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ORIGINAL ARTICLE

The importance of COX-2 expression as prognostic factor in early breast cancer

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Summary

Purpose: *A number of studies have been carried out, showing that the risk for breast carcinoma is decreased in those using non-steroidal anti-inflammatory drugs (NSAIDs). Increased cyclooxygenase-2 (COX-2) level is considered as a factor indicating poor prognosis and responsible for angiogenesis, increased cellular proliferation, apoptotic defect and aromatase enzyme induction. For this reason the level of COX-2 might have a prognostic and predictive value in breast cancer as well. This question has become the basis of the present study.*

Methods: *Eighty-eight female patients with early stage breast cancer being under adjuvant anthracycline based chemotherapy were prospectively recruited. The patient age, body weight, menopausal status, tumor size and grade as well as axillary lymph node involvement were recorded. Routine pathological examination was performed, and COX-2, CerbB2 (HER2), estrogen (ER) and progesterone receptors (PR) levels in breast cancer tissue were determined immunohistochemically.*

Results: *: Multivariate analysis confirmed the independent predictive value of both menopausal status and ER expression for overall survival (OS) (p=0.009, HR=1.92, and p=0.014, HR=0.20, respectively). A negative correlation was observed between COX-2 levels and the levels of ER and PR (p=0.006, R= -0.303, and p=0.004, R=-0.312, respectively) whereas no significant correlation was observed concerning CerbB2. No statistically significant correlation was determined between COX-2 levels and the disease-free (DFS) and OS rates.*

Conclusion: *Further studies investigating the role of COX-2 levels in breast cancer progression are needed.*

Key words: *breast cancer, COX-2, cyclooxygenase system, prognostic factors*

Introduction

Breast cancer is the most frequently encountered malignancy among women and ranks second in mortality. Exposure to high levels of estrogens for a long time is considered to be the most important risk factor for breast cancer development [1-5]. In epidemiological studies, a relationship between COX-2 levels and colorectal carcinoma was first been demonstrated. In recent years, the association between colorectal carcinoma and COX-2 has been clearly defined and many studies have been published showing that the use of NSAIDS (e.g. celecoxib), COX-2 inhibitors, prevents the development of colorectal cancer [6-8]. Advanced stage, metastasis, and decrease in survival have been shown to be associated with the increase in COX-2, and COX-2 has been defined as a factor indicating poor prognosis. Determination of high levels of COX-2 in inflammed and tumor tissues, as well as increase in estrogen synthesis due to increasing production of COX-2, its apoptotic blocking effect, and demonstration of its activity in enhancing angiogenesis and metastatic potential in cell cultures, raised the question whether the level of COX-2 might have a prognostic and predictive value in breast cancer as well [9-12]. This question has become the basis of the present study.

Methods

Patients with early stage breast cancer, admitted at the Medical Oncology Outpatient Clinic of Akdeniz University Medical School and taking adjuvant anthracycline-based chemotherapy, were prospectively evaluated.

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The age of the patients, the state of axillary lymph nodes, the grade and size of the tumor and the levels of CerbB2, ER, PR and COX-2 were recorded. Staging was done in accordance with the staging system recommended by American Joint Committee on Cancer (AJCC). Patients without menstrual bleeding for at least 1 year were considered postmenopausal, whereas the others were considered premenopausal. The state and the levels of ER, PR, CerbB2 and COX-2 receptors were assessed by use of immunohistochemical methods.

Immunohistochemical staining technique

Tissue samples from each patient case, which have been fixed with formalin, embedded in paraffin, cut to a thickness of 5μ and stained with hematoxylin-eosin (H-E), were re-examined.

COX-2 (4H12, 1:200 dilution) was immunohistochemically determined in tissue samples by means of Streptavidin-Biotin Complex method. Four-micron sections obtained from the tissues that would be immunohistochemically stained, were put onto chrome alum gelatinized sides coded as "Dako chemMate TM 500 capillary Gap microscope slides (75μ)". They were melted in an incubator at 60˚ C overnight and deparaffinized in xylol twice for 5 min each time. Afterwards, they were exposed to alcohol series with descending degrees, and hydrated in distilled water for one min. Then, the "Antigen Retrieval" procedure was applied to the sections to regain the antigen. This process was performed by boiling the sections in citrate solution (0.01 M, pH: 6.0) in a microwave oven at 90˚ C for 20 min in a way that the fluid level would cover the slides and sections won't become dry. Then, they were left for cooling at room temperature. The sections were incubated in 3% H_2O_2 solution for 5 min to block the endogenous peroxidase activity. Subsequently, the slides were washed with buffered phosphate solution and kept for 5 min. In order to prevent ground staining, the sections were kept in "blocking" solution (Dako Protein Block, Kod: X0909) for 10 min and then were dried. The tissue sections, which have been covered with primary antibody, were incubated for 1 h. Afterwards, they were incubated for 15 min in Linking Reagent (Dako) that acts as a binder between primary antiboby and enzyme carrying antibody. Sections were then incubated for 15 min in horseradish peroxidase conjugated with labeling reagent (Dako, Kod K0675) streptavidin, kept in buffered phosphate solution for 5 min and incubated in chromogenic substrate diamino benzidin/(DAB) (Dako, Kod:3466) for 5 min. Slides were counterstained with H-E (Dako), covered with Iamella and examined under light microscope. Brown color was assumed as positive.

Immunohistochemical examination

During the evaluation of COX-2 expression, the percentage and the staining intensity of the cells that showed positive immune reaction were assessed. Only the cytoplasmic staining was considered positive. All the tumor areas were assessed by low magnification (x40) and the percentage by of the positively stained cells was calculated. The intensity of staining was scored as follows: 0 (negative), + (weak), ++ (moderate), and +++ (strong). For statistical evaluation, the cases were divided into two groups, showing underexpression or overexpression. Underexpression was considered if the staining intensity was 0, +, ++, or +++ and the stained cell ratio was < 10%; or, the staining intensity was 0 or + and the stained cell ratio was < 50%. Overexpression was defined as ++ or +++ staining intensity and > 10% stained cell ratio; or, +, ++, +++ staining intensity and ≥ 50% positive cells.

Statistics

While examining the immunohistochemical parameters, total COX-2 was calculated to evaluate the COX-2 expression; to this purpose, the intensity of COX-2 was multiplied by its percentage. After the descriptive analyses had been completed, the effects of clinical and pathological variables on DFS and OS were analyzed by Cox regression analysis. Forward logistic regression criterion was used for factor selection. A p value <0.05 was considered significant for all analyses. Although the immunohistochemical variables were put in survival analysis, they were divided around the median and created binary categorical variables in case of abnormal distribution. Logarithmic transformation of interval variables was used to provide normal distribution when necessary. The relation between the immunohistochemical variables were examined by Spearman's correlation test. SPSS, version 13.00 package database was used for statistical analyses.

Results

The sociodemographic characteristics of the patEighty-eight patients with early stage breast cancer and taking anthracycline-based adjuvant chemotherapy were prospectively included in the present study. The median follow-up period was 74.2 months (range 1.9-93.7). The mean patient age was 45 years (range 29-70). The patient demographic and clinicopathological data are summarized in Table 1.

Of the patients, 50 (60%) were premenopausal and 33 (40%) postmenopausal. The median body weight was 67 kg (range 47-118), the median tumor diameter was 2 cm (range 1-6), and the median number of the positive axillary lymph nodes resected was 2 (range 0-15).

ER were positive in 62 (70%) patients and negative in 26 (30%); PR were positive in 55 (62.5%) patients and negative in 33 (37.5%), whereas CerbB2 was overexpressed in 23 (26.2%) patients and COX-2 was overexpressed in 41 (49%) patients.

No statistically significant correlation was

Table 1. Demographic data

Characteristics	*N*	*%*	*Minimum*	*Maximum*	*Median*	*Mean*
Age (years)			29	70	45	46.73
Premenopausal	50	60				
Postmenopausal	33	40				
Tumor size (cm)			1	6	2	2.44
Axillary density			0	0.81	0.1	0.14
Log axillary density			-1.59	-0.09	-0.79	-0.82
Body weight	83	94§	47	118	67	69.19
ER +	62	70				0.2
ER -	26	30				
ER total			0	0.95	0.3	0.35
ER total log			-1.78	-0.02	-0.36	-0.51
ER total X	88	100§				
PR +	55	62.5				
PR -	33	37.5				
PR total			0	0.9	0.16	0.25
CerbB 2 total			0	0.9	0.15	0.32
Grade	83	94	1	3		2.24
COX-2 +	41	49				
COX-2-	42	51				
COX-2 total expression		6	0.82	4.324		0.24

Axillary density: positivity of axilla/total number of axillary lymph nodes, expressed as ratio (continuous variable).
Log axillary density: logarithmic transformation of axillary density (positivity of axilla/total number of axillary lymph nodes).
ER total: ER staining density (the intensity of ER expression (0,1,2,3) x% of ER expression (0-100)/300).
ER total log: logarithmic ER staining density.
ER total X: median ER staining density ≤ 0.33 and >0.33, expressed as binominal variable.
PR total: PR staining density (the intensity of PR expression (0,1,2,3) x% of PR expression (0-100)/300).
CerbB2 total: CerbB2 receptor staining density (the intensity of CerbB2 expression (0,1,2,3) x% of CerbB2 expression (0-100)/300).
Cox-2 total expression: COX-2 receptor staining density (the intensity of COX-2 expression (1,1,2,3) x% of COX-2 expression (0-100)/300).
Missing values were not included in the analysis.
§Cases without missing data. ER: estrogen receptor, PR: progesterone receptor

determined between COX-2 level and age, tumor size, tumor grade, axillary lymph node involvement, body weight, ER, PR and CerbB2 in DFS analysis (Table 2),whereas there was positive correlation only with menopausal status (p= 0.013).

Whilst no statistically significant correlation was determined between COX-2 level and age, tumor size, tumor grade, axillary lymph node involvement, PR and CerbB2 in OS analysis (Table 3), there was positive correlation with menopausal status (p=0.02) and ER status (p=0.028).

When correlation analysis was carried out to evaluate the relation between immunohistochemical variables and clinical parameters, negative correlation was determined between COX-2 and ER and PR, whereas no significant correlation was determined between the other parameters (Table 4).

Discussion

In the present study, although no relation was determined between COX-2 and lymph node status, tumor grade, tumor size, DFS and OS, the correlation between COX-2 and ER and menopausal status was significant, whereas the correlation with ER and PR was negative.

In this study, breast cancer tissues from 88 patients were examined, and COX-2 expression was determined in 41 (49%) of them. The results obtained in the present study are compatible with the results of other relevant studies in the literature. COX-2 posivity is lower in breast cancer as compared with colon cancer (80-90%) [4,6,13-15].

In the study published in 2002 by Ristimaki et al. [14], 1576 patients with breast cancer were evaluated and 37.4% of the patients showed COX-2 positivity. Increased COX-2 expression was associated with large tumor size, high histological grade, negative hormone receptor status, high proliferation rate (identified by Ki-67), high p53 expression, and presence of HER-2 oncogene amplification (p < 0.0001 for all comparisons), along with axillary node metastases and ductal type

Table 2. Indicators for disease-free survival

Parameters	*p-value*	*HR*
Age	0.15	1.03
Menopause	0.01	3.02
Tumor size (cm)	0.74	1.15
Log axillary density	0.09	3.14
Body weight	0.92	1.00
ER	0.45	0.72
ER total X	0.11	0.47
PR	0.51	0.76
PR total X	0.22	0.57
Cerb 2 total X	0.64	1.21
Cerb 2 overexpressed	0.69	0.82
Grade	0.91	0.95
COX-2 total X	0.13	1.89
COX-2 percentage	0.20	1.00
COX-2 ratio	0.95	0.98
COX-2 total	0.20	2.35

Log axillary density: logarithmic transformation of axillary density (positivity of axilla/total number of axillary lymph nodes).
ER total X: median ER staining density ≤ 0.33 and >0.33, expressed as binominal variable.
PR total X: median PR staining density ≤ 0.17 and > 0.17, expressed as binominal variable.
CerbB2 overexpressed: CerbB2 overexpression status (yes vs no).
COX-2 percentage: % cells staining for Cox-2 receptor.
COX-2 total: COX-2 receptor staining density (the intensity of COX-2 expression (0,1,2,3) x% of COX-2 expression (0-100)/300).

Table 3. Indicators for overall survival analysis

Parameters	*p-value*	*HR*
Age	0.08	1.05
Menopause	0.02	3.51
Tumor size (cm)	0.55	0.71
Log axillary density	0.14	3.39
Body weight	0.69	0.99
ER	0.50	0.71
ER total	0.05	0.15
ER total X	0.02	0.24
PR	0.95	1.02
PR total	0.18	0.21
PR total X	0.63	0.77
CerbB2 total X	0.77	1.16
Grade	0.45	0.66
COX-2 percentage	0.60	1.00
COX-2 ratio	0.91	1.03
COX-2 total	0.33	2.19

Log axillary density: logarithmic transformation of axillary density (positivity of axilla/total number of axillary lymph nodes).
ER total X: median ER staining density ≤ 0.33 and > 0.33, expressed as binominal variable.
PR total X: median PR staining density ≤ 0.17 and > 0.17, expressed as binominal variable.
CerbB2 total X: CerbB2 total (CerbB2 receptor staining density expression) median ≤ 0.15 and >0.15, expressed as binominal variable.

histology (p=0.0001 and p=0.0017, respectively). COX-2 expression is more common in breast cancer with poor prognostic characteristics and is associated with unfavorable outcome.

In another study conducted by Singh-Ranger et al. [15], 39 patients with either IDC or ductal carcinoma *in situ* (DCIS) were included; COX-2 expression was determined in 36.7% of the cases with ICD and in 54.4% of the cases with DCIS. In this study, no correlation between COX-2 expression and age, tumor size, tumor grade, hormone receptor expression, lymphovascular invasion or lymph node metastasis could be demonstrated either in ICD or DCIS.

Mottolese et al. [16] reported on 186 patients with stage I and II breast cancer taking adjuvant anthracycline-based therapy; COX-2 positivity was assessed in 84.9% of them. The relationship between COX-2 level and hormone receptor status, p53, Ki 67, HER-2 overexpression, Fas and Fas ligand, as well as 5-year DFS and OR were investigated, and COX-2, p53, Fas and FasL were significant predictors of 5-year DFS (p=0.05, p=0.006, p<0.0001 and p<0.0001, respectively) and of OS (p=0.027, p=0.02, p<0.0001, respectively). In addition, COX-2 was significantly related to p53 nuclear accumulation and Ki67 proliferation index. In the present study, the effect of COX-2 on DFS and OS could not be assessed.

In a study conducted by Han et al. 178 node-positive breast cancer patients receiving anthracycline-based adjuvant chemotherapy were evaluated to see whether the COX-2 level is associated with poor prognosis [17]. In that study, COX-2 expression was determined in 39.3% of the patients and it was observed that S the phase fraction was higher in those patients and HER-2 increase, even not significant, was observed. While no correlation between COX-2 level and tumor size, histological grade and ER expression was registered, significant negative correlation was determined in DFS and OS. In multivariate analysis, COX-2 overexpression has been shown to be an important negative prognostic factor.

In a review written by Falandry et al. concerning the use of aromatase inhibitors and COX-2 inhibitors in patients with metastatic breast cancer, it was emphasized that postmenopausal women might benefit from combined therapy, however, the authors stated that the cardiovascular risk would be increased with long-term use of COX-2 inhibitors [18].

The relationship between breast cancer and increased estrogen levels has been known for a long time. High estrogen levels in postmenopausal women represent an important risk factor for

Table 4. Correlation analysis to evaluate the relationship between clinical variables and immunohistochemical parameters

Variables		*Age*	*Tumor size*	*Axillary density*	*Weight*	*ER*	*PR*	*Cerb-2*	*Grade*	*Cox2*
Age	Correlation coefficient	1.000	-0.020	-0.003	0.220	0.155	-0.105	-0.047	-0.099	-0.057
	Sign(2-tailed)	.	0.860	0.978	0.046	0.165	0.344	0.670	0.373	0.602
	N	87	79	83	83	82	83	83	83	87
Tumor size	Correlation coefficient	-0,020	1.000	0.342	-0.106	-0.082	-0.141	0.052	0.256	-0.039
	Sign(2-tailed)	0.860	.	0.002	0.352	0.475	0.214	0.46	0.023	0.734
	N	79	79	79	79	78	79	79	79	79
Axillary density	Correlation coefficient	-0.003	0.342	1.000	0.232	0.010	-0.026	-0.110	-0.113	0.001
	Sign(2-tailed)	0.978	0.002	.	0.035	0.928	0.816	0.324	0.307	0.991
	N	83	79	83	83	82	83	83	83	83
Body weight	Correlation coefficient	0.220	-0.106	0.232	1.000	-0.062	-0.011	0.068	-0.101	0.184
	Sign(2-tailed)	0.046	0.352	0.035	.	0.582	0.920	0.543	0.362	0.096
	N	83	79	83	83	82	83	83	83	83
ER	Correlation coefficient	0.155	-0.082	0.010	-0.062	1.000	0.444	0.257	-0.015	-0.303
	Sign(2-tailed)	0.165	0.475	0.928	0.582	.	0.000	0.020	0.896	0.006
	N	82	78	82	82	82	82	82	82	82
PR	Correlation coefficient	-0.105	-0.141	-0.026	-0.011	0.444	1.000	0.149	-0.117	-0.312
	Sign(2-tailed)	0.344	0.214	0.816	0.920	0.000	.	0.179	0.291	0.004
	N	83	79	83	83	82	83	83	83	83
CerbB2	Correlation coefficient	-0.047	0.052	-0.110	0.068	0.257	0.149	1.000	0.191	-0.003
	Sign(2-tailed)	0.670	0.646	0.324	0.543	0.020	0.179	.	0.084	0.981
	N	83	79	83	83	82	83	83	83	83
Grade	Correlation coefficient	-0.099	0.256	-0.113	-0.101	-0.015	-0.117	0.191	1.000	0.106
	Sign(2-tailed)	0.373	0.023	0.307	0.362	0.896	0.291	0.084	.	0.340
	N	83	79	83	83	82	83	83	83	83
Cox2	Correlation coefficient	-0.057	-0.039	0.001	0.184	-0.303	-0.312	-0.003	0.106	1.000
	Sign(2-tailed)	0.602	0.734	0.991	0.096	0.006	0.004	0.981	0.340	.
	N	87	79	83	83	82	83	83	83	88

For abbreviations see footnote of Table 1

breast cancer development. During the postmenopausal period, estrogens are synthesized from androgens via aromatase in fat tissue. Estrogens cause proliferation of breast epithelial cells and breast cancer cells by means of COX-2 and prostaglandins. Prostaglandins that are synthesized via COX-2 increase the activity of aromatase both in breast and fat tissues, thus, causing increase in estradiol synthesis and in the risk for breast cancer development. In their studies, Brueggemeier et al. showed a positive correlation between aromatase level and COX-2 in breast cancer tissue [19,20]. Further studies are needed to explain the relation between COX-2 expression and hormonal etiopathogenesis and prognosis.

Although no relation was determined between COX-2 and lymph node status, tumor grade, tumor size, DFS and OS, the correlation between COX-2 and ER and menopausal status was significant. A negative correlation was observed between COX-2 levels and levels of ER and PR. This could be attributed to the relatively limited number of patients. Although the present study has not demonstrated a relation between COX-2 levels and survival in

pre and postmenopausal women, its relation with hormone receptors' expression could be exploited as prognosticator of response to hormonal therapies. Clinical prospective studies will be needed to enlight this topic.

Conclusion

In the present study, COX-2 positivity was found in 49% of early of breast cancer patients, showing similarities with other relevant studies. It was concluded that there was a negative correlation between COX-2 level and of ER and PR levels, but no correlation was determined with other clinicopathological parameters as well as with DFS and OS.

In some of the studies, it was shown that increased COX-2 levels affect both the OS and DFS. In the present study, however, these effects were not proved. Therefore, the answer to the question whether COX-2 is a parameter indicating poor prognosis in breast cancer remains unclear. These results may be due to the limited number of patients, and studies including higher numbers of patients may reveal different results.

COX-2 has been implicated in the progression and angiogenesis of cancers. Selective COX-2 inhibitors have both apoptopic and antiangiogenic activities, and may be of use in the treatment of breast tumors which overexpress the COX-2 enzyme. Clinical trials are evaluating adjunctive therapy with a selective COX-2 inhibitor, such as celecoxib, in combination with several regimens used in the metastatic and adjuvant or neoadjuvant settings of breast cancer.

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