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The role and prognostic significance of p53 gene alterations in breast cancer

Richard M. Elledge, Suzanne A.W. Fuqua, Gary M. Clark, Pascal Pujol, and D. Craig Allred
University of Texas Health Science Center, San Antonio, Texas, USA

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Summary

Alterations in the p53 tumor suppressor gene are the most frequent genetic changes found in breast cancer, with an incidence reported in a range of 15 to 50%. The incidence of p53 alterations is approximately 15% for in situ carcinoma, while for invasive node-positive disease it is 2 to 3 times higher. This high rate of alteration suggests that the gene plays a central role in the development of breast cancer.

The p53 gene functions as a negative regulator of cell growth. Alterations in the gene lead to loss of its usual negative growth regulation and more rapid cell proliferation. Since p53 alteration can reflect a more advanced state of progression and a higher rate of proliferation, breast tumors that have a p53 alteration could have a greater probability of having micrometastasis. p53 alterations could therefore be a prognostic factor for recurrence after primary local therapy. Consistent with this hypothesis, several independent studies using different methodologies have found that breast tumors with altered p53 have a worse prognosis and are also more likely to have other poor prognostic factors.

Alterations in the p53 tumor suppressor gene are the most frequent genetic changes found in a wide variety of malignancies [1], including breast cancer. This high rate of alteration suggests that the gene plays a central role in cancer development in general, and in breast cancer in particular. 15-50% of breast cancers contain a p53 alteration, depending on the stage and method of detection [2-4]. As with other cancer types, non-invasive or less advanced breast tumors have a lower incidence of alterations [3,5] — for in situ disease, the incidence of mutation is approximately 15%, while for invasive node-positive carcinoma it is 2-3 times higher [3].

Mutation can result in a prolonged protein half-life and accumulation of the altered protein in the nucleus. Immunohistochemical (IHC) staining can detect this abnormal accumulation and is therefore thought to be an indirect indication of a mutation. 30-50% of breast tumors [2,3,5,6] have accumulation of p53 protein as measured by IHC. By DNA-based methods, fewer abnormalities are detected, between 15-45% [4,7-10]. Possible reasons for the lower detection rate by this method include presence of mutation outside the area screened, or a large portion of stromal or non-mutated tumor cells resulting in a dilution of the signal from mutated tumor DNA to below the level of detectability.

Alternatively, IHC may overestimate the incidence of mutation. Stabilization or accumulation of protein may occur for reasons other than a

Address for offprints and correspondence: Richard M. Elledge, M.D., Division of Medical Oncology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio TX 78284-7884, USA; *Tel:* 210-567-47777; *Fax:* 210-567-6687

mutation. Indeed, some have observed cytoplasmic accumulation of p53 protein, but detected no mutation by sequencing [11]. The level of p53 protein normally increases during late G_1 and S-phase [12], and also increases in response to DNA damage [13]. Cells that are in these phases of the cell cycle or are genetically damaged may have positive staining without a mutation. Accumulation of p53 could also occur as a result of binding to other cellular proteins. p53 is known to bind MDM-2 protein [14], a cellular oncoprotein, as well as several viral oncoproteins [15,16] which could have cellular homologues.

Breast tumors frequently show loss of heterozygosity (LOH) of the short arm of chromosome 17 [17,18], the region that contains the p53 locus. If p53 behaved as a classical tumor suppressor gene, one would expect that both alleles would have to be inactivated. Of tumors that have LOH of 17p, only approximately 50% or less show the remaining allele to be mutated [10,19]. The fact that the remaining allele is apparently wild-type in more than 50% of cases could have a number of explanations. First, it could simply be the result of an inability to detect a mutation experimentally, even though it is present, because of tumor heterogeneity. Second, loss of even one allele of p53 may give a cell a slight proliferative advantage over cells with two intact alleles. Third, LOH at 17p without a p53 mutation could reflect the presence of another tumor suppressor gene on this portion of the chromosome. Fourth, it might simply be the result of the random genomic instability of cancer cells.

Because the p53 gene is frequently mutated, and certain categories of mutation result from endogenous events compared to exogenous agents [1], it may be possible to extrapolate backward using the pattern of mutations occurring in a particular tumor type, in order to gain some insight as to the inciting or causative event. As an example, the mutational patterns in basal and squamous cancer of the skin are consistent with changes produced by UV light exposure, as one would expect [20]. Lung cancer also has a pattern of mutations consistent with exposure to environmental carcinogens [1]. In a recent study and review of the subject, Coles et al. [10] found a bias towards G→T transversions and a relatively high incidence of mutations involving guanosine on the non-transcribed strand in breast tumors, suggesting an exogenous carcinogen as an etiologic or promoting agent for these mutations. Though interesting, the interpretation of this data to suggest that external carcinogens play a role in breast cancer should be made with caution. The milieu and characteristics of breast tissue may simply select for these types of changes.

Since the discovery that germline p53 mutations can cause familial cancer syndromes [21], it has been of interest to determine if p53 germline mutations contribute to familial breast cancer, especially the premenopausal or bilateral type, or if these mutations are often found in women with breast cancer diagnosed at a relatively young age, in their 20's or 30's. Several studies have addressed this issue. The incidence of p53 germline mutations as a cause of familial [22-24], early onset [25], or bilateral breast cancer [26] is low, $\leq 1\%$. Because of the very low incidence of germline mutations, the screening of these groups would not be justified.

Testing for p53 alterations in breast tumors, however, may have a prognostic clinical application. Alterations in the p53 gene lead to loss of its negative growth regulatory function, and hence to more rapid cell proliferation [27]. Also, p53 alterations are more often found in invasive or advanced malignancies [3,5,28,29]. This suggests the possibility that p53 alterations occur more often as a late event in the transformation process, or are associated with an increase in metastatic potential. For these reasons, and because p53 mutations are common in breast cancer, it was our hypothesis that p53 mutations could be an indicator of the likelihood of occult distant micrometastasis in node-negative breast cancer, and thus might be a good prognostic factor in predicting future recurrence.

We have examined this hypothesis in node-negative breast tumors. p53 alterations were detected by single strand conformation poly-

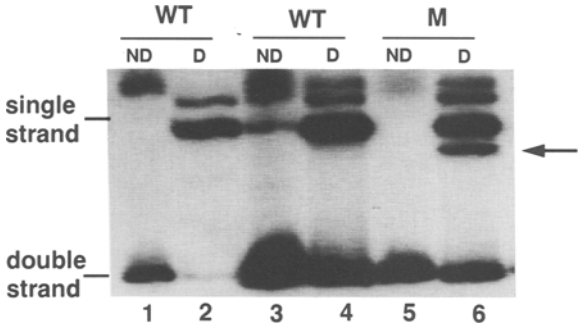

Figure 1. SSCP analysis of exon 8/9 single-strand conformers are seen at the top and double-strand conformers are seen at the bottom. Samples were run under denaturing (D) and nondenaturing (N) conditions. An abnormally migrating band, representing a GTT to GCT mutation at codon 274, is seen in lane 6. WT: wild type; M: mutation.

morphism analysis (SSCP). We focused our search for p53 mutations on exon 5 through 9 because the majority of p53 mutations in tumors have been found in this region [30]. This region of the gene is highly conserved in evolution [31], which reflects its functional significance. Three separate segments of the p53 sequence in this region were amplified by DNA PCR. These segments encompassed exons 5 and 6, exon 7, and exons 8 and 9. The amplified fragments were examined by SSCP analysis [32]. This is an electrophoretic method that can identify single base differences in amplified DNA fragments.

We used this method to search for p53 alterations in 200 node-negative tumors. Because very small tumors have a very good prognosis and are seldom treated, while patients with large tumors are now usually given adjuvant therapy independent of other prognostic factors, we limited our study to tumors in the intermediate 1 to 3 cm range. Here treatment decisions could well depend on prognostic factors.

The median follow-up for patients in our study was 71 months, and most patients, 80%, did not receive adjuvant therapy.

An example of an SSCP analysis for exons 8/9 is seen in Figure 1. Tumor DNA samples were run under both non-denaturing and denaturing conditions. Under denaturing conditions, the DNA is separated into single strands. An abnormally migrating band, representing a mutation, is seen in the far right lane. This abnormal band was cut from the gel, and the DNA cloned and sequenced. A missense mutation was found in codon 274 of exon 8. This results in a valine to alanine change in a conserved amino acid.

Sequence analysis was performed on 17 of our 28 tumors with an abnormal SSCP. The results are seen in Table 1. Most of the mutations were missense, though 2 deletions were found which resulted in a stop codon and protein truncation a short distance 3' to the deletions. Four of the tumors contained a previously described neutral polymorphism.

A total of 28 tumors, or 14%, had an abnormal SSCP. 6% (12) of the tumors had an abnormal analysis for exon 5 and 6, 1.5% (3) for exon 7, and 6.5% (13) for exons 8 and 9. The results of the SSCP analysis were correlated with clinical outcome. For each region separately, women with an abnormal SSCP had a worse disease-free survival, though this difference was not statistically significant for exon 5/6 and 8/9. There were only 3 patients with abnormality in exon 7, but all 3 recurred early. Grouping all the women with a p53 alteration together and comparing them to women with tumors not having an alteration, the difference in disease-free survival was statistically significant, $p = .01$. This is seen in Figure 2. 43% of women with a p53-altered tumor had a recurrence by 5 years, while only 21% of women without an alteration(by SSCP) had a recurrence.

The results of the SSCP analysis were compared with other established prognostic factors. p53 alterations were significantly associated with negative estrogen receptor, aneuploidy, and patient age <50 years. There was a trend towards an association of p53 alterations with high S-phase and negative PgR. Table 2 shows that in a multivariate analysis, which included age, p53, ER, PgR, tumor size, ploidy, and S-phase, only p53 status by SSCP and age <50 independently predicted the relative risk of recurrence. The relative risk of women with a p53-altered tumor

Table 1. DNA sequencing results

Exon	Codon	Change	Result
5	134	TTT → TTG	Phe → Leu
6	213	CGA → CGG	neutral polymorphism
6	213	CGA → CGG	neutral polymorphism
6	213	CGA → CGG	neutral polymorphism
6	213	CGA → CGG	neutral polymorphism
6	209	AG deletion	frameshift, truncation
7	237	ATG → ATA	met → ile
7	243	G deletion	frameshift, truncation
8	266	GGA → GAA	gly → glu
8	273	CGT → AGT	arg → ser
8	273	CGT → TGT	arg → cys
8	273	CGT → CAT	arg → hist
8	274	GTT → GCT	val → ala
8	274	GTT → ATT	val → ile
8	278* 274*	CCT → CGT GTT → ATT	pro → arg val → ile
8	285	GAG → AAG	glu → lys
8	286	GAA → AAA	glu → lys

* Two mutations in a single tumor

in this study was 2.2.

Mutations in p53 are associated with transformation of cells in culture and loss of p53's usual negative control of cell proliferation. This DNA based pilot study shows that women with small, node-negative tumors that contain a p53 alteration have a worse prognosis and higher risk of relapse at 5 years, and are more likely to have other poor prognostic factors.

By other methods, alterations in p53 are associated with a worse prognosis and poor prognostic factors. A number of immunohistological studies have also found that alterations in p53 protein are associated with a worse prognosis [5,6,33,34]. Thor found that nuclear accumulation of p53 protein was correlated with a worse metastasis-free and overall survival in both lymph node-positive and lymph node-negative patients [5]. In another study of only lymph node-negative tumors, a high level of p53 protein expression (>20% of cells staining) was associated with a significantly worse survival at 8 years than found for tumors with low or no expression [33]. In the largest study to date, including 700 node-negative patients, p53 overexpression detected in frozen sections of breast tumors was also associated with a worse overall and disease-free survival at 5 years [6]. p53 accumulation is associated with a number of negative prognostic factors including negative estrogen receptor [5,6, 33-36], negative progesterone receptor [5,33,35], HER-2 protein overexpression [33-35], and a higher proliferative fraction [6,33,37]. Interestingly, p53 status has only a weak association with tumor size in some studies [6,33-35], and in other studies none at all. In our SSCP study, an abnormal signal was associated with ER negativity, younger age, aneuploidy, and a higher proliferative fraction.

Aneuploidy is an indicator of the loss or duplication of genes within a tumor cell. It is believed that loss of the normal p53 allele, combined with the presence of a mutation in the other allele, is a mechanism which leads to uncontrolled growth in tumors. Aneuploidy can reflect this loss of heterozygosity, so the

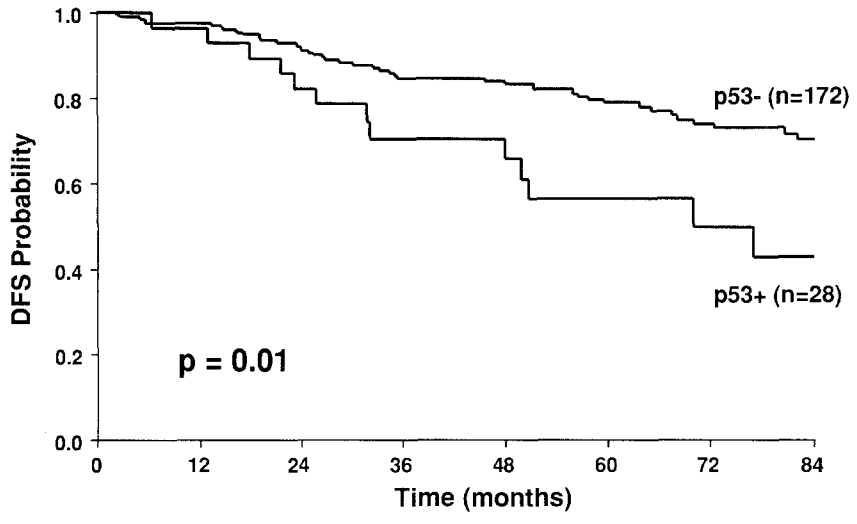

Figure 2. Disease-free survival in patients with node-negative breast cancer, tumor size 1-3 cm, as a function of a normal or abnormal SSCP analysis for p53, exons 5 through 9.

combination of aneuploidy and p53 mutations might be expected since cells with a loss of heterozygosity at the p53 locus and a mutation at that locus would gain a selective growth advantage over cells that had either, but not both. An enhanced potential to recur or metastasize could result from this combination.

SSCP-abnormal tumors had a comparatively high median S-phase fraction. The median S-phase fraction for SSCP-abnormal tumors was 13.9%, while the median was 7.6% for tumors with a normal pattern. Tumors with altered protein detected by IHC also have higher proliferative fractions [6,33,34]. This also supports the biologic hypothesis that p53 negatively regulates cell division and that mutations abrogate this regulation, giving rise to cells with a greater proliferative rate. p53 mutations may therefore result in increased proliferation or a higher S-phase fraction.

However, if increased proliferation was the only consequence of p53 mutations, it would be expected that these mutations would have no additional prognostic value over S-phase fraction. The results of this SSCP study do not support this line of reasoning. In a multivariate analysis which included S-phase fraction, an abnormal SSCP analysis predicted recurrence better than S-phase, suggesting that p53 mutations are contributing to the risk of recurrence by a mechanism other than simply increasing cell proliferation. From this, one can theorize that p53 has other cellular functions besides controlling the rate of

Table 2. Multivariate analysis of p53 alterations and prognosis in 155 node-negative breast cancer patients

Factor	p-Value	Relative risk
Age (<50 vs ≥50)	.007	2.4 (1.3 - 4.4)
p53 (abnorm vs norm SSCP)	.02	2.2 (1.1 - 4.3)
ER	.5	–
PgR	.93	–
Size	.11	–
Ploidy	.65	–
S-phase	.43	–

cell division, and that a mutation in the gene disrupts this function, resulting in a greater risk of spread or recurrence. One current hypothesis is that p53 can somehow sense the presence of genetic damage, and a functioning p53 allows the cell time to repair damaged DNA templates [38, 39].

For each of the three regions surveyed, the recurrence was greater for women with tumors that had an abnormal SSCP analysis. However, women with an alteration in exon 7 had a greater recurrence relative to those with an alteration in exons 5, 6, 8, or 9, though this was based on a small number of patients. Most mutations that cause the Li-Fraumeni syndrome have been found in exon 7 [21], though at least one has been reported in exon 5 [40]. These observations suggest that mutations in a specific area of the p53 gene, i.e. exon 7, could give rise to a more aggressive malignant phenotype than mutations in other exons. Mutations in exon 8 or 9 may not be as important in contributing to neoplastic behavior.

There are two potential limitations involving the technique of SSCP analysis. First, it will detect single base pair changes in DNA which, because of the degeneracy of the genetic code, do not necessarily result in an amino acid change, and even if a change occurs, the protein function is not necessarily altered. There are three such neutral polymorphisms known in the coding region of the p53 [41]. Two of these lie outside the area surveyed in our study, and the other is at codon 213 in exon 6. 4 tumors with this neutral polymorphism were found in this series, and others could be present. If this change has no relationship to breast cancer, deleting this type of tumor from statistical analysis would probably increase the statistical power of the findings. Alternatively, this change could be genetically linked to other alterations in the p53 gene, or in adjacent genes on the same chromosome. These linked changes could predispose to a more aggressive phenotype of breast cancer. An example of a silent (intronic) base change in p53 being linked to a change resulting in an amino acid substitution has been reported [42]. In that example, the function of the protein was not greatly altered, though more subtle alterations cannot be excluded.

Secondly, the sensitivity of SSCP analysis in any given situation is unclear. The conditions chosen for this study gave the greatest band resolution of the exons tested. By immunohistochemistry, accumulation of p53 protein leading to positive staining occurs in 15-50% of invasive breast cancers. It is thought that this accumulation of p53 protein is a result of a mutation, though epigenetic mechanisms, as previously discussed, are also possible. 176 of the 200 tumors used in this study could be evaluated by immunohistochemistry, and 49% had some positive staining [6]. It is unclear why the incidence of mutations was lower in this study in comparison to the immunohistochemical method, though the alterations that were found resulted in a worse prognosis.

It may be that mutations outside of exons 5-9 could, in part, be responsible for this discrepancy between IHC and SSCP. We attempted to survey exon 4 but were hampered by an amplification rate of only 40-50% and poor band resolution. Of approximately 50 tumors for which a signal was obtained, none were definitely abnormal, though changes could have been obscured for the technical reasons mentioned above. Others have surveyed exon 2 with SSCP, and no SSCP-abnormal breast tumors were found out of 96 tested [8].

In summary, p53 alterations play an important role in breast cancer development. Several independent studies using different methodologies have found that alterations in the p53 gene in breast tumors indicate a worse prognosis and higher risk of relapse. Unfortunately, the absence of a p53 alteration does not by itself define a node-negative group of patients whose risk of relapse is low enough that most physicians would consider not giving adjuvant therapy. The use of other factors in combination with p53 will be needed to achieve this goal.

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