Intratumoral Expression Level of Epidermal Growth Factor Receptor and Cytokeratin 5/6 Is Significantly Associated With Nodal and Distant Metastases in Patients With Basal-like Triple-Negative Breast Carcinoma

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Abstract

Triple-negative (TN) breast carcinoma, characterized by estrogen receptor, progesterone receptor, and HER2 negativity, is a group of aggressive tumors that can be further classified into 2 subtypes: basal-like, defined as CK5/6 and/or epidermal growth factor receptor (EGFR) positive by immunohistochemistry; and non–basal-like. Clinical characteristics and tumor profiles were analyzed in 105 cases of TN tumors. Among these cases, 35 had distant metastasis, 34 had axillary nodal metastasis only, and 36 were nodal negative. Our results indicate basal-like TN breast tumors with nodal and distant metastases are significantly associated with a higher intratumoral expression of EGFR and CK5/6 compared to those in the nodal negative group. High level of intratumoral EGFR and CK5/6 expression may play a role in development of nodal or distant metastases in patients with basal-like TN tumors and may be predictive of metastatic disease. Furthermore, EGFR targeted therapy may be potentially useful in the treatment of basal-like TN breast cancer.

Breast cancer is one of the most common malignant tumors in women, and it comprises a remarkably heterogeneous group of diseases. Management of breast cancer depends on clinical and pathologic features of the tumor, including patient age, tumor size, histologic type and grade, lymph node stage, and lymphovascular invasion. In addition, molecular markers such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) have been proven to provide therapeutic, predictive, and prognostic value.1,2

Triple-negative (TN) breast carcinoma is a clinical subtype of breast cancer characterized by lack of expression of ER, PR, and HER2 protein. It represents approximately 10% to 17% of all breast carcinomas and is more prevalent among young African, African American, and Latino women.3,4 As compared with other subtypes of breast carcinomas, TN tumors have an onset at a younger age (<50 years) with a higher grade and higher rate of axillary node positivity.2,3,4,5-7 They typically demonstrate a unique molecular profile, aggressive behavior, and distinct patterns of metastasis.1 The metastases tend to be more aggressive and visceral with hepatic, central nervous system, and lung being common sites. TN carcinomas lack molecular targets commonly used in targeted therapy, making them very difficult to treat, and they have worse relapse-free and overall survival.3 Developing a novel treatment strategy to treat TN tumors is crucial for improving the prognosis of patients with these tumors.

TN breast carcinomas can be further divided into 2 subtypes: basal-like and non–basal-like, although not all basal-
like tumors are TN. Basal-like tumors usually express basal cytokeratins, including cytokeratin (CK)5/6, CK14, and CK17, and epidermal growth factor receptor (EGFR). Gene expression profiling is considered the “gold standard” for the identification of basal-like tumors. Previous studies have demonstrated that CK5/6- and/or EGFR-expressing breast tumors have a persistently poorer prognosis in the longer term, and screening for those basal markers is important in determining prognosis and therapeutic strategies in patients with TN breast cancer. In this study, we investigated the intratumoral expression immunophenotype and their relation to disease progression. In practice, an immuno histochemical panel including basal cytokeratins and EGFR as “reflex” testing for TN breast cancer can be used to identify basal-like TN tumors, which are typically defined as expressing CK5/6 and/or EGFR. However, little is known about the actual intratumoral expression levels of basal CKs and EGFR in the TN tumors with a basal-like immunophenotype and their relation to disease progression. In this study, we investigated the intratumoral expression levels of these basal markers and their relation to axillary nodal and distant metastases in patients with basal-like TN breast carcinoma.

Materials and Methods

Patient Identification

Patient information was obtained based on a search of case files from 2002 to 2009 at the University of Texas Southwestern Medical Center and Parkland Memorial Hospital, Dallas. We included all patients who had a TN breast carcinoma established by immunohistochemical analysis. The criteria for determining triple negativity were based on immunohistochemical 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 (≤10% of invasive tumor cells stained) for HER2. HER2 negativity was confirmed by HER2 gene detection by fluorescence in situ hybridization assay performed by the Vysis PathVysion (Abbott Molecular, Abbott Park, IL) in conjunction with an automated fluorescence in situ hybridization assay performed by MetaSystems (Altussheim, Germany).

We identified 103 patients with primary TN breast tumor, 2 of whom had bilateral primary tumors that were analyzed separately for a total of 105 cases. A basal-like breast cancer panel was performed per a reflex protocol on the TN carcinoma specimens. Basal-like TN breast tumors were identified by CK5/6 and/or EGFR positivity; positive results were based on greater than 5% positive staining of CK5/6 or EGFR. Of the 105 cases, 73 had a basal-like breast cancer profile performed, and 69 (95%) of the 73 cases were determined to be basal-like (CK5/6+ and/or EGFR+).

Immunohistochemical Analysis (Hormone Receptors, HER2, and Basal-like Biomarkers)

Slides cut from the tumor tissue were immunostained for ER, PR, and HER2. These tests were performed by immunohistochemical analysis in conjunction with automated image analysis. Known positive and negative control tissues showed appropriate staining. Monoclonal antibodies were used for ER (1D5, prediluted, Ventana Medical Systems, Tucson, AZ), PR (PgR, prediluted, Ventana Medical Systems), and HER2 (dilution 1:1,200; DakoCytomation, Carpinteria, CA). Quantitative staining information was obtained by 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 ACIS. To assess the level of tissue ER and PR expression, ACIS provided the percentage of positively stained cells for ER and PR in the selected subregions. We used the manufacturer’s guidelines for ACIS to determine tissue ER, PR, and HER2 expression.

Immunohistochemical analysis was also used for several markers that have been reported to be useful in defining the basal-like phenotype for TN breast carcinomas, including basal CKs (CK5/6, CK14, and CK17) and EGFR. In addition, biomarkers for vascular endothelial growth factor (VEGF), BRCA-1, and phosphatase and tensin homolog (PTEN) were also performed on the TN tumors. The CK5/6, CK14, CK17, PTEN, and VEGF studies were performed by immunohistochemical analysis only. EGFR and BRCA-1 were done by immunohistochemical analysis in conjunction with automated image analysis (ACIS), and all of the results were confirmed by manual review by a pathologist (Y.P.). The information on antibodies used was as follows: CK5/6 (D5/16B4, dilution 1:50; Cell Marque, Rocklin, CA), EGFR (3C6, dilution 1:2; Ventana Medical Systems), CK14 (dilution 1:100; Biocare Medical, Concord, CA), CK17 (dilution 1:2; Biocare Medical), VEGF (dilution 1:300; Zymed/Invitrogen, Carlsbad, CA), BRCA-1 (dilution 1:600; GeneTex, Irvine, CA), and PTEN (dilution 1:100; Zymed/Invitrogen). Positivity of these markers was defined as more than 5% of invasive tumor cells stained.

Statistical Analysis

The patients with basal-like TN breast carcinoma were divided into the following subgroups: axillary node negative, axillary node positive, and distant metastasis. The unpaired t test was used to compare clinicopathologic characteristics and expression levels of the basal markers in the 3 groups. All
analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

**Results**

Results of ER, PR, and HER2 confirmed TN status of the tumors in this study. The clinicopathologic characteristics of all TN tumors and the subgroup of the basal-like TN tumors are listed in **Table 1**. By looking at all of the TN tumors, we found that tumors were significantly larger in the distant metastasis group (6.24 ± 1.14 cm) compared with the node-positive (3.15 ± 0.37 cm) and node-negative (2.47 ± 0.24 cm) groups (P < .05 and P < .001, respectively). In the basal-like TN tumor subgroup, the tumor sizes in the distant metastasis and node-positive groups (5.54 ± 1.4 and 3.6 ± 0.4 cm, respectively) were significantly larger than in the node-negative group (2.4 ± 0.26 cm; P < .05 for both) **Figure 1**.

We also found in the TN tumors that the distant metastasis group had a significantly higher percentage of axillary lymph nodes involved by carcinoma (42.05% ± 7.22%) compared with the node-positive group (23.39% ± 5.45%; P < .05) when an axillary dissection was performed. The axillary dissection was done in 26 (76%) of 34 cases in the axillary node–positive group and 21 (60%) of 35 cases in the distant metastasis group. For the basal-like TN tumor subgroup, axillary dissection was done in 21 (78%) of 27 cases in the axillary node–positive group and in 11 (50%) of 22 cases in the distant metastasis group and showed similar findings, with the distant metastasis group showing a significantly higher percentage of axillary lymph nodes involved by carcinoma (43.82% ± 9.87%) compared with the node-positive group (22.2% ± 5.11%; P < .05) **Figure 2**.

We compared the frequency of the basal CKs (CK5/6, CK14, and CK17), EGFR, PTEN, VEGF, and BRCA-1 in the axillary node–negative, axillary node–positive, and distant metastasis subgroups of the basal-like TN tumors and there was no significant difference in the frequency of expression of these biomarkers between groups.
We then investigated whether the intratumoral expression level of these biomarkers differed in the axillary node–negative, axillary node–positive, and distant metastasis subgroups. Overall, CK5/6 was expressed in 43 (62%) of the 69 basal-like TN tumors. We found that among the CK5/6+ cases, the intratumoral expression of CK5/6 (percentage of positive cells) was significantly higher in the axillary node–positive group compared with the axillary node–negative group (59.06% ± 9.26% vs 20.79% ± 7.45%; P < .005) Figure 3. There was a borderline statistically significant difference in the CK5/6 expression level between the axillary node–negative group (49.30% ± 13.26% vs 20.79% ± 7.45%; P = .057; Figure 3). The mean CK5/6 expression levels in the axillary node–positive and distant metastasis groups were similar and showed no significant difference (P = .53), so we combined the 2 groups. We found that the combined nodal and distant metastasis group had a significantly higher expression level of CK5/6 compared with the axillary node–negative group (55.31% ± 7.45% vs 20.79% ± 7.45%; P < .01; Figure 3).

Overall, EGFR was expressed in 63 (91%) of the 69 basal-like TN tumors. We found that among the EGFR+ cases, the intratumoral expression of EGFR was significantly higher in the distant metastasis group compared with the axillary node–negative group (64.20% ± 7.19% vs 42.63% ± 7.00%; P < .05) Figure 4. Although the EGFR expression level in the axillary node–positive group was not significantly higher than that in the axillary node–negative group (56.92% ± 6.49% vs 42.63% ± 7.00%; P = .14; Figure 4), there appeared to be a trend of higher expression in the axillary node–positive group. As with CK5/6, the EGFR expression levels in the axillary node–positive and distant metastasis groups were not significantly different (P = .45), and we combined the 2 groups as a whole. The combined nodal and distant metastasis group had significantly higher expression of EGFR compared with the axillary node–negative group (60.27% ± 4.80% vs 42.63% ± 7.00%; P < .05; Figure 4). There was no significant difference in the intratumoral expression of CK14, CK17, PTEN, VEGF, or BRCA-1 in the 3 subgroups.

Discussion
Basal-like TN tumors are gaining an increasing amount of recent attention in part owing to recognition as a distinct entity, but most important owing to the overall poor prognosis that the diagnosis indicates. Additional markers are being actively sought after to further define tumor prognosis and therapy options. Numerous clinical trials are currently focusing on identifying possible therapeutic targets for TN tumors.5 Some reports have demonstrated that TN tumors have a good response to adjuvant anthracycline-based chemotherapy.5,13 However, it has been shown that patients with a basal-like TN phenotype had a significantly poorer response to the chemotherapy, making alternative therapy options much desired for these patients.13,17,18
Historically, CK5/6 is the most commonly used basal CK for determining basal-like carcinoma. CK5/6 is one of the most commonly positive basal CKs in basal-like breast cancers, with previous studies reporting percentages of positivity at 61% to 62%. In our study, CK5/6 expression was expressed in 62% of the basal-like TN carcinomas.

In this study, we showed that the expression of EGFR level in basal-like TN tumors was significantly higher in the nodal and distant metastases group compared with that in the node-negative group. A previous study also demonstrated that patients with TN breast carcinoma with EGFR immunoreactivity of 50% or more had significantly worse disease-free and overall survival. An association of a high EGFR intratumoral level with shorter survival was seen not only in TN breast carcinoma but also in non-TN breast carcinomas. EGFR is a 170-kDa membrane-bound tyrosine kinase. The EGFR protein product has an important role in cell proliferation, migration, and protection against apoptosis mediated by subsequent activation of intracellular pathways. The poorer prognosis of breast carcinomas expressing EGFR is likely connected to these functions. Targeted anti-EGFR antibodies (eg, cetuximab) and EGFR tyrosine kinase inhibitors (eg, gefitinib) may provide a possible treatment modality. Clinical trials using cetuximab, a chimeric monoclonal antibody against EGFR, are currently being tested in patients with metastatic TN tumors. Results have suggested that cetuximab and gefitinib have a low response rate as single agents, but in combination may provide more promising results.

Our results indicate that CK5/6 and/or EGFR positivity offered excellent detection of TN tumors with a basal-like phenotype, which is consistent with reports in the literature. We found that the CK5/6 and EGFR immunohistochemical markers were also helpful in identifying patients with a higher risk of metastatic disease. Overall, basal-like TN tumors have a worse prognosis. Developing a novel treatment strategy to treat basal-like TN tumors is crucial for improving the prognosis of patients with these tumors.

In our study, approximately one third (22/69) of basal-like TN carcinomas developed distant metastasis. The lung was the most common site of distant metastatic disease (Table 1) for basal-like and non–basal-like TN tumors, which is consistent with that reported in the literature, although number of cases is limited. Earlier identification of these patients to give aggressive therapy to them is critical. It can be challenging for pathologists to diagnose metastatic basal-like TN carcinomas presented as an unknown primary tumor because the tumors lack expression of ER, PR, and HER2 protein. In addition, other currently used markers to identify breast primary tumors such as mammoglobin and gross cystic disease fluid protein have a low sensitivity. Our study showed that CK5/6 was expressed in 62% of the basal-like TN tumors and EGFR in 91%. These findings suggest that immunohistochemical studies for CK5/6 and EGFR along with a morphologic comparison of the primary breast tumor may be helpful to detect metastatic basal-like TN breast tumors, and patients with metastatic disease may benefit from treatment with anti-EGFR drugs. However, it must be noted that negative CK5/6 or EGFR staining does not rule out basal-like TN tumors.

We also found that larger tumors and a higher percentage of axillary lymph nodes involved by carcinoma were strongly associated with distant metastasis in TN and basal-like TN breast cancers. Previous studies have shown that TN tumors are associated with increased tumor size and positive axillary lymph nodes. Also, nodal status and tumor size have been inversely associated with disease-free and overall survival in TN tumors. Large tumors and a high percentage of positive lymph nodes may be predictive of metastatic disease, and these tumors may warrant a more aggressive treatment plan.

To determine if the increased expression of EGFR and CK5/6 was independent of the increased tumor size in terms of their prognostic values, a multivariate logistic regression analysis was performed (SAS version 9.2, SAS Institute, Cary, NC). Before the multivariate analysis, a univariate analysis with factors including expression levels of EGFR, CK5/6, CK14, CK17, VEGF, PTEN, and BRCA-1; tumor size; and age was performed for selecting potential independent risk factors for metastatic disease in basal-like TN tumors. The levels of EGFR and CK5/6 expression, tumor size, and age were selected among all the factors listed. Subsequently, the multivariate analysis was performed and revealed that increased tumor size correlated strongly with nodal and distant metastases (P < .05). However, association of the metastases with levels of EGFR and CK5/6 expression and age did not reach statistical significance. The findings of increased EGFR and CK5/6 intratumoral expression in nodal and distant metastases cases were not independent of the tumor size. However, because the number of cases in our study was limited, a larger case series to study EGFR and CK5/6 is needed to better delineate their roles as potential prognostic markers.

Overall, we believe that these markers are still of prognostic and predictive value. Numerous previous studies have shown that increased tumor size was significantly associated with poor prognosis in patients with all types of breast cancers. Therefore, tumor size as an independent risk factor is not specific for basal-like TN tumors. Basal-like TN tumors, before being resected, are sometimes treated with neoadjuvant therapy that may alter tumor size in resection specimens. More important, EGFR may serve as a potential therapeutic target for treatment of basal-like TN breast cancers. However, we are not recommending routinely testing for EGFR and CK5/6 in breast cancer specimens at this point because further work is needed to better understand the roles of EGFR and CK5/6 in tumor metastasis, such as a larger case series, and...
validation of these markers in a prospective trial before they can be applied clinically.

In summary, our results indicate that basal-like TN breast carcinomas with nodal and distant metastases are significantly associated with higher intratumoral expression of EGFR and CK5/6 compared with those in the node-negative group. High intratumoral EGFR and CK5/6 expression levels may have a role in the development of nodal or distant metastases in patients with basal-like TN tumors and may be predictive of metastatic disease. Furthermore, EGFR-targeted therapy may be potentially useful in the treatment of basal-like TN breast cancer. In addition, larger tumors and a higher percentage of axillary lymph nodes are more likely to be associated with distant metastases in TN and basal-like TN breast cancers.

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