each sample showing a mobility shift. For all samples, we performed PCR-SSCP using DNA from matched lymph nodes without metastasis to eliminate any polymorphism.

Direct sequencing Samples with mobility shifts altered from the normal controls were reamplified without [γ-^{32}P]ATP and purified using the QIAquick-spin PCR purification kit (Qiagen Inc., Chatsworth, CA). Purified DNA was used as the template for double-strand cycle sequencing with ^{32}P-end labeled primers according to the instructions supplied with the kit (Promega, Madison, WI). Sequence primers corresponding to a more internal sequence except for exons 6 (antisense) and 8 (sense) were used as follows:

exon 5: 5′-GCAGTACTCCCCTGCCCTCAACAA-3′ (sense)
5′-TCACCATCGCTATCTGAGCAGCGC-3′ (antisense)
exon 6: 5′-TCTTAGGTCTGGCCCCTCCTCAGC-3′ (sense)
5′-CAGACCTCAGGCGGCTCATA-3′ (antisense)
exon 7: 5′-CTCCTAGGTTGGCTCTGACTGTAC-3′ (sense)
5′-TGGCTCCTGACCTGGAGTCTTCCA-3′ (antisense)
exon 8: 5′-AGTGGTAATCTACTGGGACGG-3′ (sense)
5′-TTGCTTACCTCGCTTAGTGCTCCC-3′ (antisense)

Products were electrophoresed on 8% polyacrylamide gel containing 7 *M* urea at 50 W for 1.5 h, dried, and autoradiographed with an intensifying screen at −80°C for 12 h. Sequencing was performed on both sense and antisense strands at least twice.

Immunohistochemistry Nuclear accumulation of p53 protein was detected using the polyclonal antibody CM1 (Novocastra Laboratories, Newcastle, England), which recognizes both wild and mutant forms of the p53 protein in formalin-fixed, paraffin-embedded tissues.[2, 18] Sections (3 μm) were dewaxed, rehydrated and stained with standard avidin-biotin-immunoperoxidase using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). CM-1 was used at a dilution of 1:1000 and incubated overnight at +4°C. Diaminobenzidine (0.03% in phosphate-buffered saline) was the chromogen. Phosphate-buffered saline containing 1% bovine serum albumin was used as the negative control instead of primary antiserum.

Estrogen receptor ER levels were determined using a dextran-coated charcoal separation method (Biomedical Laboratories, Tokyo). A concentration greater than 14 fmol/mg of ER protein was considered to be positive.

Statistical analysis The association of nuclear p53 protein expression or gene mutations with tumor size, histological grade, TNM stage, nodal status and mitotic index was analyzed by the chi-square test.

RESULTS

Nuclear staining of cells was considered positive for nuclear accumulation of p53 protein in the immunohistochemical study (Fig. 1). The percentage of p53-positive cells was estimated semiquantitatively, and tumors were

assigned to two categories; positive or negative. There were 20–50% positive cells in 6 samples, and more than 50% in 10 samples, making a positive rate of 32%. No nuclear staining was seen in any of the negative control sections or surrounding non-cancerous breast tissues. There was a statistically significant association between p53 protein accumulation and a higher histological grade ($P<0.001$) or mitotic index ($P<0.01$) (Table I). A

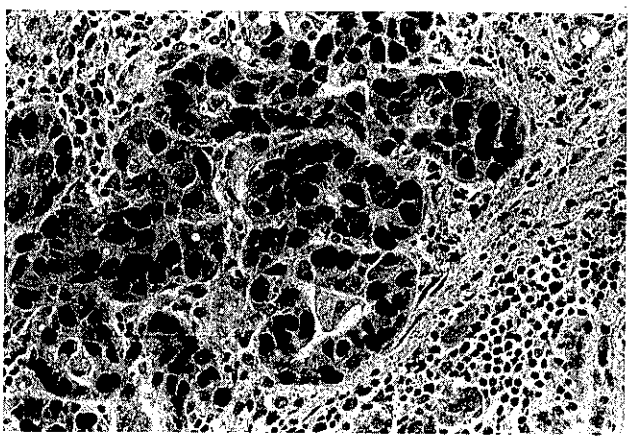

Fig. 1. Invasive ductal carcinoma showing nuclear staining with p53 CM1 (×380).

Table I. Association of p53 Nuclear Staining with CM1 and Clinicopathological Parameters

	16 tumors with positive staining	34 tumors with negative staining	*P* value
Tumor size (mm)			
0–20	5	9	NS
21–50	9	16	
51–100	2	9	
Lymph node metastasis			
positive	4	16	NS
negative	12	18	
Estrogen receptor			
positive	8	22	NS
negative	8	12	
TNM stage			
I and II	13	23	NS
III and IV	3	11	
Histologic grade			
I	0	12	<0.001
II	8	19	
III	8	3	
Mitotic index			
0–9	5	25	<0.01
10–20	2	4	
>20	9	5	

NS: not significant.

single specific amplification product from each sample was detected in all cases by preliminary PCR studies. In total, 13 (26%) of the 50 breast carcinomas revealed mobility shifts in PCR-SSCP analysis (Fig. 2). The position and type of the p53 gene mutation was identified by direct sequencing (Fig. 3) as listed in Table II. The position and incidence of the mutations were distributed as follows: exons 4 (0 sample), 5 (3 samples), 6 (3 samples), 7 (2 samples) and 8 (5 samples) and they were all point mutations. Each of the mutations altered the coding sequences and caused amino acid substitutions in highly conserved regions of the p53 gene. The most frequent pattern of mutations was a G:C to A:T transition (6 samples). Of the 13 point mutations, 5 (38.4%) occurred in a CpG dinucleotide sequence. There was a statistically significant association between p53 gene mutation and a higher histological grade ($P<0.05$) or mitotic index ($P<0.0001$) (Table III). Of 13 specimens with gene mutations, 11 (84.6%) showed accumulation of p53 protein. However, of 37 specimens in which gene mutations were not seen in exons 4, 5, 6, 7 and 8, 5

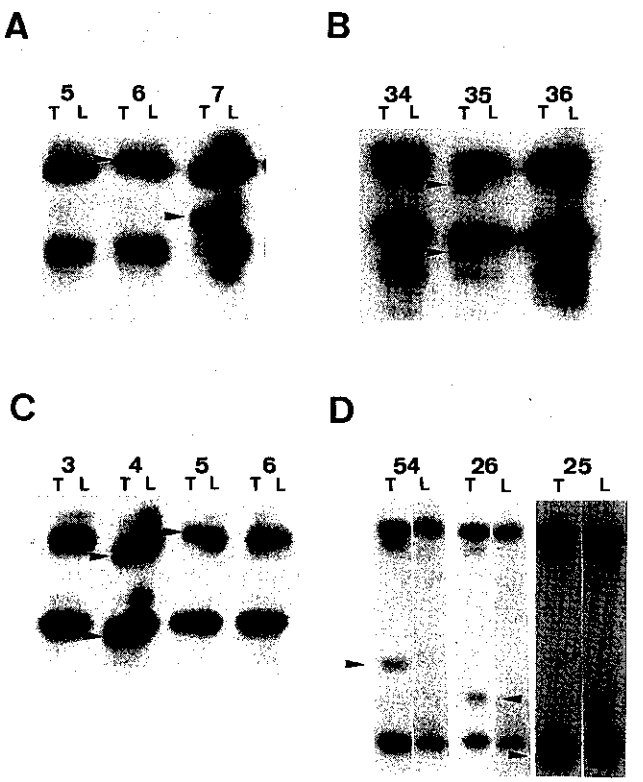

Fig. 2. Detection of p53 gene mutations in breast cancer by PCR-SSCP analysis for: A, exon 5; B: exon 6; C: exon 7; and D: exon 8. The patient's number is shown at the top of each lane. T; tumor DNA, L; lymph node DNA. Arrowheads denote the bands with mobility shifts relative to controls.

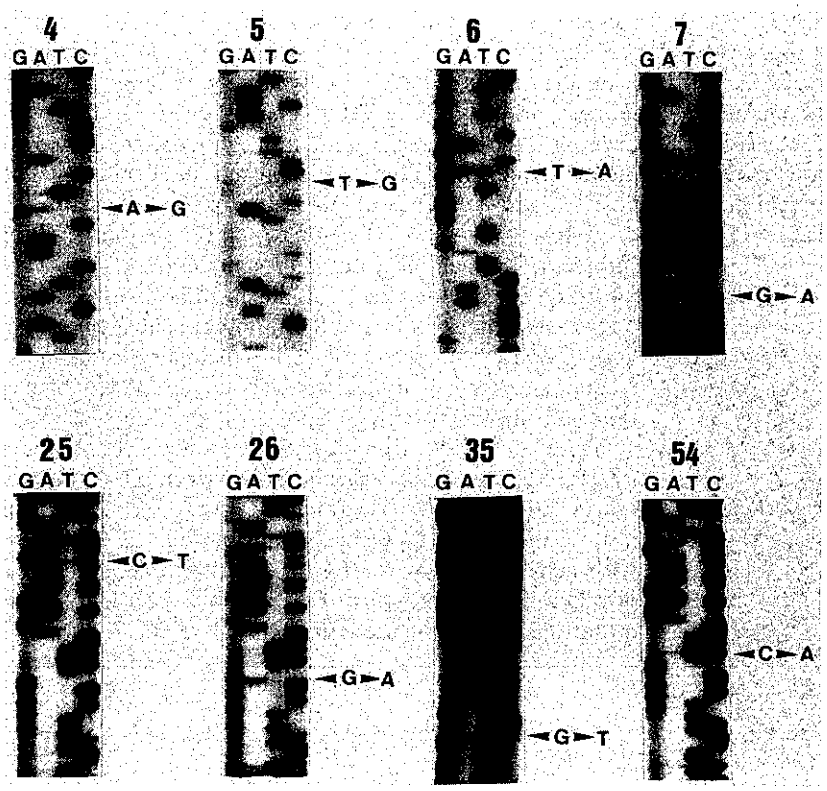

Fig. 3. Direct DNA sequencing of PCR-amplified fragments. The sequences of the coding strands are shown for patients 4, 5, 6, 7, 35, and of the non-coding strands for patients 25, 26, 54. The patient's number is shown at the top of each lane.

Table II. Description of the p53 Mutations in Human Breast Cancer

Case No.	Codon	Base change (amino acids)
I. G:C to A:T transitions		
7	175[a]	CGC (Arg) → CAC (His)
15	214	CAT (His) → TAT (Tyr)
16	273[a]	CGT (Arg) → TGT (Cys)
25	273[a]	CGT (Arg) → CAT (His)
26	282[a]	CGG (Arg) → TGG (Trp)
52	282[a]	CGG (Arg) → TGG (Trp)
II. A:T to G:C transition		
4	234	TAC (Tyr) → TGC (Cys)
41	145	CTG (Leu) → CCG (Pro)
III. Transversions		
5	242	TGC (Cys) → GGC (Gly)
6	145	CTG (Leu) → CAG (Gln)
17	216	GTG (Val) → GAG (Glu)
35	202	CGT (Arg) → CTT (Leu)
54	280	AGA (Arg) → ATA (Ile)

a) Mutations at CpG sites.

(13.5%) showed nuclear accumulation of p53 protein which was detected by immunohistochemistry. The concordance between gene mutations and nuclear accumulation of p53 protein was 85.5%. There was a highly significant association between the presence of p53 gene mutation and nuclear accumulation of p53 protein ($P<0.001$) (Table IV).

DISCUSSION

It has not been clearly elucidated whether the immunohistochemical detection of p53 protein accumulation is associated with gene mutations in breast cancers. Thompson *et al.* reported marked heterogeneity of staining in breast tumors, which may cause major problems in detailed analyses of p53, as a result of sampling from different areas of tumors and the homogenization of tissues for DNA or mRNA studies.[12] Our immunohistochemistry data also revealed staining heterogeneity. If the proportion of tumor cells (containing p53 mutations) is relatively low compared to normal cells (containing wild-type p53), molecular analysis may not detect the mutations in the tumor cells. Therefore, to analyze the direct association between immunohistochemical and molecular biological detection of p53 abnormalities on archival paraffin-embedded tissues, we performed microdissection to enrich the tumor cells and to sample precisely the areas in which p53 protein accumulation could be detected by immunohistochemistry. There was a highly significant association between the presence of p53

Table III. Association between p53 Gene Mutations and Clinicopathological Parameters

	p53 gene mutations		*P* value
	present	absent	
No. of samples	13	37	
Tumor size (mm)			
0–20	2	12	NS
21–50	10	15	
51–100	1	10	
Lymph node metastasis			
positive	4	16	NS
negative	9	21	
Estrogen receptor			
positive	5	25	NS
negative	8	12	
TNM stage			
I and II	11	25	NS
III and IV	2	12	
Histologic grade			
I	0	12	<0.05
II	7	20	
III	6	5	
Mitotic index			
0–9	2	28	<0.0001
10–20	2	4	
>20	9	5	

NS: not significant.

Table IV. Association between Accumulation of p53 Protein and p53 Gene Mutations

	p53 gene mutations		*P* value
	present	absent	
p53 protein accumulation			
positive	11	5	<0.0001
negative	2	32	

gene mutations and nuclear accumulation of p53 protein, and the concordance was 85.5%. This is somewhat higher than that of bladder cancer reported by Esrig *et al.*[19] although the gene was analyzed using fresh-frozen material. However, of 16 samples in which p53 protein accumulation was detected, 5 (31.2%) showed no gene mutation. There are several possible reasons for this.

1) Although 98% of p53 gene mutations in diverse types of cancer have been found in exons 5–8,[20] it could not be ruled out that mutations exist in exons other than 5–8.

2) PCR-SSCP analysis is a sensitive method for detecting genomic mutations,[21] but not all mutations are detected by this means. Also, if mutations are present in primer sequences, they would be undetectable.

3) The antibody used in this study recognizes both the wild type and the mutant forms of p53 protein. It is possible that the immunohistochemical assay sometimes detected the accumulation of wild-type p53 protein. Binding of wild-type p53 protein to a cellular oncogene (such as MDM2 gene products) or protein of some viral antigen (such as simian virus 40 T antigen, papilloma virus E6 and adenovirus E1A) may alter the rate of p53 protein degradation, resulting in an increased half-life. This may enable detection by immunohistochemistry.

Conversely, of the 13 samples with mutations, only two did not show p53 protein accumulation. Therefore, immunohistochemical analysis may be more sensitive than gene analysis by PCR-SSCP and direct sequencing. However, the possible pitfalls in screening p53 alterations by immunohistochemistry alone must be emphasized. Nonsense, frameshift, and silent mutations in the p53 gene do not result in overexpression of the protein.[22] Moreover, the antigenic determinant of the p53 protein may be lost during fixation and embedding. On the other hand, there was a statistically significant association between p53 abnormalities (gene mutations or protein expression) and higher histological grade or mitotic index. These results are broadly consistent with the report by Tsuda *et al.*[7] Histological grade, mitotic index or p53 protein expression are known to be indicators of poor prognosis.[4, 14, 15] In addition, histological grade is known to be a predictor of aggressive biological behavior of invasive breast cancer.[23] Therefore, p53 abnormalities might be associated with an aggressive biological phenotype of breast cancer. We are planning to evaluate the possible prognostic value of p53 gene abnormalities by examining 5-year survival rates of our cases. Surgical treatment of breast cancer is changing from aggressive Halstead, Patey or Auchincloss methods to limited lumpectomy, quadrantectomy or simple mastectomy. It is useful for surgeons to obtain pre-operative information on the biological phenotype of breast cancer. We found that immunohistochemical analysis may be more sensitive than PCR-SSCP analysis with direct sequencing in detecting p53 mutations or discordant cases in which there is an accumulation of wild-type p53 protein. Although we have to bear in mind the possible pitfalls of immunohistochemical analysis, this method is useful for secreening p53 abnormalities which might be associated with an aggressive phenotype on archival paraffin-embedded tissues, as well as conventional histopathological analysis (especially histological grade and mitotic index).

ACKNOWLEDGMENTS

We thank Mr. T. Kodama and Mr. T. Nitanda for their assistance. This work was supported in part by the Kodama Memorial Fund for Medical Research.

(Received February 21, 1994/Accepted May 23, 1994)

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