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Mutation detection by highly sensitive methods indicates that p53 gene mutations in breast cancer can have important prognostic value

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ABSTRACT **Human cancer cells with a mutated p53 tumor-suppressor gene have a selective growth advantage and may exhibit resistance to ionizing radiation and certain chemotherapeutic agents. To examine the prognostic value of mutations in the p53 gene, a cohort of 90 Midwestern Caucasian breast cancer patients were analyzed with methodology that detects virtually 100% of all mutations. The presence of a p53 gene mutation was by far the single most predictive indicator for recurrence and death (relative risks of 4.7 and 23.2, respectively). Direct detection of p53 mutations had substantially greater prognostic value than immunohistochemical detection of p53 overexpression. Analysis of p53 gene mutations may permit identification of a subset of breast cancer patients who, despite lack of conventional indicators of poor prognosis, are at high risk of early recurrence and death.**

Mutations eliminating or altering the p53 protein function are the single most common genetic alteration observed in human cancers (1). The product of the p53 gene is hypothesized to maintain genomic stability by (*i*) blocking cell replication after DNA damage until the damage is repaired or (*ii*) initiating apoptosis if the damage is too extensive for repair (2). The p53 gene product acts as a transcriptional regulator, enhancing the expression of genes that contain specific p53 binding sites and interacting with a variety of transcription factors to inhibit the expression of other genes (3). Cells lacking normal p53 function have a selective growth advantage and are more resistant to ionizing radiation and some widely used anticancer drugs than cells with normal p53 function (3, 4). Thus, cells with mutated p53 genes might be expected to be more aggressive clinically than cells with normal p53 function.

Many studies have examined the association between breast cancer prognosis and p53 expression in breast cancer detected immunohistochemically. About one-third of such studies found such an association, but differences in technique and variability in the frequency and intensity of immunoreactivity with p53-specific antibodies make it difficult to compare results among laboratories (reviewed in refs. 5 and 6).

There have been few studies of the prognostic significance of p53 gene in *mutations* in breast cancer. Mazars *et al.* (7) and Tsuda *et al.* (8) found, in French and Japanese women, respectively, significant associations between the presence of a p53 mutation and low estrogen receptor (ER) and/or progesterone receptor (PR) concentrations, markers of poor prognosis in breast cancer patients. Marchetti *et al.* (9) reported a significant association between p53 mutations and a high proliferative activity of the tumors determined by immunohistochemistry with the monoclonal antibody Ki-67. Three other studies which correlated p53 mutations directly with breast cancer prognosis reported relative risks for disease-free and overall survival between 2.2 and 3.3 in multivariate analyses (refs. 10–12; see Table 5 for details).

In an analysis of 53 consecutive breast cancers in Midwestern White women, we previously reported that patients with a p53 gene mutation had a significantly worse prognosis than patients whose breast cancer did not have a p53 gene mutation (13). However, the number of tumors with mutations was small and the *P* value for shorter recurrence times was barely significant ($P = 0.05$). The analysis is now extended to 97 breast cancers from Midwestern White women received consecutively from the surgical pathology suite at Rochester Methodist Hospital. The results document that a p53 gene mutation is an independent, prognostic marker of early relapse and death from breast cancer.

METHODS

Forty-four primary breast cancers collected consecutively from the Surgical Pathology Suite, Rochester Methodist Hospital, were analyzed. These 44 patients and the patients previously studied (13) have not been part of a clinical study and received the appropriate therapy according to the tumor/node/metastasis (TNM) stage of their disease. Most of the postmenopausal patients with receptor-positive tumors received adjuvant hormonal therapy and most of the patients with advanced disease (TNM stage > IIb) received adjuvant combination chemotherapy. Touch preparations from the partially thawed surface of frozen tumor tissue were made as described (14). Tumor cell clusters ranging from 30 to 100 cells were lysed by the addition of proteinase K.

All coding exons (exons 2–11) were examined in the initial sample of 53 breast cancers from Midwestern Caucasian women. In a total of 194 breast cancers from various populations, all exons of the p53 gene were examined by direct sequencing or by dideoxy fingerprinting (15). About 20% of the mutations were located outside exons 5–8, but none were present in exons 2, 3, and 11 (16). Furthermore, we found no mutations in the putative promoter region of the p53 gene and in exon 1 in a subset of 58 breast cancers from this group (16). Thus, in this series only exons 4–10 and adjacent splice regions were amplified by nested amplification as previously described (14, 17). The separate exons of all samples were screened for mutations by dideoxy fingerprinting (15), a method which detects virtually 100% of mutations in the p53 gene and other genes (15, 18, 19). Regions that screened positively were directly sequenced by genomic amplification with transcript sequencing (GAWTS) (20, 21). Twenty-two tumors were screened by dideoxy fingerprinting *and* sequenced in both directions; 22 additional tumors were screened and the precise mutation in dideoxy fingerprinting positive samples was defined by sequencing. Dideoxy fingerprinting did not miss any mutation in the 22 patients for which both methods were

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Abbreviations: ER, estrogen receptor; PR, progesterone receptor.

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Table 1. Mutations in exons 4–10 in the p53 gene in breast cancers from Midwestern white women

Tumor no.	TNM stage[a]	Nucleotide change[b]	Position and result of change[c]	Immunohisto-chemistry[d]	Mutation type	Allelic status[e]
62T[fg]	IIIB	AGT(A → G)TTT	E6/205 Y → C	+/+/+	Missense	Hemizygous
64T	IIA	GAG(G → T)GGG	E10/360 G → V	+/+/+	Missense	Hemizygous
79T[fg]	IIB	TGG(G → A)CGG	E7/244 G → D	+/+/+	Missense	Hemizygous
81T[h]	IIB	TGT(G → A)TAA	E7/238 C → Y	+/+/+	Missense	Hemizygous
83T	IIB	TGT(G → A)TAA	E7/238 C → Y	+/+/−	Missense	Heterozygous
85T[f]	IIB	GTG(C → T)GTG	E8/273 R → C	+/+/+	Missense	Hemizygous
95T[fg]	IIIB	ACACT(T)GGAA	E7/257 1-bp insertion	−/−/−	Insertion frameshift	Hemizygous
104T	I	AGT(G → A)TGG	E6/216 V → M	+/+/−	Missense	Heterozygous
118T[i]	IIIA	TGT(T → G)CCG	E10/341 F → C	+/+/+	Missense	Hemizygous
129T	I	CGCTG(C)CCCCAC	E5/176 1-bp deletion	−/−/−	Deletion frameshift	Hemizygous
131T	IIB	TCC(T)GCA	E7/242 1-bp deletion	−/−/−	Deletion frameshift	Hemizygous
162T[f]	IIA	TAG(TGG)TAA	E8/262 3-bp deletion	+/+/+	Deletion in-frame	Hemizygous
165T[hj]	IIB	aca(g → a)TAC	I4/last 3′ base splice junction	+/+/−	Splice site	Heterozygous

[a]Staging according to American Joint Committee of Cancer (third edition).
[b]Small characters represent intronic sequence.
[c]Position is given as exon (E) or intron (I) no./codon no. Resultant amino acid changes are indicated in single-letter code.
[d]Immunohistochemical staining with three different antibodies (PAb1801/PAb240/PAb421). Tumors with ≥5% cells with nuclear expression of p53 with any of the three antibodies were considered immunohistochemically positive.
[e]The hemi- or heterozygous status of a tumor cell cluster was judged on the basis of the presence or absence of wild-type sequence at the site of the mutation.
[f]Tumors of patients with local or systemic recurrence of the disease.
[g]Tumors of patients who died of their disease.
[h]Excluded from the survival analyses because no follow-up was available.
[i]Excluded from the survival analyses (bone metastasis at diagnosis and no follow-up available).
[j]The mutation alters the splice acceptor site. The 3′ splice site is determined by a scanning mechanism which searches for the first AG dinucleotide located 3′ of the lariat branch point (24). The next available AG dinucleotide occurs after a pyrimidine-rich sequence 21 nucleotides downstream (5′-TACTCCCCTGCCCTCAACAAG/ATGTTTTG-3′). If splicing occurred with this site, a 7-aa in-frame deletion would result. Since all seven in-frame deletions of 7 aa or less in our total sample of 112 p53 mutations found in 298 breast cancers were associated with immunohistochemical staining, the 7-aa deletion is a likely explanation for the overexpression of p53 protein and the seemingly anomalous result of a normally null mutation at the last base of the intron occurring in the heterozygous form.

employed. All mutations were confirmed by amplifying and sequencing DNA from another cell cluster. PCR and sequencing primers were described previously (13, 16).

Clusters of tumor cells in touch preparations were stained for expression of p53 antigen with three monoclonal antibodies (PAb1801, PAb240, and PAb421) as described (17). All staining reactions were confirmed by analysis of a second touch preparation.

Recurrence of disease was defined as either local recurrence or metastatic spread of the disease. Recurrence and death were documented either by physical exams every 6 months or by yearly questionnaires sent to patients followed at clinics other than the Mayo Clinic.

Time-to-recurrence and survival-time distributions were estimated by the method of Kaplan and Meier (22) and compared between women with a p53 mutation or overexpression of p53 and women without a mutation or overexpression of the protein or between women with or without any of the other risk factors by the exact log-rank statistics method (STATXACT; ref. 23). To adjust the prognostic significance of

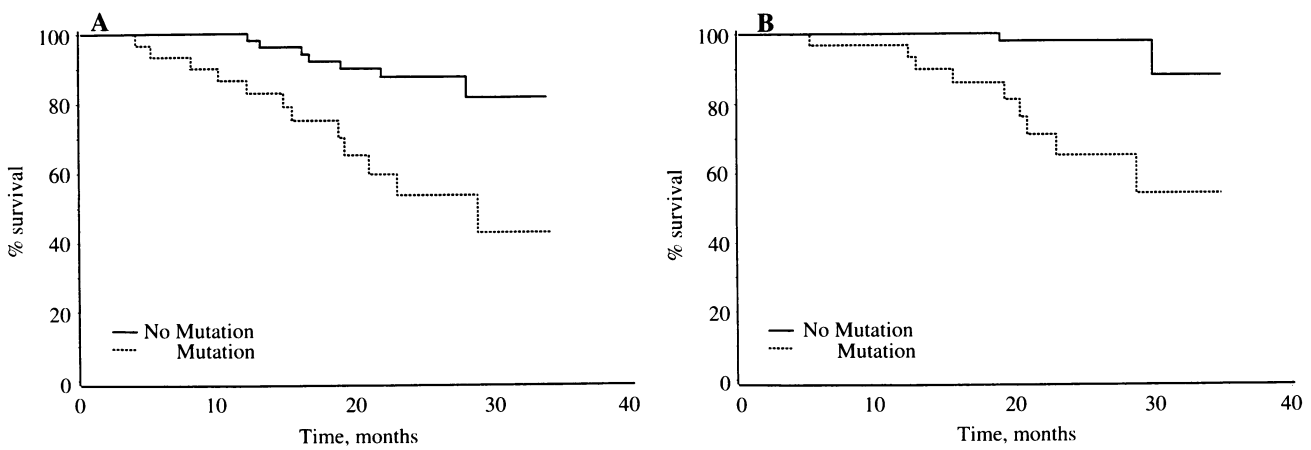

FIG. 1. Distribution of time to breast cancer recurrence (*A*) and time to death (*B*) according to the presence (dotted line) (n = 32) versus the absence (solid line) (n = 58) of a p53 gene mutation as estimated by the method of Kaplan and Meier (22) (*Methods*). There was no difference in the duration of follow-up between patients with or without mutation.

p53 mutations for the prognostic factors of ER status, PR status, nodal status, tumor stage, and tumor size, a stratified log-rank statistic was calculated by stratifying on the prognostic factor. Because the number of recurrences and deaths was small, we used STATXACT statistical software (23) to compute exact P values for the log-rank statistics as well as the stratified log-rank statistic. The relative risk was assessed by the Cox multivariate proportional hazard model.

RESULTS AND DISCUSSION

To accurately estimate the magnitude of p53 gene mutation on prognosis, it is important to detect almost all mutations. Three aspects of the methodology utilized in this study helped to assure that almost all the mutations were detected (see *Methods*): (*i*) the p53 gene was amplified from pure tumor cell clusters prepared by touch preparations; (*ii*) regions of the gene were screened with a method demonstrated to detect virtually all mutations; and (*iii*) exons 4 and 10 were analyzed in addition to exons 5–9.

Thirteen mutations (29.5%) were present among the 44 new Midwestern breast cancer samples studied in exons 4–10 and adjacent intronic regions (Table 1). Four mutations of the total number of mutations were microdeletions/insertions (31%), confirming the unusual abundance of this type of alteration we had previously found (38%) in the initial 53 tumors in our series (13). The basis for the greater frequency of microdeletions in the U.S. Midwest population is not clear, but both endogenous and exogenous factors have been suggested as origins of deletions and insertions (for review see refs. 25 and 26). Time to recurrence for the combined series of 90 women (7 patients had to be excluded because of the lack of follow-up data) was estimated by the method of Kaplan and Meier (22) and compared between women with a mutation and women without a mutation by the exact log-rank statistic (see *Methods*). Of 32 patients with a p53 mutation, 13 relapsed and 10 died in the period of 24 months of follow-up (Fig. 1). Of 58 patients without p53 mutation, only 7 relapsed and 2 died. The presence of a mutation was highly associated with early recurrence ($P = 0.0008$) and with death ($P < 0.0001$). In our cohort, with a short median follow-up of 24 months, a p53 mutation was by far the single most predictive indicator for recurrence and death (Table 2). Established clinical risk factors such as the presence of lymph node metastases, a large tumor size, and an advanced TNM stage did not show any significant association with survival, presumably because the sample size was small and the follow-up was short. The prognostic value of p53 mutations was independent of ER, PR, and lymph node status in multivariate analyses (disease-free survival, $P = 0.015$, relative risk = 4.71; overall survival, $P = 0.007$, relative risk = 23.2; Tables 3 and 4). Null mutations (nonsense, frameshift, and splice-site mutations) and missense mutations were equally associated with shorter survival ($P = 0.83$) and shorter time to recurrence ($P = 0.80$).

Table 2. Univariate analyses of prognostic factors for overall and disease-free survival in 90 breast cancer patients (average follow-up, 24 months)[a]

Prognostic indicator	Disease-free survival, *P* value[b]	Overall survival, *P* value[b]
ER (neg vs. pos)	0.0350	0.0550
PR status (neg vs. pos)	0.0170	0.0170
Lymph node status (pos vs. neg)	0.7960	0.2790
Lymph node status (>4 pos vs. <4 pos)	0.9700	0.3000
TNM stage[ac] (IIB–III vs. I + IIA)	0.41	0.67
Tumor size (>2 cm vs. <2 cm)	0.2700	0.1400
p53 IHC ≥ 2 antibodies[d]	0.0360	0.0070
p53 IHC PAb1801	0.0360	0.0070
p53 IHC PAb240	0.0160	0.0020
p53 IHC PAb 421	0.0060	0.0050
p53 mutation	0.0008	0.0001

IHC, immunohistochemstry; pos, positive; neg, negative.
[a]Patients with stage IV disease ($n = 2$) and without follow-up ($n = 4$) were excluded from the analyses.
[b]Exact log-rank statistics.
[c]American Joint Committee on Cancer (Third Edition).
[d]We determined the prognostic significance of p53 expression as detected by staining with three antibodies and combinations of two of the three antibodies because there was some variability in staining certain samples with all three antibodies.

Table 3. Bivariate analyses of the prognostic value of p53 mutations after adjustment for one other risk factor

Adjustment	p53 mutation: disease-free survival, *P* value	p53 mutation: overall survival, *P* value
ER status	0.040	0.006
PR status	0.030	0.006
Lymph node status	0.002	0.004
p53 IHC ≥2 antibodies	0.007	0.012

Overexpression of p53 antigen as assessed by nuclear staining with two or more p53-specific monoclonal antibodies or with every single antibody used was also a predictor of early recurrence and shorter survival, but the *P* values were 10–100 times less significant than in the presence of a mutation (Table 2). The greater prognostic value of a p53 mutation relative to positive nuclear immunostaining for p53 overexpression most likely reflects the relatively high number of null mutations in this patient population. Forty-four percent of the 34 mutations were either frameshift, nonsense, or splice junction mutations expected to lead to a truncated p53 protein which would not be detectable immunohistochemically.

Three other groups have found correlations between the presence of p53 mutations and breast cancer and prognosis with median follow-ups ranging from 32 to 60 months, reporting relative risks for disease-free and overall survival ranging between 2.2 and 3.3 in multivariate analyses (Table 5). The much higher prognostic value of p53 mutation in our cohort may be related to a specific characteristic of our population but more likely reflects the highly sensitive method used for mutation detection. The advantages of our approach are (*i*) the use of touch preparations of fresh or partially thawed frozen samples of breast cancer tissue, yielding clusters of pure tumor cells (14); (*ii*) the use of dideoxy fingerprinting, a screening method which detects virtually 100% of mutations (15, 18, 19), and (*iii*) the inclusion of exons 4, 9, and 10, which yield 20% more mutations than are observed when analysis is limited to exons 5–8 (16). Other studies are needed to confirm the

Table 4. Multivariate analyses of recurrence and death in 90 breast cancer patients

Prognostic factor	Early recurrence: Relative risk	Early recurrence: *P* value	Early death: Relative risk	Early death: *P* value
Negative ER status	0.96	0.950	0.63	0.633
Negative PR status	1.30	0.708	1.36	0.740
Positive lymph node status	1.55	0.442	2.25	0.34
p53 mutation	4.71	0.015	23.20	0.007

Table 5. Prognostic value of p53 mutations in breast cancers in three previous studies

Study	No. of patients	Investigated exons	Median follow-up, months	Screening method	Disease-free survival		Overall survival	
					Relative risk	*P* value	Relative risk	*P* value
Ref. 10	200 (node neg.)	5–9	60	SSCP[a]	2.2	0.01	NA	NA
Ref. 11	109	5, 7, 8	32	CDGE[b]	NA	NA	3.3	0.001
Ref. 12	163	5–8	48	CDGE[b]	2.2	0.01	2.9	0.02

NA, not available.
[a]Abnormal mobility by single-strand conformation polymorphism (SSCP) analysis. The mutation was not defined by sequencing.
[b]gel electrophoresis. The mutations were defined by sequencing.

general applicability of our results in an ethnically and geographically homogeneous population.

It is of interest to determine whether p53 gene mutation is a reliable indicator of poor prognosis in both lymph node-negative and lymph node-positive breast cancer patients, because the latter group of patients usually receives adjuvant chemotherapy. Previous evidence links mutations in p53 to increased resistance to irradiation and/or chemotherapeutic drugs, probably due to a defect in the p53-dependent apoptotic pathway induced by anticancer agents in oncogenically transformed cells (4, 27, 28). Thus, patients with p53 mutant tumors might not benefit as much from chemotherapeutic regimens as do patients with tumors containing wild-type p53.

In our sample of 53 node-positive patients, of 18 patients with a p53 gene mutation, 8 relapsed and 4 died. Of 35 node-positive patients without mutation, 7 relapsed and only 1 died ($P = 0.0025$ and 0.0003, respectively). In the 27 axillary node-negative patients in this series, of 9 patients with a p53 gene mutation, 4 relapsed and 2 died. Of 18 patients without a mutation, only 2 relapsed and none died ($P = 0.082$ and 0.049, respectively). The proportion of relapses in patients with a p53 gene mutation is similar in node-negative and node-positive patients, hinting that the prognostic significance of p53 mutations is the same for both nodal groups. However, the small sample size of the group with negative axillary lymph nodes yields results of only suggestive prognostic significance. A larger study is needed to confirm the findings within patients with negative axillary lymph nodes.

In conclusion, our data indicate that p53 gene mutation is highly associated with poor prognosis of breast cancer patients. The basis of this association is unclear but may be related to increased proliferative capacity of tumor cells with p53 mutant protein and/or the greater resistance of such cells to growth inhibition by a variety of chemotherapeutic agents.

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