Cyclooxygenase-2 expression in non-metastatic triple-negative breast cancer patients

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Abstract. Triple-negative breast cancer (TNBC) is characterised by lack of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER)2/neu gene amplification. TNBC patients typically present at a younger age, with a larger average tumor size, higher grade and higher rates of lymph node positivity compared to patients with ER/PR-positive tumors. Cyclooxygenase (COX)-2 regulates the production of prostaglandins and is overexpressed in a variety of solid tumors. In breast cancer, the overexpression of COX-2 is associated with indicators of poor prognosis, such as lymph node metastasis, poor differentiation and large tumor size. Since both TNBC status and COX-2 overexpression are known poor prognostic markers in primary breast cancer, we hypothesized that the COX-2 protein is overexpressed in the primary tumors of TNBC patients. The purpose of this study was to determine whether there exists an association between TNBC status and COX-2 protein overexpression in primary breast cancer. We prospectively evaluated COX-2 expression levels in primary tumor samples obtained from 125 patients with stage I-III breast cancer treated between February, 2005 and October, 2007. Information on clinicopathological factors was obtained from a prospective database. Baseline tumor characteristics and patient demographics were compared between TNBC and non-TNBC patients using the Chi-square and Fisher's exact tests. In total, 60.8% of the patients were classified as having ER-positive tumors, 51.2% were PR-positive, 14.4% had HER-2/neu amplification and 28.0% were classified as TNBC. COX-2 overexpression was found in 33.0% of the patients. TNBC was associated with COX-2 overexpression (P=0.009), PR expression (P=0.048) and high tumor grade (P=0.001). After adjusting for age, menopausal status, body mass index (BMI), lymph node status and neoadjuvant chemotherapy (NACT), TNBC was an independent predictor of COX-2 overexpression (P=0.01). In conclusion, the association between TNBC and COX-2 overexpression in operable breast cancer supports further investigation into COX-2-targeted therapy for patients with TNBC.

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Introduction

Breast cancer is a heterogeneous disease that is defined and classified using clinical and pathological characteristics, including patient age, tumor size, axillary node involvement, histological grade, estrogen receptor (ER) and progesterone receptor (PR) status and human epidermal growth factor receptor (HER)2/neu (also referred to as ERBB2) amplification (1). Advances in molecular biology techniques have expanded the classical description of breast cancer tumors into distinct subtypes. Three of these subtypes represent ER-negative tumors [triple-negative breast cancer (TNBC), basal-like (BL) and HER2/neu-positive] and two are characterized by ER-positivity (luminal A and B tumors). Tumors classified as luminal A express ER, with or without PR, and lack human HER2 expression; luminal B tumors express ER and HER2, with or without PR expression. BL and triple-negative tumors are highly concordant; both are characterized by a lack of expression of ER, PR and HER2 and are indistinguishable by standard immunohistochemical staining of formalin-fixed and paraffin-embedded samples; therefore, both are classified as TNBC (2). TNBC cases represent 10-30% of all breast cancers. TNBC patients typically present at a younger age (<50 years), with larger average tumor size, higher grade and higher rates of axillary lymph node positivity compared to ER-positive patients (3-6). TNBC is more prevalent among premenopausal African-American patients compared to postmenopausal African-American and non-African-American patients (7,8).

Due to the lack of estrogen and HER2 expression and the aggressive nature of TNBC, effective management of TNBC patients remains a challenge in the clinical practice. Triple-negative patients are typically treated sequentially with a combination of chemotherapy, surgery and radiation. Triple-negative tumors respond favorably to neoadjuvant chemotherapy (NACT) (9-12) and TNBC patients achieve high pathological complete response (pCR) rates following systemic therapy compared to non-TNBC patients (10). Although there is no reported difference in the rates of local relapse in TNBC vs. non-TNBC patients (13,14), the mean time to recur-

rence is shorter for TNBCs. TNBC patients typically recur less than 3 years post-diagnosis (10,13) with propensity for distant recurrence to the spinal cord, meninges, brain, liver and lungs (11). The recurrence rate peaks at ~3 years and diminishes steadily over the next 5 years, accompanied by a very low risk thereafter. Although pCR predicts excellent survival regardless of receptor status, TNBC patients with residual disease following NACT exhibit significantly shorter disease-free and overall survival compared to patients with non-TNBC tumors and residual disease following NACT (10,13). These data highlight the urgent need for improved prognostic tools and novel targeted therapies for TNBC patients.

Cyclooxygenase-2 (COX-2) is an inducible, proinflammatory enzyme that catalyzes key steps in the conversion of arachidonic acid to prostaglandins and thromboxanes. COX-2 expression is induced in tumor cells and is regulated by transcriptional and translational processes that are mediated by cytokines, growth factors and oncogenes (15). Studies using *in vitro* breast cancer cell lines and *in vivo* mouse models have demonstrated that COX-2 overexpression plays key roles in tumorigenesis by stimulating epithelial cell proliferation, inhibiting apoptosis, stimulating angiogenesis, increasing multidrug resistance and enhancing cell motility and invasion (15-23). The evidence supporting the role of COX-2 in breast cancer progression has also been demonstrated in clinical studies; patients with COX-2-expressing primary tumors exhibited shortened disease-free and overall survival (24-30).

The majority of the studies employed immunohistochemistry and monoclonal antibodies to assess COX-2 protein expression in primary breast carcinomas and COX-2 positivity was identified in 33-58% of the cases (24-29,31-34). COX-2 expression is frequently associated with high histological grade and large tumor size (24-28,30,32,33) and, to a lesser degree, negative ER status (24,26,27,30,31,33). The association between COX-2 expression and HER2/neu expression is not conclusive; in two large studies, COX-2 was significantly associated with increased HER2/neu expression (27,33), while no significant association was observed in a number of other studies (24,25,28,30-32). These disparate results emphasize the need for standardized, reproducible assays for COX-2 assessment, which may enable meaningful associations and interpretations.

Triple-negative status and COX-2 expression have both been significantly associated with an unfavorable outcome for non-metastatic breast cancer patients (30,35). The purpose of our study was to determine whether COX-2 protein expression in primary breast cancer is associated with TNBC. Since COX-2 is overexpressed in breast cancer, it may serve as a possible target for chemoprevention in TNBC, for which, apart from systemic chemotherapy, targeted therapy is currently non-existent.

Materials and methods

Patients. We reviewed the data collected from 125 female patients with stage I-III breast cancer, who were treated at The University of Texas MD Anderson Cancer Center between February, 2005 and October, 2007. The data are derived from an Institutional Review Board (IRB)-approved protocol (DR070276; The University of Texas MD Anderson Cancer Center). The patients were enrolled as a part of two University of Texas MD Anderson Cancer Center IRB-approved research protocols (nos. LAB04-0657 and 04-0698; Principal Investigator, A. Lucci). The patients enrolled in these IRB-approved protocols provided written informed consent for the collection of tissue, blood and bone marrow at the time of their primary surgery for breast cancer. Enrollment was strictly voluntary and the patients did not receive a stipend for participating in this study. The investigators were blinded to individual patient results through the use of a random number system as the unique patient identifier. Patients with bilateral breast cancer or any other malignancy within 5 years of the diagnosis of the current cancer were considered ineligible and were excluded from these studies.

Information on prognostic markers (e.g., ER, PR, HER2 and Ki-67 proliferation index) and other clinical variables, such as age at diagnosis, race, lymph node metastasis, menopausal status, lymphovascular invasion and nuclear grade was obtained from clinical records.

Staging and classification. The primary TNM stage [primary tumor (T), regional nodes (N) and distant metastases (M)] and tumor grade were designated according to the criteria set by the American Joint Commission on Cancer (36) and Black's nuclear grading system (37), respectively. Clinical stage was defined as the TNM stage determined at the time of the first diagnostic procedure confirming the invasive component of the tumor. Axillary lymph node status was determined using ultrasound and fine-needle aspiration. Pathological stage was determined following primary tumor and lymph node removal. Clinical stage was used for analysis for patients who received NACT. The response to NACT was termed as pCR only when there was no evidence of residual invasive disease in the excised tumor and lymph nodes following completion of chemotherapy (10,38).

Immunostaining procedures. The tumor sections were immunostained for ER and PR using previously published procedures (32). The immunostaining results for HER2 were scored as 1+ when <10% of the tumor cells exhibited complete membranous staining; as 2+ when weak-to-moderate membranous staining was present in >10% of the tumor cells; and as 3+ when strong complete membranous staining was present in >30% of the tumor cells. All the 2+ and 3+ cases were evaluated by fluorescence *in situ* hybridization for HER2 gene amplification using the PathVysion HER2 DNA probe kit (Abbott Laboratories, Abbott Park, IL, USA). A HER2/chromosome 17 centromere ratio of >2.2 was considered as positive for HER2 gene amplification. TNBC was defined by absence of ER and PR expression and HER2 gene amplification in the primary tumor. The tumors were immunostained as previously described and deemed COX-2 positive when ≥5% of the tumor cells immunostained for COX-2 (32). The tumors were considered Ki-67 positive when ≥35% of the tumor cells exhibited Ki-67 staining.

Statistical analysis. The primary tumor characteristics and patient demographics (including pCR) were tabulated and compared between TNBC and non-TNBC patients using the Chi-square and Fisher's exact tests. Odds ratios (ORs) were calculated to assess the association between COX-2 expression and TNBC status. The ORs were adjusted for important

clinical and pathological prognostic markers [e.g., age, body mass index (BMI), menopausal status, lymph node status and lymphovascular invasion]. The adjusted ORs for COX-2 expression and their 95% confidence intervals (CIs) were reported using logistic regression. The statistical analyses were performed by statisticians using STATA 13 software (StataCorp, College Station, TX, USA) and P<0.05 was considered to indicate a statistically significant difference.

Results

Clinicopathological characteristics. The clinical characteristics of the 125 patients and the pathological characteristics of their tumors are presented in Table I. The mean age was 53.4 years (range, 25-92 years) and the mean BMI was 28.6 kg/m^2. Of the 125 patients, 90 (72.0%) were Caucasian, 13/125 (11.0%) were African-American and 19/125 (15.0%) were Hispanic. A total of 84 (68.0%) of the 125 patients were postmenopausal.

In total, 39/125 patients (31.2%) had T1, 53/125 (42.4%) had T2, 10/125 (8.0%) had T3 and 23/125 (18.4%) had T4 tumors; 68/125 patients (54.4%) had axillary lymph node metastasis, 60/125 (48.8%) had high-grade (grade 3) tumors and lymphovascular invasion was present in 42/125 (34.7%) of the patients. ER, PR and HER2/neu gene amplification were present in 60.8% (76/125), 51.2% (64/125) and 14.4% (18/125) of the patients, respectively. Based on the information of the three tumor markers, 28.0% (35/125) patients were classified as TNBC. Ki-67 immunostaining is an index of high cellular proliferation, but it is not routinely performed for all patients. Ki-67 data were available for analysis in 51/125 of our sample cohort and 52.9% (27/51) exhibited a high proliferation index (≥35% of tumor cells were Ki-67-positive). A total of 42 patients (34.7%) received NACT; 8/125 (6.4%) achieved pathological partial response or pCR. COX-2 expression assessment was available for 106 patients in this study. Using a 5% threshold for COX-2 expression, 35/106 (33.0%) patients had primary tumors that were COX-2-positive.

Association of COX-2 expression with clinicopathological characteristics. The unadjusted ORs between COX-2 expression and primary tumor characteristics are shown in Table IIA. COX-2-expressing tumors (≥5% of the cells expressing COX-2) were three times more likely to be TNBC (OR=3.34, 95% CI: 1.40-8.22; P=0.009) and four times more likely to be high-grade (OR=4.09, 95% CI: 1.58-10.82; P=0.001) compared to COX-2-negative tumors (COX-2 expression in <5% of the cells). No significant associations were observed between COX-2 expression and ER positivity (P=0.10), HER2/neu gene amplification (P=0.18), or Ki-67 index (P=0.09). However, PR positivity was associated with COX-2 expression (OR=0.43, 95% CI: 0.19-0.99; P=0.048). After adjusting for age, BMI, menopausal status, lymph node status and lymphovascular invasion, the multivariate analysis demonstrated that TNBC patients were more likely to exhibit COX-2 expression (OR=3.48, 95% CI: 1.28-9.44; P=0.01) (Table IIB).

Association of TNBC with clinicopathological characteristics. In our study, TNBC was associated with high tumor grade (OR=8.89, 95% CI: 3.12-28.55; P<0.001) (Table IIIA). We identified a negative association between TNBC and lymphovascular invasion (OR=0.43, 95% CI: 0.06-0.73; P=0.006). We found no significant association between TNBC and positive axillary lymph node status (OR=1.16, 95% CI: 0.49-2.78; P=0.70), or pCR (OR=2.77, 95% CI: 0.48-15.72; P=0.15) (Table IIIA). The multivariate analysis demonstrated that TNBC was significantly associated with high tumor grade (OR=6.30, 95% CI: 2.15-18.41; P<0.001) (Table IIIB).

Table I. Clinicopathological characteristics of the 125 breast cancer patients.

Characteristics	Number of patients, no. (%) (n=125)
Mean age, years	53.4
Mean BMI, kg/m^2	28.6
Race	
Caucasian	90 (72.0)
African-American	13 (11.0)
Hispanic	19 (15.0)
Other	3 (2.0)
Postmenopausal	84 (68.0)
Tumor size	
T1[a]	39 (31.2)
T2[b]	53 (42.4)
T3[c]	10 (8.0)
T4[d]	23 (18.4)
Axillary LN metastasis	68 (54.4)
High-grade tumor[e]	60 (48.8)
LVI	42 (34.7)
ER	76 (60.8)
PR	64 (51.2)
HER-2/neu amplification	18 (14.4)
TNBC[f]	35 (28.0)
NACT	42 (34.7)
COX-2 expression (n=106)[g]	35 (33.0)
Ki-67 (n=51)[h]	27 (52.9)

[a]≤2 cm. [b]>2 and ≤5 cm. [c]>5 cm. [d]Any tumor size, but extending to the overlying skin or chest wall. [e]Grade 3. [f]Absence of ER and PR positivity and HER2 gene amplification. [g]Presence of COX-2 expression in ≥5% of cells as determined by immunohistochemistry. [h]≥35% of tumor cells exhibiting Ki-67 staining. BMI, body mass index; LN, lymph nodes; LVI, lymphovascular invasion; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer; NACT, neoadjuvant chemotherapy; COX-2, cyclooxygenase-2.

Discussion

Previous studies demonstrated that COX-2 expression (24,25,27-29,35,39,40) and TNBC (7,8,10,13,14,41-44) are independent predictors of poor prognosis in stage I-III breast cancer. Based on this information, we hypothesized

Table II. Association of COX-2 expression with clinicopathological characteristics.

A, Unadjusted measures of association of COX-2 expression with ER, PR, HER2 gene amplification, TNBC, high tumor grade and Ki-67.

Characteristics	OR	95% CI	P-value
ER	0.49	0.22-1.15	0.10
PR	0.43	0.19-0.99	0.048[a]
HER2	0.23	0.03-1.93	0.18[b]
TNBC	3.34	1.40-8.22	0.009[a]
High grade	4.09	1.58-10.82	0.001[a]
Ki-67	2.78	0.71-11.29	0.09

B, Adjusted ORs from a multivariate logistic regression analysis with COX-2 expression as the dependent variable.

Characteristics	OR	95% CI	P-value
Age	0.99	0.94-1.04	0.66
BMI	0.95	0.88-1.02	0.16
Postmenopausal	2.03	0.56-7.35	0.28
LN status[c]	2.22	0.89-5.53	0.09
LVI	1.25	0.43-3.60	0.68
TNBC	3.48	1.28-9.44	0.01[a]

[a]Denotes statistical significance (P<0.05). [b]Fisher's exact test. [c]Presence of axillary lymph node metastasis. COX-2, cyclooxygenase-2; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer; OR, odds ratio; CI, confidence interval; BMI, body mass index; LN, lymph node; LVI, lymphovascular invasion.

Table III. Association of TNBC with clinicopathological characteristics.

A, Unadjusted measures of association of TNBC with high tumor grade, LVI, LN status and pCR.

Characteristics	OR	95% CI	P-value
High grade	8.89	3.12-28.55	<0.001[a]
LVI	0.43	0.06-0.73	0.006[a]
LN status	1.16	0.49-2.78	0.70
pCR	2.77	0.48-15.72	0.15

B, Adjusted ORs from a multivariate logistic regression analysis with TNBC as the dependent variable.

Characteristics	OR	95% CI	P-value
High grade	6.30	2.15-18.41	<0.001[a]
LVI	0.28	0.08-0.92	0.036[a]
LN status[b]	1.53	0.49-4.76	0.46
pCR	2.38	0.24-24.14	0.46

[a]Denotes statistical significance (P<0.05). [b]Presence of axillary lymph node metastasis. TNBC, triple-negative breast cancer; LVI, lymphovascular invasion; LN, lymph node; pCR, pathological complete response; OR, odds ratio; CI, confidence interval.

that increased COX-2 expression may be associated with TNBC status. A total of 33.0% of our sample cohort exhibited COX-2 expression (using the 5% threshold), which is similar to the results of previous studies (24-29,31-34). In congruence with previously published reports, we observed a significant association between COX-2 expression and high tumor grade (26-28,30,32,33) and also identified a significant association between COX-2 expression and TNBC. The association between TNBC status and high tumor grade (OR=8.89, 95% CI: 3.12-28.55; P<0.001) was in agreement with results of larger studies (12,13,15,16,18,22). We did not identify a significant association between TNBC and positive axillary lymph node status, as reported in several (8,11,44), but not all (8,10,13) studies.

Preliminary reports indicated that COX-2 expression predicts outcome in early-stage (stage I/II) and node-negative patients (25,28). Although we did not determine the prognostic significance of COX-2 expression in the present study, it is intriguing to consider the potential COX-2-mediated mechanisms involved in disease progression. In addition to the well-documented *in vitro* and *in vivo* COX-2-mediated tumorigenic/angiogenic effects, COX-2 expression has also been shown to correlate with increased resistance to radiation therapy (45,46) and poorer response to chemotherapeutic agents (47). In addition, a 2009 report published by our group demonstrated that COX-2 expression in the primary tumor predicted the presence of bone marrow micrometastasis; the bone marrow tumor cells also exhibited COX-2 overexpression (32), suggesting that, in addition to tumor cell dissemination to distant sites by lymphatic spread, COX-2-expressing tumor cells may disseminate through the hematogenous route.

It is difficult to draw statistical conclusions regarding the association of COX-2 positivity with different races or ethnicities and adjust for potential confounders. Our patient sample size was limited to patients with non-metastatic breast cancer who were consented and treated at a single tertiary care hospital. Our findings should now be evaluated in a multicenter setting with larger patient samples. The cross-sectional nature of this study may lead to some bias due to the unequal distribution of confounders. This study was a secondary analysis of data; therefore, our results must be interpreted with caution, since the primary study was designed to assess the association between COX-2 expression in the primary tumor and detection of microscopic disease in the bone marrow and peripheral blood at the time of primary surgery. It is possible that NACT resulted in widespread changes within the tumor bed that may have interfered with the detection of COX-2 expression in the tissue acquired at the time of surgery. This scenario may be avoided by measuring COX-2 expression in tissues obtained prior to chemotherapy initiation.

In conclusion, we report that COX-2, an extensively investigated marker of poor prognosis in patients with non-metastatic breast cancer, is associated with TNBC and high tumor grade.

Due to the aggressive nature of TNBC, assessing the COX-2 expression in TNBC patients may provide valuable prognostic information and assist in identifying those TNBC patients at higher risk for recurrence. Since TNBC patients have only limited treatment options, clinical trials investigating COX-2 suppression using COX-2 inhibitors are required.

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