Hormone Receptor Status Rather Than HER2 Status Is Significantly Associated With Increased Ki-67 and p53 Expression in Triple-Negative Breast Carcinomas, and High Expression of Ki-67 but Not p53 Is Significantly Associated With Axillary Nodal Metastasis in Triple-Negative and High-Grade Non–Triple-Negative Breast Carcinomas

Jeong S. Han, MD, PhD,1 Dengfeng Cao, MD, PhD,2 Kyle H. Molberg, MD,1 Venetia R. Sarode, MD,1 Roshni Rao, MD,3 Lisa M. Sutton, MD,1 and Yan Peng, MD, PhD1

Key Words: Ki-67; p53; Triple-negative; Breast cancer; Axillary nodal metastasis

DOI: 10.1309/AJCP9DV3EVZUATFV

Abstract

Triple-negative (TN) breast carcinoma is associated with a higher recurrence rate and shorter survival and lacks the benefit of specific therapy. TN tumors usually express high levels of Ki-67 and p53 that are considered prognostic markers for breast cancer. We compared Ki-67 and p53 expression between TN and high-grade non-TN invasive carcinomas in a total of 214 cases and investigated an association between their expression and axillary nodal metastasis in these tumors. Our findings demonstrate that TN tumors are associated with significantly higher expression of Ki-67 and p53 compared with non-TN tumors, which may contribute to the poorer prognosis in TN tumors. Hormone receptor negativity rather than HER2 negativity is associated with the significantly increased Ki-67 and p53 expression in TN tumors. Furthermore, a high expression level of Ki-67 but not p53 is more likely to be associated with axillary nodal metastasis in these cases.

Human breast carcinomas represent a collection of diverse tumors that vary in their natural history and responsiveness to therapy. Previous studies have classified breast carcinomas into different subtypes mainly based on their molecular and immunohistochemical profiles of hormonal receptors and human epithelial growth factor receptor 2 (HER2) overexpression.1,4 Triple-negative (TN) breast carcinoma is characterized by negativity for estrogen receptor (ER), progesterone receptor (PR), and HER2. TN breast carcinoma is a high-risk tumor that lacks the benefit of specific therapy that targets these proteins. TN breast cancer is associated with a higher histologic grade, shorter survival, and a higher recurrence rate.5 TN tumors usually express high levels of Ki-67 and p53 that are currently considered prognostic markers for patients with breast cancer.1,2

Ki-67, proliferation index, is a nonhistone nuclear protein that is closely linked to proliferating cells. High Ki-67 expression has been shown to be associated with a higher histologic grade, larger tumor size, the presence of axillary lymph nodal metastasis, and shorter disease-free and overall survival in patients with breast cancer.2,3,6-8 In addition, a positive correlation between Ki-67 expression and pathologic tumor response to neoadjuvant chemotherapy has been reported, implicating its role in response to treatment.9 Some researchers used meta-analysis to unify various results of previous studies; they identified 524 genes that were significantly...
associated with breast cancer survival and found that 70% of these genes were strongly correlated with proliferation. This meta-analysis highlighted the important role of proliferation in breast cancer prognosis.

The p53 gene encodes for a 53-kDa nuclear phosphoprotein, which has been implicated in controlling cell-cycle regulation, differentiation, DNA repair, and apoptosis. Unlike normal p53, nonfunctional mutated p53 accumulates in the nucleus of tumor cells, and, therefore, it can be detected by immunohistochemical analysis. Multiple studies have shown that p53 overexpression in breast cancer is associated with a worse outcome. Recent studies suggested that p53 status might have a different predictive value for the efficacy of anthracycline/alkylating agent–based chemotherapy regimen between TN and non-TN breast cancers.

The aims of our study were to compare Ki-67 and p53 expression between TN (histologic grade II or grade III tumors) and high-grade (grade II or grade III) non-TN invasive breast carcinomas, respectively, and to investigate an association between their expression and axillary nodal metastasis with an attempt of exploring a cutoff value of these markers in predicting nodal metastasis in these cases. We also further analyzed differential expression of Ki-67 and p53 in subgroups of non-TN tumors (ER+/PR+/HER2−, ER+/PR+/HER2+, and ER−/PR−/HER2+−) to determine which biomarker (hormone receptors vs HER2) was correlated with Ki-67 and p53 overexpression.

**Materials and Methods**

This study was approved by the institutional review board. A total of 214 cases of high-grade carcinoma of the breast from the surgical pathology files of the University of Texas Southwestern Medical Center, Dallas, and Parkland Memorial Hospital, Dallas, between 2003 and 2009 were included in this study. Clinical information was obtained from electronic medical records. Clinical characteristics and immunohistochemical tumor profiles were analyzed in 81 TN tumors and 133 non-TN tumors. Of the 133 non-TN tumors, 18 (13.5%) were ER+/PR−/HER2+, 18 (13.5%) were ER+/PR+/HER2+, and 97 (72.9%) were ER+/PR+/HER2−. The expression of Ki-67 and p53 was compared between the TN and non-TN groups. In addition, we compared the Ki-67 and p53 expression between the TN tumors with nodal metastasis (32/81 [40%]) and the non-TN tumors with nodal metastasis (63/133 [47.4%]). Subsequently, the TN and non-TN tumors were combined as a whole (n = 214) and then divided into 2 subgroups based on the percentage of Ki-67+ and p53+ tumor cells (high Ki-67 or high p53 group, >10%; low Ki-67 or low p53 group, ≤10%), and ER expression and the percentage of positive axillary lymph nodes were compared between the 2 subgroups as well.

**Immunohistochemical Assay and Image Quantitation Methods**

We used quantitative immunohistochemical analysis to determine ER, PR, HER2, Ki-67, and p53 expression in the breast tumors. Immunohistochemical staining was performed using a TechMate 1000 automated immunostainer (Ventana Medical Systems, Tucson, AZ). A known positive control section was included in each run to ensure proper staining. Rabbit immunoglobulin fraction (normal) or nonspecific IgG1 monoclonal diluted with phosphate-buffered saline was used as a negative control. Monoclonal antibodies were used for ER (1D5, prediluted, Ventana Medical Systems), PR (PgR, prediluted, Ventana Medical Systems), HER2 (dilution 1:1,200; DakoCytomation, Carpinteria, CA), Ki-67 (MIB-1, prediluted, Ventana Medical Systems), and p53 (DO-7, 1:16,000; DAKO).

Quantitative information for staining was obtained using the automated microscopy method, Automated Cellular Imaging System (ACIS, Clarient, San Juan Capistrano, CA). The ACIS system consisted of an automated robotic bright-field microscope module, a computer, and a Windows NT–based software interface. The robotic microscope module scanned the immunohistochemically stained slides, and the computer monitor displayed the digitalized tissue images. After viewing the high-magnification images on the ACIS computer, several subregions of the digitalized tissue images were selected for analysis by the ACIS. To assess the level of tissue ER and PR expression, the ACIS provided the percentage of positively stained cells for ER and PR in the selected subregions. We used the manufacturer’s guidelines for the ACIS to determine tissue ER, PR, and HER2 expression.

When the percentage of cells staining positive for ER and PR was 5% or more, the tumor was considered positive for ER and PR expression. Ki-67 and p53 were considered as high expression when positive cells were more than 10% and as low expression when positive cells were 10% or less. To assess HER2 overexpression, the ACIS provided an average score for 5 selected subregions of the tissue with the highest staining intensity for HER2. Greater than 30% of tumor cells stained with an average intensity score of 2.0 or more were considered to have HER2 overexpression (3+). A score of more than 1.4 to less than 2.0 was considered a borderline result (2+, 10%-30% of tumor cells stained), and a score of less than 1.4, negative (0 or 1+, <10% of tumor cells stained). All of the results were confirmed by manual review by a pathologist (Y.P.).

The criteria for determining triple negativity were based on immunohistochemical analysis and image quantitation of ER, PR, and HER2. Negative results were based on less than 5% positive staining of ER or PR, and staining of 0 or 1+ for HER2. All positive, borderline, or negative HER2 results by immunohistochemical analysis were confirmed by fluorescent in situ hybridization. Positivity of Ki-67 and p53 was determined...
by the presence of nuclear staining. Grading was based on the percentage of stained tumor cells. A favorable prognostic category for both biomarkers is considered to be less than or equal to 10% of tumor cells staining positively.

**HER2 Fluorescent In Situ Hybridization**

Two of the unstained slides were cut at a 4-μm thickness. Dual-color fluorescent in situ hybridization was performed with the HER2 probe labeled with spectrum red and chromosome 17-specific centromere (D17Z1) probe labeled with spectrum green on sections cut from the same block. Deparaffinization, in situ hybridization, and staining were performed using the PathVysion kit (Abbott-Vysis Laboratories, Abbott Park, IL) per the manufacturer’s protocols. Fluorescent signals in at least 60 nonoverlapping interphase nuclei with intact morphology were scored with a 100× objective, using a triple band-pass filter that permits simultaneous blue, green, and red colors. Only tumor cells from the site designated on the H&E-stained slide by the pathologist were scored for the number of red (HER2) and green (chromosome 17) signals. A case was scored as amplified if the ratio of the number of fluorescent signals of HER2 to chromosome 17 was greater than 2.2.

**Statistical Analysis**

Tumor size, mean age, and the expression of Ki-67 and p53 were compared between the TN and non-TN groups by 1-way analysis of variance with the Tukey post hoc test. Ki-67 and p53 expression was individually compared with ER and HER2 expression using the Pearson correlation test. Numbers of lymph nodes with metastasis were compared between the high p53 group and the 3 subgroups of non-TN tumors are summarized in Table 1.

Subsequently, the non-TN tumors were further classified into 3 subgroups based on their hormone receptor (ER and/or PR) and HER2 status as follows: ER+/PR±/HER2–, ER–/PR+/HER2+ and ER+/PR+/HER2+. Mean age of the patients, tumor size, and Ki-67 and p53 expression in the TN tumor group and the subgroups is shown as mean percentage ± SEM in Table 1 and in graphs in Figure 1.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Non-TN Tumors</th>
<th>TN Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>ER+/PR±/HER2– (n = 97)</td>
<td>58.3 ± 1.44</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>ER+/PR±/HER2– (n = 18)</td>
<td>1.99 ± 0.19</td>
</tr>
<tr>
<td>Ki-67 (%)</td>
<td>ER+/PR±/HER2+ (n = 18)</td>
<td>23.98 ± 2.51</td>
</tr>
<tr>
<td>p53 (%)</td>
<td>ER+/PR+/HER2+ (n = 18)</td>
<td>9.06 ± 2.15</td>
</tr>
<tr>
<td></td>
<td>ER–/PR+/HER2+ (n = 81)</td>
<td>53.9 ± 4.83</td>
</tr>
</tbody>
</table>

* Data are shown as mean ± SEM; P values obtained by 1-way analysis of variance.

© American Society for Clinical Pathology

### Table 1

Mean Age, Tumor Size, Ki-67 Activity, and p53 Expression in TN Tumors and Non-TN Tumors in Three Subgroups

<table>
<thead>
<tr>
<th></th>
<th>Non-TN Tumors</th>
<th>TN Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>ER+/PR±/HER2– (n = 97)</td>
<td>58.3 ± 1.44</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>ER+/PR±/HER2– (n = 18)</td>
<td>1.99 ± 0.19</td>
</tr>
<tr>
<td>Ki-67 (%)</td>
<td>ER+/PR±/HER2+ (n = 18)</td>
<td>23.98 ± 2.51</td>
</tr>
<tr>
<td>p53 (%)</td>
<td>ER+/PR+/HER2+ (n = 18)</td>
<td>9.06 ± 2.15</td>
</tr>
<tr>
<td></td>
<td>ER–/PR+/HER2+ (n = 81)</td>
<td>53.9 ± 4.83</td>
</tr>
</tbody>
</table>

ER, estrogen receptor; PR, progesterone receptor; TN, triple negative (for ER, PR, and HER2).
Although this did not reach statistical significance. Expression of p53 in the ER–/PR–/HER2+ subgroup was significantly higher than that in the ER+/PR±/HER2+ group (68.2 ± 9.03 vs 25.7 ± 8.2; \( P < .05 \)).

**Correlation of Hormone Receptor and HER2 With Ki-67 and p53 Expression**

The preceding results demonstrated that hormone receptor (ER and/or PR) status rather than HER2 status was significantly associated with increased Ki-67 and p53 expression in the TN tumors. The Pearson correlation test was performed to further investigate the association between hormone receptor and HER2 status and Ki-67 and p53 expression. ER expression showed a significant negative correlation with Ki-67 expression (\( r = –0.527; \ P < .0001 \)) and with p53 (\( r = –0.534; \ P < .0001 \)). HER2 overexpression showed no significant correlation with Ki-67 expression (\( r = 0.033; \ P = .63 \)) or with p53 expression (\( r = 0.078; \ P = .25 \)). These findings further support our hypothesis that hormone receptor status, but not HER2 status, is closely associated with Ki-67 and p53 expression in TN breast cancers.

**Comparison of Percentage of Positive Axillary Nodes and ER Expression of Tumors Between the High and Low Ki-67/p53 Groups**

To determine a cutoff value of Ki-67 and p53 expression in predicting axillary nodal metastasis in these cases, we combined the TN and non-TN tumors as a whole (n = 214) and then divided them into 2 subgroups based on the percentage of Ki-67+ or p53+ tumor cells: high Ki-67 or p53 group, more than 10%; low Ki-67 or p53 group, 10% or less. The percentages of positive axillary lymph nodes when an axillary dissection was performed were compared between the 2 subgroups. In addition, comparison of ER expression between the 2 subgroups was also conducted to further investigate the relationship between ER and Ki-67/p53 expression.

The 44 tumors with low Ki-67 expression level showed significantly higher ER expression compared with the 170 tumors in the high Ki-67 group (69.3% ± 5.6% vs 41.4% ± 3.6%; \( P = .0003 \); unpaired \( t \) test) \( \text{Figure 2A} \). The 127 tumors with a low p53 expression level showed significantly higher ER expression compared with the 87 tumors in the high p53 group (65.8% ± 3.7% vs 19.8% ± 4.0%; \( P = .0001 \); unpaired \( t \) test) \( \text{Figure 2B} \). These results provide further evidence that ER expression is negatively correlated with high Ki-67 and p53 expression.
The high Ki-67 group included 170 cases. Results of sentinel lymph node biopsy (SLNB) and/or axillary lymph node dissection (ALND) were available for review in 144 of these 170 cases. A total of 1,305 lymph nodes from the 144 cases were examined, and 386 of them were positive for macrometastasis (386/1305 [29.6%]). The low Ki-67 group had 44 cases, and results of SLNB and/or ALND were available for review in 36 of them. A total of 302 lymph nodes from the 36 cases were examined, and 49 of them were positive for macrometastasis (49/302 [16.2%]). The percentage of positive lymph nodes in the high Ki-67 group (29.6%) was significantly higher than that in the low Ki-67 group (16.2%).

The high p53 expression group had 87 cases, and results of SLNB and/or ALND were available for review in 70 of them. A total of 694 lymph nodes from the 70 cases were examined, and 183 of them were positive for macrometastasis (183/694 [26.4%]). The low p53 group had 127 cases, and results of SLNB and/or ALND were available for review in 110. A total of 913 lymph nodes from the 110 cases were examined, and 252 of them were positive for macrometastasis (252/913 [27.6%]). There was no significant difference in the percentage of positive lymph nodes between the high p53 and low p53 groups (P = .61; Fisher exact test)

**Discussion**

Our study demonstrated that Ki-67 and p53 expression was significantly higher in TN breast cancers compared with high-grade non-TN tumors. This finding suggests that Ki-67 and p53 expression may have a role in a worse prognosis of TN breast cancers. It is conceivable that tumors with a worse outcome may have a higher proliferation index, Ki-67, and higher probability of mutation in tumor suppressor genes, such as p53. Because we specifically excluded low-grade (grade I) invasive carcinomas in this study, the higher expression of Ki-67 and p53 in TN tumors compared with expression in the non-TN tumors was less likely owing to their high histologic grade, which is commonly seen in TN tumors, and was more likely associated with the TN status of these breast cancers. Although previous studies showed that TN tumors had higher Ki-67 and p53 expression than non-TN tumors, the authors did not exclude low-grade breast carcinomas in their studies for the comparison.

Subsequently, we investigated whether hormone receptor negativity or HER2 negativity was associated with significantly increased Ki-67 and p53 expression in TN tumors. Our results revealed that hormone receptor (ER and/or PR) negativity, but not HER2 negativity, was significantly associated with high Ki-67 and p53 expression in TN tumors. Our analysis of subgroups based on hormone receptor and HER2 status indicated that Ki-67 and p53 expression was negatively correlated with hormone receptor status.
correlated with ER expression, but not with HER2 overexpression. Previous studies done in all types of breast carcinomas, including low-grade carcinomas, revealed that Ki-67 expression had a negative correlation with ER expression that supports our findings, but they did not examine the relationship between p53 and ER expression and the relationship between Ki-67/p53 and HER2 overexpression.17-19 Owing to their higher expression in TN tumors, Ki-67 and p53 may be used as predictors for response to chemotherapeutic agents. Ki-67 overexpression has been implicated in a better response to chemotherapy agents.7 The posttreatment Ki-67 expression level is currently considered to have significant prognostic and predictive value.6,20,21 Although no consensus has been established so far, a few specific chemotherapeutic agents have been shown to be particularly effective in tumors with high Ki-67 expression.9,22 It has been shown that breast tumors with a p53 mutation were generally ER– and were associated with decreased disease-free survival.11,14 The underlying mechanism that may involve this association is unclear. A few molecules, such as ellipticine, PRIMA-1, RITA, and MIRA-1, have been used for the restoration of p53 function, and some of them demonstrated antitumor activity during in vitro or in vivo experiments.11

Our findings support the currently proposed molecular classification of breast cancer by gene expression profile,4,23 in which breast carcinomas are classified into ER+ (luminal A and B) and ER– (HER2+ and TN) groups. Different types of tumors were shown to have different tumor biology, different prognoses, and different responses to therapy. ER status may have a more important role than HER2 status in predicting prognosis of TN and non-TN breast cancers. In the luminal subgroup that encompasses tumors that express ER and genes related to activation of the ER pathway, the luminal B subtype of breast cancer expresses lower ER positivity and higher Ki-67 level and has a worse prognosis compared with the luminal A subtype. HER2+ tumors were originally considered to be associated with a worse prognosis in hormone receptor–negative tumors. However, recent studies have shown that treatment with trastuzumab appeared to eliminate the disparity in outcomes.24 The lack of specific therapy in TN tumors highlights the importance of further biologic characterization of these tumors, which will lead to the development of effective treatment strategies. Our findings reinforce the emerging notion that breast cancer should be classified according to its gene expression profile, in order to make accurate predictions about the outcome of the disease and select the optimal treatment for patients with breast cancer.
Our study also indicated that the Ki-67 and p53 expression levels in TN tumors with nodal metastasis were significantly higher than those in non-TN tumors with nodal metastasis. To determine a cutoff value of Ki-67 and p53 expression in predicting axillary nodal metastasis in these cases, we combined the TN and non-TN tumors as a whole and then divided them into 2 subgroups: high Ki-67 or p53 (>10%) and low Ki-67 or p53 (≤10%). We found that the percentage of lymph nodes with metastasis was significantly higher in tumors with a high Ki-67 expression level compared with those with low Ki-67 expression, while there was no significant difference in the frequency of lymph node metastasis between the high p53 and low p53 groups. Some previous studies revealed that a high Ki-67 expression level was associated with more frequent lymph node metastasis in patients with all types of breast cancer, but they did not investigate an association between p53 expression and nodal metastasis.\(^7\)\(^{25,26}\) Our findings demonstrated that a high expression level of Ki-67 (>10%) but not p53, although both are considered prognostic markers for breast cancer, was significantly associated with axillary nodal metastasis in patients with TN and high-grade non-TN tumors, indicating that Ki-67 has a better prognostic value than p53 for these breast carcinomas. Previous studies have demonstrated that TN breast tumors are more frequently associated with lymph node metastasis compared with non-TN tumors. Our results suggest that high Ki-67 expression may contribute to the higher risk for nodal metastasis in TN tumors. The overall prognostic value of p53 appears to be weaker than Ki-67 because a variety of studies have found conflicting data.\(^8\)\(^{27,28}\) The more consistent results regarding the prognostic value of Ki-67 over p53 may be due to its significant association with higher nodal metastasis.

To our knowledge, this study is the first to demonstrate that ER negativity rather than HER2 negativity is significantly associated with increased Ki-67 and p53 expression in TN breast carcinomas, and a high expression level of Ki-67, but not of p53, is significantly associated with axillary lymph node metastasis in TN and high-grade non-TN breast carcinomas.

Although there appears to be significant prognostic and predictive value to an immunohistochemical assessment of Ki-67 and p53, it is still not recommended for routine clinical practice. This resistance is primarily due to continued unresolved issues regarding these markers. First, the cutoff values for Ki-67 and p53 are variable in different studies, making it difficult to conduct a comparative analysis. Standardized staining methods and image analysis are also required to achieve consistent measurement in different laboratories. Another potential problem is that the p53 overexpression detected by immunohistochemical analysis may not necessarily correlate directly with p53 mutations. Mutations of p53 are heterogeneous, and some of them may not produce a stable protein that is detectable by immunohistochemical analysis.\(^11\) Wild-type p53 may accumulate in some conditions by binding to other cellular proteins, producing positive immunohistochemical results.\(^11\) In fact, it has been shown that correlation between immunohistochemical analysis and sequencing of p53 in breast cancers is less than 75% and that the immunohistochemical method has a lower sensitivity and specificity compared with sequence-based analysis.\(^29\) Numerous proteins have been shown to interact with p53 and modify its activity. The p53 signaling pathway is closely linked to other signal transduction pathways such as BRCA1.\(^30\) Although the direct mechanism is not evident at this time, our results suggest potential association between the ER signaling pathway and Ki-67 and p53 regulation.

TN breast carcinomas are associated with significantly higher expression of Ki-67 and p53 compared with non-TN tumors, which may contribute to the poorer prognosis in TN tumors. Hormone receptor (ER and/or PR) negativity rather than HER2 negativity is significantly associated with the increased Ki-67 and p53 expression in TN tumors. Furthermore, high expression level of Ki-67 (>10%), but not of p53, although both are considered prognostic markers for breast cancer, is significantly associated with axillary nodal metastasis in patients with TN and high-grade non-TN tumors, indicating that Ki-67 has a better prognostic value than p53 for these breast carcinomas.

References


