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ST6Gal1, Cox-2 and HB-EGF mRNA Expression in Breast Cancer Samples from Kashan, Iran

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Abstract

Background: ST6Gal1, Cox-2 and HB-EGF genes are involved in different tumors and their enhanced expressions often correlate with poor prognosis. In this study we assay the expressions of these genes by reverse transcriptase-PCR in 54 breast cancer samples.

Methods: Tissue samples were either formalin-fixed for histopathological examination or frozen for reverse transcriptase-PCR. Image program was used for the densitometry of the image of the gels and the expression of different genes was normalized with beta actin expression. The student's t-test and correlation matrix were used for data analyses.

Results: We observed significantly higher expressions of ST6Gal1 (P= 0.040), Cox-2 (P= 0.001) and HB-EGF (P= 0.009) in the tumor region compared to the margin samples. A significant correlation was found between HB-EGF and Cox-2 expression (P= 0.001). There was a positive correlation between total score, tumor size, histology grade and nuclear grade but there was a reverse correlation between age and tumor size, histology grade and total score.

Conclusion: Expressions of ST6Gal1, Cox-2 and HB-EGF in breast tumor samples in this and a number of other studies emphasize their role as important markers in breast cancer. The use of medications to inhibit either their individual expressions or the possible inhibition of all three genes may improve patient survival and prevent metastasis.

Keywords: Breast cancer, ST6Gal1, Cox-2, HB-EGF, RT-PCR

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Introduction

Breast cancer is one of the most common cancers and the main cause for death among women worldwide.[1] The clinical outcome of breast cancer is affected by prognostic and predictive factors. During the past

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few years, numerous studies have been undertaken concerning the biological factors that affect tumor behavior and explain metastasis in breast cancer.

The enzyme, β-galactoside α2–6-sialyltransferase (ST6Gal1) facilitates the α2–6 linkage of sialic acids to N-acetyllactosamine structures (Galβ1–4GlcNAc) which is a Golgi-mediated process. Alternative α2–6 sialylation can have a wide array of biologic and pathogenic consequences, including alterations in immune response and embryogenesis, as well as a role in the development and progression and metastasis of some cancers.[2] Increased expression of ST6Gal1 has been reported in carcinomas of the colon, breast and cervix, choriocarcinomas, acute myeloid leukemia, and brain tumors.[2]

Cyclooxygenase (Cox) is an enzyme involved in the biosynthetic prostaglandin pathway.[3] In humans, two separate genes produce the constitutive (Cox-1) and the inducible Cox-2 forms.[3] Cox-1 is constitutively expressed in most tissues and is responsible for normal physiologic or ''housekeeping'' processes.[3] Cox-2 is not usually expressed in normal tissue but is induced by different factors that include bacterial endotoxins, cytokines, growth factors, and oncogenes.[3] Cox-2 gene expression is associated with cellular growth and differentiation. Cox-2 expression and elevated prostaglandins play a critical role in tumorigenesis.[3] Cox-2 expression has been reported in a variety of solid tumors, including colorectal, breast, lung, and ovarian cancers.[3] In rat models, specific Cox-2 inhibitors have been shown to prevent mammary tumor development,[3] while forced overexpression of Cox-2 in transgenic mice induced tumorigenesis.[3] Overexpression of Cox-2 could inhibit apoptosis and enhance invasiveness.[3]

Heparin-binding EGF-like growth factor (HB-EGF) is a 20-22 kDa protein member of the epidermal growth factor (EGF)-like growth factor family that binds to and activates the EGF receptor (EGFR) and its associated receptors ERBB2, ERBB3 and ERBB4. It has been reported that expression of HB-EGF is altered in multiple types of cancers including breast cancer.[4] In addition to the increased expression of HB-EGF in tumor versus nontumor tissue, its subcellular localization and release of N- and C-terminal fragments are involved in oncogenic behaviors.[4] It has also been demonstrated that HB-EGF is a potent inducer for angiogenesis *in vivo*.[4]

This study examined expressions of the ST6Gal1, Cox-2 and HB-EGF genes at the mRNA level for the first time in Iranian breast cancer samples from Kashan, Iran. The relation of different parameters with the expression of these genes has been analyzed using SPSS software. These findings might help to indicate a role for ST6Gal1, Cox-2 or Hb-EGF as markers of tumor progression in breast cancer.

Materials and Methods

Tissue samples

We used 54 breast cancer tissues from patients who underwent surgery for their primary tumors at the Kashan University of Medical Sciences hospitals. Corresponding control (margin) specimens were obtained from the same patients during the surgery and lacked macroscopic tumor involvement. After excision, one part of the tissue samples was snap-frozen in liquid nitrogen and stored at -80°C until analyzed. The remainder of the tissue samples was formalin-fixed and paraffin-embedded for routine histopathological examination. Breast cancer type was determined according to WHO.[5] Nottingham modification of the Bloom-Richardson scoring system was used for breast cancer total score.[6]

Reverse transcriptase-PCR (RT-PCR) analysis

Total RNA was extracted from the margin and tumor region of the breast cancer samples using an RNX-plus solution (RTPL12; CinnaGen Co., Tehran, Iran). Briefly, 200-300 mg of tissue sample were minced and homogenized in 1 ml of RNX-plus solution with a glass/Teflon potter homogenizer. After incubation at room temperature for 5 min, 200 µl of chloroform was added, mixed and the sample was incubated on ice for 15 min. After centrifugation at 13000 rpm at 4°C for 15 min, the aqueous phase was transferred

to a new tube and an equal volume of isopropanol was added, mixed and the sample was incubated on ice for 15 min. The sample was centrifuged at 13000 rpm at 4°C for 15 min, after which the supernatant was discarded and the pellet washed with 1 ml of 75% ethanol, and centrifuged at 8000 rpm at 4°C for 10 min. The pellet was dried for a few minutes and dissolved in 50 µl of DEPC treated water. The amount of RNA was quantified by measuring the absorbance at 260 nm.

cDNA synthesis

The synthesis of cDNA and PCR was performed by using the 2-step RT-PCR Kit (Vivantis Technologies, Kuala Lumpur, Malaysia). We mixed 2 µg of total RNA with 1 µl of 40 uM oligo d(T), 1 ul of 10 mM dNTP mix and water up to 10 µl, incubated the mixture at 65°C for 5 min, and subsequently chilled it in ice for 2 min. This mixture was added to 10 µl of a cDNA synthesis mix that included 2 µl of 10X buffer M-MulV, M-MulV reverse transcriptase (100 u), which was subsequently mixed and incubated at 42°C for 60 min. The reaction was terminated by incubating the tube at 85°C for 5 min. The tube was chilled in ice and briefly centrifuged to pellet the solution in the tube.

PCR

We used RT-PCR to amplify 2 µl of the cDNAs using primers for ST6Gal1 (forward: CATC-CAAGCGCAAGACTGACG and reverse: TGTGCCCTGGTTGAGATGCTTC) to amplify a 125 bp fragment; for Cox-2 (forward: GCGTCAGGAGCACGTCCAGG and reverse: GCTGTCTGAGGGCGTCTGGC) to amplify a 72 bp fragment; and for HB-EGF (forward: GATGGTTGTGTGGTCATAGGT and reverse: TGGCTGCAGTTCTCTCGGC) to amplify a 449 bp fragment, in a 15 µl reaction volume. As an internal control, we amplified b-actin cDNA by using primers (forward: ATGATATCGC-CGCGCTCG and reverse: GTTGGTGACGATG CCGTGCT) to amplify a 435 bp fragment. The PCR reaction contained: 1X Taq polymerase buffer; 1.7 mM MgCl2; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; and 0.5 U recombinant DNA polymerase (CinaGen Co., Tehran, Iran). The PCR conditions were as follows: 5 min at 95°C, 1 min at 94°C, 45 sec at 61°C and 45 sec at 72°C, for 36 cycles and 5 min at 72°C at the end. All samples were analyzed in triplicate.

The PCR product was electrophoresed in a 1.5% agarose gel. The gel was stained with ethidium bromide, after which pictures were taken under the UV light and Image J software was used to measure the density of the PCR product bands in the scan. The results of different gene expressions were normalized by beta actin expression. The mean value of St6Gal1, Cox-2 and HB-EGF expressions in the tumor region was compared to that in the margin region using the student's t-test. Pearson's correlation index was used to determine the presence of any mutual relationship between different gene expressions and histopathological parameters by using SPSS software.

Table 1. Breast cancer subtypes in samples.

Breast cancer type	Number	%
Invasive ductal carcinoma (IDC)	38	70
In situ ductal carcinoma	5	9
Infiltrating lobular carcinoma	4	7
Ductal and lobular carcinoma	3	6
Other	4	7
Total	54	100

Results

From 54 breast samples, 38 were diagnosed with invasive ductal carcinoma (IDC) of NOS (70%) and the remainder comprised other types (Table 1).

We compared expression of the ST6Gal1 genes at the mRNA level in tumor and margin samples (other sample types were removed from the study). The average age of the patients was 50 (21-78) years. The mean value of ST6Gal1 expression in the tumor region (165±179) was higher than in the margin region (73±116), which was significant (P=0.040; Table 2). Significantly higher expressions of Cox2 (P=0.001) and HB-EGF (P=0.009) genes were seen in the tumor samples compared to the margin regions (Table 2).

The relation between different gene expressions

Table 2. The mean values and standard deviations of tumor and margin samples.

	HB-EGF		Cox-2		ST6Gal1	
	Margin	Tumor	Margin	Tumor	Margin	Tumor
Mean	137.59	423.79	155.87	340.9	73	165
STDEV	130.26	487.95	123.22	225.35	116	179
t-test	0.009		0.001		0.040	

(ST6Gal1, Cox-2 and HB-EGF) and histopathology parameters (age, tumor size, IDC, histology grade, total score, nuclear grade and lymphovascular invasion) was analyzed using correlation matrix. For some of the samples we did not have all of the information, therefore the number of data samples entered into the regression was 30. We observed a significant correlation between some of the parameters. The histopathological parameters that had significant correlations are shown in Table 3. Among the histopathological parameters there was a significant direct correlation between total score and tumor size, histology grade and nuclear grade. There was a reverse correlation between age and tumor size, histological grade and total score.

Among different genes there was only a significant correlation between Cox-2 and HB-EGF expression (P=0.001) since the Pearson correlation coefficient for HB-EGF and Cox-2 was 0.833, which was significant at the one percent level.

Discussion

Our results showed higher levels of ST6Gal1, Cox-2 and HB-EGF mRNA in the tumor regions compared to the margin regions of breast cancer samples. Although the expression of these genes was detected in margin samples, the expressions were significantly enhanced in the tumor region. Our results agreed with those that detected a higher expression of these genes in the tumor region compared to normal tissue.

Higher expression of ST6Gal1 has been reported in carcinoma cells from different origins. For instance, the activity of human ST6Gal1 is only low or not present in normal colonic mucosa cells but high in metastasizing colorectal carcinomas.[7] Wang et al. have investigated the expression of ST6Gal1 in normal and cancer cervical tissue samples by real-time relative RT-PCR. There was significantly increased ST6Gal1 mRNA expression in cancerous tissues compared to normal tissues.[8] In cervical cancer patients, the expression of ST6Gal1 was increased in those with lymph node metastases compared to patients without lymph node metastases. It has been suggested that ST6Gal1 is important for lymph node metastases in cervical cancer patients and there is a crucial relevance for the presence of poor prognostic factors such as deep stromal invasion and lymph–vascular space involvement to lymph node metastases.[9] ST6Gal1 expression in human breast carcinomas is reportedly associated with poor prognosis.[10] Other studies suggest that transcriptional regulation of the ST6Gal1 gene is altered during malignant transformation, which verifies the results of the above mentioned studies.[11] Transfection of MDA-231 cell lines with ST6Gal1 expression vector has been shown to increase its migration and tumorogenicity.[12] There are several recognized substrates upon which ST6Gal1 is known to act: β_1 integrin,[13] E-selectin, ICAM-1, and VCAM-1.[14] Forced expression of ST6Gal1 in an ovarian cancer cell line (OV4) resulted in sialylation of β_1 integrins, and induced greater cell adhesion to and migration toward, collagen I. Similarly, ST6Gal1 expressing cells were more invasive through Matrigel. ST6Gal1 mediated sialylation of β_1 integrins in ovarian cancer cells might contribute to peritoneal metastasis by altering tumor cell adhesion and migration through the extracellular matrix.[15] These results suggested that cell surface α-2,6-sialylation contributed to cell–cell and cell–extracellular matrix adhesion of tumor cells.

The role of ST6Gal1 in resistance to

Table 3. Correlation between different histopathological parameters in breast cancer

		Age	Tumor Size	History Grade	Total Score	Nuclear Grade
Age	Pearson Correlation	1	-0.472**	-0.581**	-0.632**	
	Sig. (2-tailed)		0.008	0.001	0	
Tumor	Pearson Correlation	-0.472**	1	0.368*	0.429**	0.509**
	Sig. (2-tailed)	0.008	1	0.001	0	
History Grade	Pearson Correlation	-0.581**	0.368*	1	0.764**	0.522**
	Sig. (2-tailed)	0.001	0.045		0	0.003
Total Score	Pearson Correlation	-0.632**	0.429*	0.764**	1	0.735**
	Sig. (2-tailed)	0	0.018	0		0
Nuclear Grade	Pearson Correlation		0.509**	0.522**	0.735**	1
	Sig. (2-tailed)	0	0.004	0.003	0	

**: Correlation is significant at the 0.01 level (2-tailed).; *: Correlation is significant at the 0.05 level (2-tailed).

chemotherapy has been studied. According to reports higher expression of ST6Gal1 in ovarian tumor cells confers a survival advantage in the presence of cisplatin. These collective findings support a role for ST6Gal1 in chemoresistance and highlight ST6Gal1 as a potential therapeutic target for platinum resistant tumors.[16]

Many studies demonstrated an association between certain pathological processes such as oncogenic transformation, tumor metastasis, and invasion with enhanced sialyltransferase (ST) activity. A few inhibitors have been developed to modulate this activity and alleviate the disease processes caused by ST.[17, 18]

In the current study higher levels of Cox-2 and HB-EGF mRNA expressions were detected in the tumor region compared to the margin of breast cancer samples. A positive correlation, in addition to their higher expression, was observed between the expressions of these two genes.

Increased expression of Cox-2 in premalignant and malignant tissues has been reported in other studies and correlated with worse breast cancer prognosis.[19] Cox-2 is not expressed constitutively but several factors such as growth factors, oncogenes, cytokines, and tumor promoters stimulate Cox-2 transcription via protein kinase C (PKC) and Ras-mediated signaling.[3] The mutant p53 is unable to inhibit expression of Cox-2 *in vitro*[20] whereas the wild-type has this capability. Consistent with this finding, an elevated level of Cox-2 in different cancers including breast cancer with mutant rather wild-type p53 has been observed.[21] Overexpression of Cox-2 leads to increased amounts of prostanoids such as PGE2 in tumors that can stimulate cell proliferation and motility while inhibiting immune surveillance and apoptosis.[22] Importantly, PGE2 can also induce angiogenesis by stimulating the production of proangiogenic factors that include vascular endothelial growth factor.[23] Taken together, these findings suggest that the balance between activation of oncogenes and inactivation of tumor suppressor genes modulates the expression of Cox-2 in tumors.

HB-EGF expression is altered in different tumors including breast cancer.[4, 24] The HB-EGF expression, subcellular localization and N- or C-terminal fragments mediate oncogenic behavior.[4] Although in cancer HB-EGF is typically expressed in epithelial cells but its expression in the stroma and endothelium[25] has been reported as well. One study compared gene expression using real-time quantitative reverse transcription (RT)-PCR. The results showed that HB-EGF upregulated in inflammatory breast cancer when compared to non-inflammatory breast cancer.[26] The higher expression of HB-EGF enhanced tumor invasion.[27]

Our results showed a correlation between Cox-2 and HB-GF expressions in breast cancer samples. A crosstalk between Cox-2 and HB-EGF has been previously reported.[28] Increased amounts of Cox-2 were observed in breast cancers that overexpress HER-2/neu because of enhanced Ras signaling.[29] Therefore the combination of an inhibitor of Cox-2 and an inhibitor of EGFR tyrosine kinase was more effective in suppressing tumor growth.[30]

DNA damage or p53 can stimulate Cox-2 expression through the Ras/Raf/MAPK cascade.[20, 31] HB-EGF, which is a p53 downstream target gene, can induce Cox-2 expression as well. It has been suggested that Cox-2 is an ultimate effecter in the p53/HB-EGF/Ras/Raf/MAPK/Cox-2 pathway.[31]

The current study results have shown a positive correlation between histology grade, tumor size, total score and nuclear grade. Histology grade is determined based on factors such as tumor size, total score and nuclear grade therefore the observe correlation was expected. However there was a reverse correlation of age with histology grade, total score and nuclear grade. These results have supported several studies and the general belief that breast cancer in younger women is generally considered to be unfavorable. In a study in 1997 it was found that the incidence of grade III infiltrating ductal carcinoma was higher in women under the age of 35. There was a lower rate of axillary lymph node metastases, vascular invasion and lymphoplasmacytic stromal reaction with increasing age. The researchers suggested that there might be age-related changes in the histology of breast cancer and, in some cases, less aggressive features in the elderly.[32]

Invasive breast cancer that occurs in women less than 35 years of age has a more aggressive biological behavior and is associated with a worse prognosis than in older premenopausal women.[33] Although the estrogen-receptor positive tumors have a significantly worse disease-free survival in younger patients, but by contrast, among older patients the disease-free survival has been shown to be similar irrespective of estrogen-receptor status.[34] Overall, in breast cancer, young age is - among multiple unfavorable risk factors such as positive axillary lymph nodes, high nuclear grade, and large tumor that show poorer local control and disease-free survival.[35] The reverse correlation of age with total score in breast cancer has been reported in several studies, however this correlation does not exist in other type of cancers. For example in prostate cancer the rate of metastasis increases in older patients; tumor grade also increases slightly with age as well.[36]

Upregulation of the Cox-2, HB-EGF and ST6GALNAC5 genes have been reported in breast cancer cells that have the ability to pass the blood-brain barrier.[37] According to a study, Cox-2 and HB-EGF genes induce cancer cell mobility and invasiveness, acting as genetic mediators in the spread of breast cancer to the brain.[37] These studies highlight the role of cell-surface glycosylation in organ-specific metastatic interactions. The up-regulated genes in breast cancer can offer useful diagnostic or prognostic markers and form the basis of novel therapeutic strategies.

Conclusion

Our results indicated that breast cancer tissue had higher expressions of ST6Gal1, Cox-2 and HB-EGF. The higher expression and positive correlation between Cox-2 and HB-EGF in this study re-emphasized the benefit of using inhibiting drugs to target genes (Cox-2 and HB-EGF) or Cox-2, HB-EGF and ST6Gal1 in the control of cancer growth.

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Conflict of Interest

No conflict of interest is declared.

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