Estimating Loss of the Wild-Type p53 Gene by In Situ Hybridization of Fine-Needle Aspirates from Breast Carcinomas

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TP53 mutations have been found in 16–64% of breast carcinomas. The aim of our study was to investigate loss of the wild-type TP53 gene by in situ hybridization (ISH) of fine-needle aspirates (FNAC) from breast carcinomas. The material consisted of FNAC from 33 breast carcinomas, with histologic specimens from 19 of the cases. Routine diagnostic smears were used for cytologic grading. ISH of the wild-type TP53 gene and chromosome 17 was performed on air-dried smears. Hybridization signals were counted in at least 100 nuclei, and the percentage for each signal number was calculated. FNAC from four fibroadenomas as well as cell preparations from five lymphocyte cultures were used as normal/benign controls. Cutoff for defining loss of p53 gene signals was set at 20% of cells with zero and one gene signal only. Concomitant p53 protein expression was determined on 20 histologic sections and eight additionally available air-dried smears.

Loss of wild-type p53 gene was found in 20 carcinomas (60.6%). The rate of signal loss varied from 0.4% to 75.3% of the cells. All tumors with aneusomy of chromosome 17 revealed loss of p53 gene signals, as did 42% of the disome cases. Loss of wild-type p53 gene was present in 10 of 16 grade 1 cancers (62.5%), eight of 13 grade 2 tumors (61.5%), and two of four grade 3 cases. Signal loss did not correlate with p53 protein expression.

In conclusion, subpopulations with loss of the wild-type p53 gene are a common finding in breast carcinomas; they are detected in more than 60% of the tumors, including grade 1 cancers. Diagn. Cytopathol. 1999;20:266–270. © 1999 Wiley-Liss, Inc.

Alterations in the p53 gene occur in about 60% of all human cancers.[1,2] Commonly, one allele harbors a mutation that inactivates the normal function, and the second allele is lost through deletion or gene conversion, resulting in a loss of heterozygosity (LOH) or a reduction to homozygosity in the tumor cells.[3] The majority of the mutations are found in the domain that contains the sequence-specific DNA binding activity of the p53 protein, and they result in loss of DNA binding.[4] More than 80% are missense point mutations and deletions that do not cause a downstream frameshift. Most alterations have been found in exons 5–8, and they are most often accompanied by p53 protein overexpression in the tumors. Mutations outside exons 5–8 are chiefly nonsense point mutations and deletions causing a downstream frameshift or stop codon, and these tumors are usually p53 protein negative.[1,5,6]

Wild-type p53 protein is a transcription factor that regulates entry into the S-phase of the cell cycle and has the ability to induce apoptosis in malignant cells.[3] Loss of these functions may result in genomic instability with accumulation of DNA damage, gene amplifications, and aneuploidy[7] Tumor cells lacking normal p53 function thus have a selective growth advantage and might be expected to be more aggressive clinically than cells with normal p53 function.

Mutations in the p53 gene have been found in 16–64% of breast carcinomas.[8–16] The p53 gene is located at 17p13.[17] Structural abnormalities of chromosome 17 are common in breast cancer cells,[18–22] reflecting alterations in the p53 and neu genes, among others. Numeric aberrations of chromosome 17 occur most often in high-grade, highly aneuploid tumors.[23] Some studies have found that LOH at 17p13.1 correlates with p53 mutations,[6,24] whereas others have not.[9,25] Both overexpression of the p53 protein and alterations in the p53 gene have been associated with a worse prognosis in breast cancer.[18,26–28]

The aim of our study was to investigate the extent of loss of the wild-type p53 gene by in situ hybridization (ISH) of interphase cells on fine-needle aspirates (FNAC) from breast

Table I. Procedure of In Situ Hybridization[a]

1. Aspirates fixed in methanol/acetic acid (3:1) for 40 min at $-20°C$
2. Dehydration at room temperature (RT) in 70%, 80%, and 96% alcohol, allowed to dry
3. Denaturation in $2\times$ SSC, 70% formamide at pH 7.0 and 70°C for 2 min
4. Dehydration at $-20°C$; 2 min each in 70%, 80%, and 96% alcohol; allowed to dry
5. Denaturation of α-satellite probe (chromosome 17) 5 min in water bath at 70°C
6. Pre-warming of p53 probe at 37°C for 5 min
7. p53 gene and α-satellite probes applied to separate areas of the slide and incubated at 37°C for 16 hr (overnight)
8. Post-hybridization wash (0.60$\times$ SSC, pH 7.0) at 72°C for 5 min
9. Washed in PBS with 0.25% Triton-X 100 for 2 min at RT
10. Incubated with mouse anti-digoxigenin for 3 hr at RT
11. Incubated with biotinylated anti-mouse (ABC kit) for 30 min at RT
12. Washed with TBS, 5 min at RT
13. Incubated with ABC complex for one hour at RT
14. Washed with TBS, 5 min at RT
15. DAB solution 10 min
16. Wash in running tap water, counterstain with hematoxylin and mount

[a]SSC, standard sodium citrate; PBS, phosphate-buffered saline; TBS, tris-buffered saline; ABC, avidin-biotin-complex; DAB, diaminobenzidine.

Fig. 1. Breast carcinoma cells without significant loss of wild-type p53 gene signals. Most nuclei harbor two small, dark (brown) signals ($\times1,000$).

carcinomas. The results were correlated with overexpression of p53 protein, numeric abnormalities of chromosome 17, and cytologic grading.

Materials and Methods

The material consisted of fine-needle aspirates from 33 breast carcinomas and histologic paraffin-embedded material from 20 of the cases. Cytologic grading was performed according to the method of Robinson et al.[29] on routine diagnostic smears. In cases where additional air-dried smears were available, they were put aside and kept at $-20°C$. ISH was performed according to the manufacturer's instructions, with the modifications shown in Table I. A digoxigenin-labeled α-satellite probe for chromosome 17 (Oncor, Gaithersburg, MD) and a unique sequence probe (17p13.1) for detecting wild-type TP53 gene (Oncor, catalog no. p5106–DG.5) were used. An ABC (avidin-biotin-complex) technique (Vector Laboratories, Burlingame, CA) with diaminobenzidine (DAB) was used for visualization.

The number of signals for the p53 gene (Fig. 1) and chromosome 17 was counted in at least 100 well-preserved tumor cell nuclei. Only signals in clearly discernible nuclei were counted, and the percentage of cells with varying signal number was recorded. Smears from four fibroadenomas and five lymphocyte cell cultures were used as benign/normal controls. For each of the benign specimens, the maximum and mean percentage of nuclei with none and one signal, respectively, of the p53 gene and chromosome 17 were assessed, to reveal the magnitude of insufficient hybridization. The sum of the maximum percentage of cells harboring zero and one signal of wild-type p53 gene in benign cells was used to define a cutoff of 20% for loss of
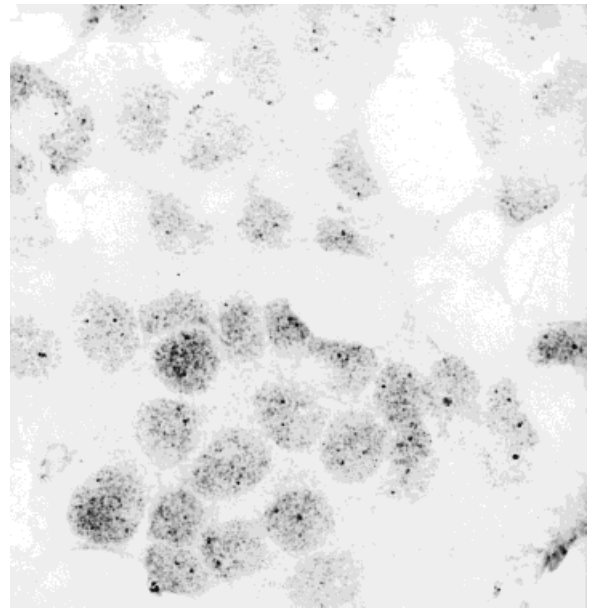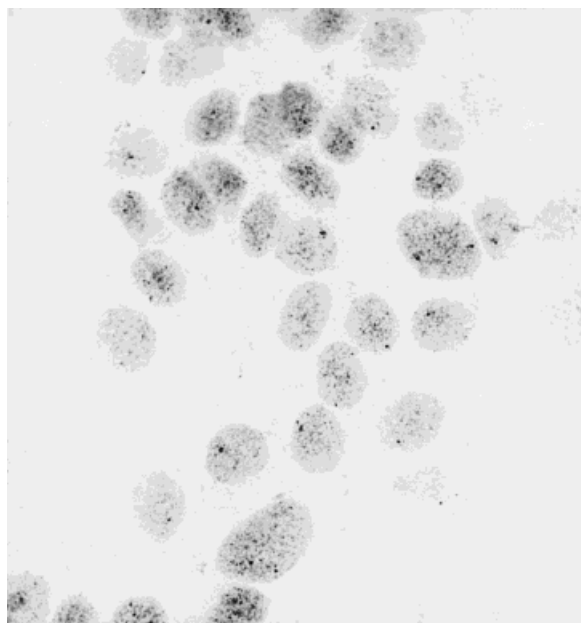
p53 gene signals in the carcinoma cells. Whenever there was extensive numeric gain of chromosome 17, the ratio between p53 gene and chromosome 17 signal numbers was estimated, to reveal the relative loss of wild-type p53 gene signals. The p53 hybridization signals were significantly smaller than the centromere signals and had to be counted under oil immersion ($\times100$). We applied a stringent post-hybridization wash with $0.6 \times$ SSC instead of $0.2 \times$ SSC (standard sodium citrate), as proposed from Oncor for p53 determination. In chromosome 17, the rate of insufficient hybridization was used only as a quality control. We corrected for this rate when determining whether a tumor had a normal or abnormal number of chromosome 17. Specimens with $\geq70\%$ two-signal nuclei were regarded as disome for chromosome 17. Both loss and gain of chromosome numbers outside this range were regarded as aneusomy.[23] In general, counting of both the centromere and the gene signals takes approximately 15 min.

Concomitant p53 protein expression could be evaluated in 28 of the 33 cases. Twenty histologic specimens and eight air-dried smears were analyzed using a monoclonal antibody for p53 from Ventana (Ventana Medical Systems, Inc., Finland; catalog no. 250–1542), prediluted and with microwave pretreatment. Staining was performed on the Ventana ES automated immunostaining system. A biotinylated secondary antibody and avidin–horseradish peroxidase with DAB werre used for visualization. Staining for p53 protein was considered positive when $>10\%$ of tumor cell nuclei were positive. A known p53 protein–positive breast carcinoma was used as control.

Table II. Estimates on Insufficient Hybridization of Wild-type p53 Gene in Fibroadenoma Cells and Lymphocytes

Percentage of p53 gene signals	Lymphocyte preparations	Fibroadenoma cells
Maximum of zero signals	11%	8.6%
Mean of zero signals	5.1%	7.7%
Maximum of one signal	12.4%	11.1%
Mean of one signal	7.6%	8.5%
Maximum of zero plus one signal	20.9%	18.8%
Mean of zero plus one signal	12.7%	16.2%

Table III. Relationship of Numerical Aberrations of Chromosome 17, Cytologic Grading, Immunohistochemical Determination of p53 Protein and Loss of Wild-type p53 Gene Signals

	Loss of p53 gene signals	No loss of p53 gene signals
Aneusomy chrom 17	9	
Disomy chrom 17	11	13
Grade 1	10	6
Grade 2	8	5
Grade 3	2	2
P53 protein positive	6	9
P53 protein negative	11	2

Table IV. Comparison of Loss of Wild-type p53 Gene Signals and Cytologic Grading in Breast Carcinomas with Disomy of Chromosome 17

Cytologic grading	Loss of p53 gene signals	No loss of p53 gene signals
1	7	6
2	3	5
3	1	2

Fig. 2. Breast carcinoma cells with significant loss of wild-type p53 gene signals. Many nuclei harbor only one gene signal. Some nuclei have no p53 gene signal, whereas some tumor cells have kept both wild-type signals (×1,000).

Results

The maximum and mean percentages of fibroadenoma cells and lymphocytes showing only one or zero wild-type p53 gene signals are shown in Table II. The largest percentage of cells with one plus zero gene signal was 20.9%; this percentage was found in one of the lymphocyte preparations. According to these figures, 20% of cells showing loss of gene signals was chosen as the cutoff for defining loss of the wild-type p53 gene on the carcinoma specimens. ISH revealed aneusomy of chromosome 17 in nine cases (27%). Three of these showed hyposomy with signal loss (80%, 40%, and 28% monosomy, respectively), whereas six had signal gain. Loss of wild-type p53 gene signals (Fig. 2) was found in 20 of 33 carcinomas (60.6%). In two of these 20, the percentage of signal loss was proportional to the loss of chromosome signals (the cases with 80% and 40% monosomy of chromosome 17). Two cases with extensive gain of chromosome 17 signals had a relative loss of p53 gene signals.

Loss of wild-type p53 gene signals varied from 0.4% to 75.3%, with a mean of 22.5% and a median of 14%. Significant loss of both gene signals was found in six cases. A mixed loss was shown in eight specimens, whereas six tumors mainly had loss of one gene signal. Grade 1 tumors had a mean loss of 23.9%, a median of 14%, and a maximum of 75.3%. Grade 2 cancers showed a mean loss of 20.1%, a median of 8.4%, and a maximum of 58%. The relationships between p53 gene loss/no loss and numeric aberrations of chromosome 17, cytologic grading, and immunohistochemically determined p53 protein are shown in Table III. Chromosome 17 disomy was found in 13 of 16 grade 1 tumors, eight of 13 grade 2 tumors, and three of four grade 3 cancers. Characteristics of p53 gene signal loss and grading in tumors with disomy of chromosome 17 are shown in Table IV.

Discussion

Determination of the loss of the wild-type p53 gene provides an indirect estimate of p53 mutations and deletions, but without any specifications as to type of abnormality. Our results yielded a rather high rate of loss of wild-type p53 (60.6%). They are in concordance with the results of Deng et al.,[9] who found p53 mutations in 57% of breast cancers, and with those of Singh et al.,[12] who found 64% loss of the p53 allele. A number of studies with lower percentages[8,10,13,15,16] have mainly screened for mutations in exons 5–8, which may underestimate the mutation rate by approximately 20%.[5]

On the basis of the results from the controls, we defined a cutoff of 20% signal loss to correct for insufficient hybridization. This is the same level as set by Li et al.,[30] whereas two other studies defined the cutoff at 40% and 50%, respectively.[31,32] They both used FISH and had a somewhat higher rate of insufficient hybridization. In contrast to FISH,

nonfluorescent ISH does not give a cell-to-cell relationship between the p53 gene and chromosome 17 signal number but rather the rate of carcinoma cells lacking wild-type p53 within the individual tumors.

Subpopulations with loss of wild-type p53 are apparently common, even in low-grade tumors. The extent of loss was highly variable in both grades 1 and 2 carcinomas, but the mean loss and median loss were about the same in both groups. Most cases revealed a moderate loss of the wild-type p53 gene, with approximately 20% of tumor cells showing signal loss. The highest rate was found in a grade 1 cancer with 75% signal loss, mostly of both alleles, whereas there was disomy of chromosome 17.

All tumors with aneusomy of chromosome 17 revealed loss of the wild-type p53 gene. This finding is consistent with previous findings that aneusomy of chromosome 17 is a feature of malignancies with extensive chromosomal abnormalities.[23] In addition, 42% of carcinomas with disomy of chromosome 17 showed loss of wild-type p53 gene. The same pattern was observed by Matsyama et al.[33] in a study on urothelial tumors. According to Yin et al.,[34] wild-type p53 restores cell cycle control in vitro in cells with mutant p53 alleles. It is possible, therefore, that tumor cells lacking only one wild-type allele maintain a normal cell cycle control, whereas this control will be lost in cancer cells lacking both wild-type alleles. A dominant loss of both gene signals or a mixed loss was found in 14 of our cases (seven grade 1, five grade 2, and two grade 3).

Fifty-three percent of the immunocytochemically analyzed tumors revealed overexpression of p53 protein. In 60% of these tumors, however, no loss of the wild-type p53 gene was found. Conversely, 65% of carcinomas harboring loss of wild-type p53 were negative for the p53 protein. Thorlacius et al.[28] found that 58% of p53-protein-positive cases did not harbor p53 missense mutations, whereas Singh et al.[12] detected protein-positive results in 50% of the p53 mutants. Other researchers have found a somewhat lower figure, with about 30% discrepancies between p53 mutations/no mutations and p53 protein status.[13,16]

Possibly, our results are better compared with LOH at 17p13. Tsuda et al.[6] ascertained that 83% of tumors with p53 mutations revealed LOH at 17p13. In their study, Singh et al.[12] found no correlation between p53 protein overexpression and LOH at 17.13. Several factors might influence discrepancies between immunocytochemically determined p53 protein expression and p53 gene abnormalities. Most important is probably the rate of nonsense mutations and deletions that do not result in an abnormal p53 protein, which may account for approximately 20% of the aberrations.[1,5] In addition, specimen-fixation artifacts, different epitope specificity of antibodies, and aberrant overexpression of wild-type protein may contribute to divergence in the results.

In conclusion, subpopulations with loss of the wild-type p53 gene are a common finding in breast carcinomas; they are seen in more than 60% of these tumors, including grade 1 tumors. Preoperative investigation of this feature might have a role in singling out tumors with possibly more aggressive behavior and aid in deciding on the optimal primary treatment of the patients.

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