Expression of cyclooxygenase-2 and its relation to histological grade, inducible nitric oxide synthase, matrix metalloproteinase-2, CD-34, Caspase-3, and CD8 in invasive ductal carcinoma of the breast

Esin Atik, MD, Bulent Akansu, MD, Sevgi Bakaris, MD, Nedim Aban, MD.

ABSTRACT

الأهداف: تقدير ظهور السكلواكسجينز (COX-2) في السرطان القنوي الجائر عن طريق الكيمياء المناعية النسيجية، و العلاقة المحتملة مع الدرجة النسيجية، و إنزيم حامض النتريك (iNOS)، و الإنزيم الفلزي (MMP2)، و المتغيرات المناعية الأخرى CD8, Caspase-3, CD-34.

الطريقة: اشتملت هذه الدراسة الاستعادية الارشيفية على 50 مريضة و أجريت في جامعة مصطفى كمال – هاتاي – و جامعة سوطسو إمام – كهرمان ماراس – تركيا. تم إجراء تقنية الصبغة الروتينية بالهيتاموكسيلين واستعمال COX-2 و iNOS و MMP2 و CD-34 و CASP-3 و CD8 على أنسجة البرافين المطمورة.

النتائج: بلغ معدل قيمة COX-2 حوالي 274.02±54.49، و قيمة MMP-2 كانت 263.42±54.30، بينما معدل قيم iNOS كانت 258.10±56.05. أظهرت صبغة CD-34 نتائج إيجابية مثل 26.18±8.00. يقدر معدل قيم CASP-3 284.06±41.2 و CD8 كانت 164.17±69.5. يظهر ذلك علاقة عكسية بين تفاعل CASP-3 و CD8 و تبلغ العلاقة p=0.01، r=-0.33. يوجد علاقة عكسية بين تفاعلات حامض النتريك iNOS و عمر المريض (r = -0.29, p=0.03). هناك علاقة إيجابية أيضاً مع COX-2، و MMP-2 p=0.00. و لكن لا يوجد أي علاقة بين مع COX-2، و المتغيرات الأخرى.

خاتمة: يعد ظهور COX-2 من المتغيرات المهمة للسرطان القنوي الجائر في الصدر. وجدنا علاقة إيجابية بين COX-2 و MMP-2، بينما لم نستطيع إيجاد علاقة مباشرة بين COX-2، و iNOS، و CD-34، و CASP-3، و CD8.

Objectives: To assess by immunohistochemistry the cyclooxygenase-2 (COX-2) expression in invasive ductal carcinoma and its possible correlation with the histological grade, inducible nitric oxide synthase (iNOS), matrix metalloproteinase-2 (MMP2), and other common immunohistochemical parameters (CD-34, Caspase-3, and CD8).

Methods: This was a retrospective pathology archive study including 50 female patients and was performed in Mustafa Kemal University, Hatay, and Sutcu Imam University, Kahraman Maras, Turkey. The routine hematoxylin-eosin staining and COX-2, iNOS, MMP-2, CD-34, CASP-3, and CD8 immunoperoxidase techniques were performed on paraffin-embedded tissues.

Results: The mean value of COX-2 was 274.02±54.49 and MMP-2 was 263.42±54.30, whereas the mean iNOS values were 258.10±56.05. CD-34 staining also yielded positive results as 26.18±8.00. The mean value of CASP-3 was 284.06±41.2 and CD8 was 164.17±69.5. This reveals an inverse correlation between CASP-3 reactivity and CD8 (Spearman correlation [r]= -0.33, p=0.01). There was also an inverse correlation between iNOS reactivity and patients age (r = -0.29, p=0.03). There was a positive correlation with COX-2 and MMP-2 (p=0.00), but there was no relation with COX-2 and other parameters.

Conclusion: COX-2 expression is an important parameter for invasive ductal carcinoma of the breast. We found a positive correlation between COX-2 and MMP-2, whereas, we could not show direct correlation between COX-2 and iNOS, CD-34, CASP-3, and CD8.

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From the Department of Pathology (Atik, Akansu), Department of General Surgery (Aban), Faculty of Medicine, Mustafa Kemal University, Hatay, and Department of Pathology (Bakaris), Faculty of Medicine, Sutcu Imam University, Kahraman Maras, Turkey.

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Address correspondence and reprint request to: Dr. Bulent Akansu, Department of Pathology, Faculty of Medicine, Mustafa Kemal University, Hatay, Turkey. Tel. +90 505 3949679. Fax. +90 (326) 2455305. E-mail: bulentakansu@yahoo.com

Invasive breast cancer is the most common carcinoma in women in developed and developing countries that causes significant mortality and morbidity.[1] In recent years, the observations on the use of non-steroidal anti-inflammatory drugs (NSAIDs) for chemo prevention of some cancers such as breast carcinoma are popular. The main target of NSAID activity is the cyclooxygenase (COX) enzyme.[2-10] Nitric oxide (NO) and its metabolites are implicated in carcinogenesis and metastasis. Both stimulatory and inhibitory effects of NO have been reported in relation to breast cancer, and its role in the development of malignancies and metastasis remains uncertain.[11] Matrix metalloproteinases (MMPs) area family of over 20 proteolytic enzymes that play key roles in extracellular matrix degradation. Matrix metalloproteinases-2 (type IV collagenase) has been the most closely linked to breast cancer. The major activity of MMP-2 is the hydrolysis of gelatine and type-IV collagen, being the principal structural components of basement membranes.[12,13] Angiogenesis is a prerequisite for tumor growth and progression. This can be visualized by immunohistochemical staining with antibodies such as CD-34.[14] Apoptosis or programmed cell death is crucial for the development and homeostasis of multicellular organisms, whereas an imbalance in apoptosis can lead to diseases such as autoimmunity and cancer. A key component of this regulatory network is a proteolytic system involving a family of proteases called caspases.[15] The aim of our study was to assess by immunohistochemistry the COX-2 expression in invasive ductal carcinoma and its possible correlation with the histological grade, inducible nitric oxide synthase (iNOS), matrix metalloproteinase-2 (MMP-2), and other common immunohistochemical parameters (CD-34, Caspase-3 [CASP-3], and CD8) for further treatment and prevention modalities.

Methods. This was a retrospective pathology archive study including 50 female patients with a mean age of 54.9±12.0 years who were diagnosed as invasive ductal carcinoma in the Pathology Departments of Mustafa Kemal University and Sutcu Imam University, Hatay, Turkey between January 2003 and December 2004. Only patients that underwent modified radical mastectomy procedure were included in this study. The ethical committee on human research at our institution approved the protocol for all human research. These cases were graded according to a modified form of

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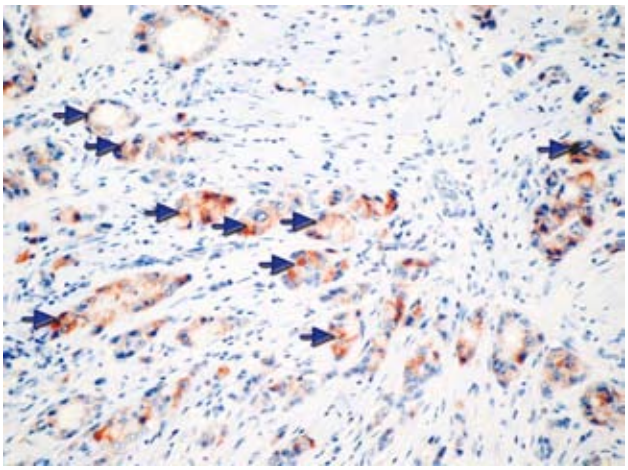

Figure 1 - Immunopositivity of cyclooxygenase-2 (COX-2) (arrows) in an invasive ductal carcinoma (x200).

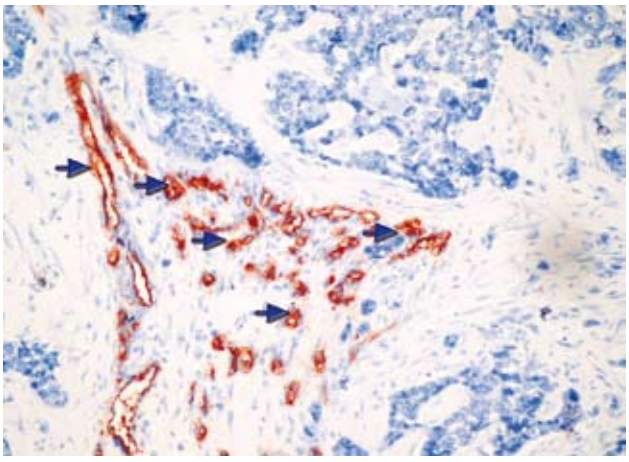

Figure 2 - Immunopositivity of CD-34 (arrows) in an invasive ductal carcinoma (x200).

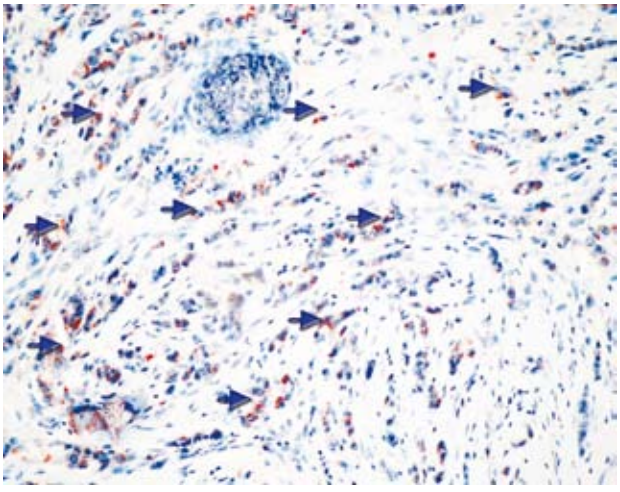

Figure 3 - Immunopositivity of inducible nitric oxide synthase (iNOS) (arrows) in invasive ductal carcinoma (x200).

the Bloom Richardson Histological Grading Index.[1] Routine hematoxylin-eosin staining and COX2, iNOS, MMP-2, CD-34, CASP-3 and CD8 immunoperoxidase techniques were performed on paraffin–embedded tissues (**Figures 1-3**). Immunohistochemical staining was carried out by deparaffinization, dehydration, and incubation in citrate buffer. The antigen staining was performed with Ultra Vision Polyvalent, HRP-AEC kit (Neomarker-Biogen, Lab Vision Corp. Californa, USA). A labelled streptoavidin-biotin-peroxidase (immunoenzymatic) antigen detection system and AEC chromogen were used. For the evaluation of CD-34, positive tumor areas with a high density of vascularization were chosen. Positive stained vessels were counted in 5 different areas at a magnification of x40 by an Olympus BX51 (Olympus Corp., Tokyo, Japan)) microscope in each case. For the evaluation of COX-2, iNOS, MMP-2, CASP-3, and CD8, we counted the positive stained cells per field of at least 5 dense stained fields, at a magnification of X40 by an Olympus BX51 light microscope. The mean value of positive stained cells were independently calculated by 3 pathologists, and the results were averaged.

The statistical procedures were carried out using the software Epi INFO version 3.3 and $p<0.05$ was considered statistically significant. For the comparison of the findings, Spearman's correlation test was performed.

Results. Descriptive statistics of age, histological grade, lymph nodes, COX-2, iNOS, MMP-2, CD-34, CASP-3, and CD8 are shown in **Table 1**. This reveals an inverse correlation between CASP-3 reactivity and CD8 (Spearman correlation r = -0.33, $p=0.01$). There was also an inverse correlation between iNOS reactivity and patients age (Spearman correlation r = -0.29, $p=0.03$). There was a positive correlation with COX-2 and MMP-2 ($p=0.00$), but there was no relation with COX2 and other parameters. While there was a positive correlation with lymph node positivity and histological grade ($p=0.007$), a positive correlation was found between lymph node positivity and CD-34 reactivity and patients age ($p=0.007$, $p=0.01$).

Table 1 - Descriptive statistics of age, histological grade, lymph nodes, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), matrix metalloproteinase-2 (MMP-2), CD-34, Caspase-3 (CASP-3), and CD8 by the subgroups.

Variables	Number of patients	Mean ± standard deviation
Age	50	54.86 ± 11.95
Histological score	50	1.92 ± 0.69
Lymph node	50	3.52 ± 8.86
MMP-2	50	263.40 ± 54.36
CASP-3	50	284.00 ± 41.22
iNOS	50	258.00 ± 56.05
CD-34	50	26.18 ± 8.00
CD-8	50	164.17 ± 69.59
COX-2	50	274.02 ± 54.49

Discussion. Cyclooxygenase-1 and COX-2 are the 2 isoforms of COX enzymes, which synthesize prostaglandins[4,5,10,16] that are important for wound healing, cardiovascular diseases, inflammation, and development, and growth of malignant lesions.[2,3,5] Cyclooxygenase-1 is expressed on normal cell membranes, whereas COX-2 is located in the cytoplasm and known to induce inflammatory and neoplastic processes.[2,3,5] Cyclooxygenase-2 has been reported as upregulated in breast cancer and many other solid tumors,[4,5,9,17] and involved in malignant transformation and tumor progression by affecting mitosis, apoptosis, cell proliferation, adhesion, angiogenesis, and immunity.[2,3,9,16,17] Elevated COX-2 levels seem to be correlated with poor prognosis in cancer patients, including malignant breast tumors.[2-4,17] Cyclooxygenase-2 is accepted as a target for prevention and treatment of these tumors.[2-4,16] There are several studies that confirm the benefit of regular use of aspirin and other NSAID's in breast cancer.[2,3] Treatment with COX-2 inhibitors was found useful for reducing occurrence and growth of breast tumor in experimental animal models.[2-4,17] These findings indicate the importance of COX-2 in breast cancer progression and show that COX-2 inhibitors may be used for prevention of cancer.[2-4,9] Several studies revealed that elevated COX-2 levels are related with aggressiveness of breast cancer, large tumor size, high proliferation rate, axillary node involvement and ductal histology in their study of 1576 invasive breast cancer cases.[11-13] Cho et al[2] found a significant correlation between COX-2 expression and tumor size (malignant tumors stage).[2] The COX-2 upregulation mechanism in breast cancer is not still understood, but it may be related to the activation of various oncogenes.[2,3] Estrogens are synthesized from androgens by the way of aromatase enzyme.[9] There is evidence on the importance of intratumoral aromatase in breast cancer incidence and progression.[9] Cyclooxygenase-2 expression is found correlated with aromatase expression in breast cancer.[9] Chemopreventive and therapeutic effects of selective and non-selective COX-2 inhibitors were assessed in various experimental studies.[4,8] Shim et al[7] concluded that it was difficult to interpreted any statistically significant correlation with age and tumor size, since COX-2 expression was prevalent.[7] We found a correlation between COX-2 and MMP-2, which were

consistent with other studies. But, there was no direct correlation between COX-2 and INOS, CD34, CASP-3, and CD8. These findings suggest that there should be an indirect correlation between COX-2 expression and histological grade, and lymph node metastasis. Matrix metalloproteinases-2 is a zinc-dependent proteolytic enzyme, which degrades type-IV collagen in basement membranes and is important for invasion and metastasis.[5,12,13,18] Elevated MMP-2 expression and activity was found in breast cancer and this was thought to be related to lymph node and distant metastasis, and so with shortened survival.[5,12,13,18] Matrix metalloproteinases-2 expression is associated with poor prognosis in primary tumors.[12,13,18,19] It is widely assumed that degradation of extracellular matrix by MMP promotes metastasis.[13,18,19] The main component of basement membrane is type-IV collagen.[13,18,19] Cancer cells must pass the basement membrane for metastasis. Type-IV collagen is degraded mostly by MMP-2 and MMP-9. Therefore, MMPs are important factors for metastasis.[13,18,19] Also, there may be a direct relationship between COX-2 and MMP-2, since COX-2 transfection increases activated MMP-2 amounts in colon and breast cancer.[5,17] As consistent with the literature, we found a positive correlation between COX-2 and MMP-2 expression. Costa et al[16] found a significant association between COX-2 expression and microvessel count, lymph node involvement, and apoptotic index, but not with other clinicopathological features such as age, tumor grade, Transforming growth factor alpha, estrogen receptor, p53, c-Erb-B2, and MIB-I. Apoptosis, in other terms programmed cell death, is executed by the action of caspases and is important for homeostasis and development of an organism.[15] Any disorder in this mechanism can cause autoimmune diseases and cancer.[15] Mimori et al[20] observed cancer specific methylation in CASP-3, therefore, methylation of CASP-3 might be a carcinogen alone without involvement of other caspase related up-stream molecules in breast cancer cells. In invasive breast cancer, inflammation mostly consists of diffuse T-cell and macrophage infiltration. However, perivascular and perilobular B and T-cell clusters can be seen at the tumor edge.[21] Lee et al[21] showed that perivascular inflammation clusters were related to increased stromal vascularity. The effect of growth-stimulatory and inhibitory mediators released from T-cells or tumor cells on cancer growth is important due to the recent studies.[22] Nevertheless, no obvious correlation can be detected between any lymphocyte subgroups (CD3+, CD4+, CD8+, CD20+, granzyme-B+) and tumor infiltration.[23] These findings suggest that there is no discrete specific immune response to the tumor.[23] Robinson et al[24] did not found any significant difference in the CD8 cell subgroups among patients with multiple primary tumors and between each group of patients and healthy subjects, but found that CD3, CD4, and CD4/CD8 ratios were significantly lower in cancer patients than in healthy controls.[24] Also, the mean CD4/CD8 ratio was significantly lower in patients with metastatic disease than in controls or in patients without metastasis.[24] The CD4/CD8 ratio was found increased in patients without relapse compared to patients with relapse.[24] Patients without axillary lymph node involvement had a higher CD4/CD8 ratio than patients with metastasis as well.[24] The CASP-3 expression was inversely correlated with CD8 expression due to our results. If we assume that CASP-3 expression was directly related with malignancy, our findings on the CD8 expression are not meaningful. Usually, CD8 values can be contradictory in different studies.

In clinical and experimental studies, the quantity of vascularization had been shown as associated with tumor aggressiveness and metastasis in breast, prostate, bladder carcinomas, and malignant melanoma.[14] According to several studies, increased vascularity is associated with increased metastasis and poor prognosis.[21] Intratumoral microvessel density shows the tumor angiogenesis directly and can be visualized by immunohistochemical stains like anti-CD34.[14] Harris et al[25] showed that vascular endothelial growth factor (VEGF) overexpression was related with enhanced NOS activity, and suppression of tumor cell apoptosis.[25] We detected a positive correlation between lymph node metastasis and vascularization, and age of patients. This situation is evidence of poor prognosis and metastatic potential, and in concurrence with the literature. The effect of NO on tumor progression or inhibition is contradictory. Enhanced concentrations can cause cytotoxicity, whereas low concentrations can prevent cellular damage.[26] Increased vascularity and apoptotic index in iNOS positive tumors brings to mind that iNOS has the same effect in breast cancer. Thus, an increase in iNOS positive stromal cell counts can be related with enhanced vascularity, invasion of tumor cells, and metastasis.[26] Furthermore, the recent studies exhibit the promoting role of NO in tumorigenesis.[11] Carcinogenesis is a complex and multistep process. It is not an unexpected result that, both positive and negative effects of NO were shown, since 3 different NO isoforms are present in mammary glands.[11] The effect of iNOS can be different as related to the concentration of NO that is produced, and it can be changed in the local environment of different tumor and cell types.[26] We found an inverse correlation between iNOS reactivity and patient age. The increased iNOS positivity in younger patients can be interpreted as related with the decrease of immunity in older patients.

The retrospective nature of this study and the limited number of patients included has limited this study.

In conclusion, this study shows that COX-2 expression is an important parameter for invasive ductal carcinoma of the breast. We found a positive correlation between COX-2 and MMP-2, whereas, we could not show direct correlation between COX-2 and iNOS, CD-34, CASP-3, and CD8. However, one should bear in mind that these parameters may also be important. When the mechanism of the effect of all these parameters in tumor promotion is understood exactly with further studies, it will be possible to define new therapeutic targets and produce more intensive therapeutic agents. Moreover, elevated expression of COX-2 may be involved in the carcinogenesis of the breast and may be a useful target for chemoprevention of breast cancer.

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