EpCAM and COX-2 expression are positively correlated in human breast cancer

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Abstract. Upregulation of the epithelial cell adhesion molecule (EpCAM) is involved in tumor progression. Cyclooxygenase (COX)-2 is the key enzyme catalyzing prostaglandin synthesis and is involved in breast cancer progression and metastasis. However, the prognostic value of EpCAM and its putative correlation with COX-2 in breast cancer have yet to be elucidated. The aim of the present study was to assess the clinical relevance of the relationship between EpCAM and COX-2, via examining the putative correlation between EpCAM and COX-2 expression in various types of human breast cancer. A total of 134 breast cancer tissue samples was examined in the present study. Immunohistochemistry approach was used to detect EpCAM and COX-2 expression in the tissue microarrays. Spearman's correlation analysis was performed to evaluate the correlation between the protein expression and clinicopathological parameters present in patients with various tumor subtypes, with the aim to potentially establish a relationship between EpCAM/COX-2 and clinical prognosis. Expression of EpCAM and COX-2 was revealed to be associated with tumor progression, and poor prognosis in breast cancer. The present findings demonstrated that EpCAM was involved in the regulation of COX-2 expression, and a positive correlation between the proteins was associated with poor prognosis in patients with breast cancer. The present results suggest that EpCAM and COX-2 may have potential as prognostic biomarkers in the diagnosis and treatment of patients with breast cancer.

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Introduction

Breast cancer has one of the highest mortality rates among malignant tumors in women worldwide, and since it is also characterized by high incidence and morbidity rates, it poses a major public health concern (1,2). Tumor metastasis is one of the main causes underlying cancer-associated mortality. Although only 5-10% of newly diagnosed breast cancer patients exhibit metastasis to distant organs, the risk of metastasis in patients with localized primary disease, following successful primary tumor resection and adjuvant therapy, remains high (3,4). Several diagnostic biomarkers and therapeutic targets, such as the estrogen receptor (ER) and progesterone receptor (PR), and the human epidermal growth factor receptor (HER) 2 are already being used in clinical practice; however, variations among individual patients hinder the diagnosis and effective treatment of breast cancer (5). Therefore, the need to identify reliable biomarkers for the diagnosis, prognosis and treatment of patients with breast cancer is urgent.

Epithelial cell adhesion molecule (EpCAM) is a glycosylated, type I transmembrane protein, which is overexpressed in several neoplasms, such as breast cancer, hepatocellular carcinoma (6), glioma (7) and colorectal cancer (8). Since EpCAM has been associated with cancer progression and prognosis, it is used as a diagnostic and prognostic marker for various types of disease (9).

Our previous studies have indicated that EpCAM may serve a regulatory role during epithelial-mesenchymal transition (EMT) in breast cancer cells (10), whereas knockdown of EpCAM inhibited breast cancer cell growth and metastasis via inhibition of the Ras/Raf/extracellular signal-regulated kinase signaling pathway and matrix metalloproteinase (MMP)-9 (11). These results suggested that EpCAM may serve a role in the regulation of cancer cell growth and may hold potential as a prognostic marker in breast cancer.

Cyclooxygenase (COX)-2 is the key enzyme regulating prostaglandin synthesis and is involved in inflammatory processes. COX-2 is expressed in several tissues, and its expression is induced and regulated by tumor promoters, cytokines, endotoxins, growth factors and prostaglandins (12). High levels of prostaglandins, resulting from the overexpression of COX-2, have been implicated in the pathogenesis of

numerous malignancies, including colon, breast, and lung cancer, and have been associated with carcinogenesis, particularly neoangiogenesis and tumor progression (13-18). However, the relationship between EpCAM and COX-2 in breast cancer has yet to be elucidated.

In the present study, an immunohistochemical approach was used to evaluate the expression of EpCAM and COX-2 in tissue samples derived from patients with breast cancer, and to determine whether a correlation can be established between them. The results revealed that the expression of EpCAM exhibited a statistically significant, positive correlation with COX-2 expression, thus suggesting a combined prognostic value for EpCAM and COX-2 in breast cancer.

Materials and methods

Tissue microarray (TMA) and immunohistochemistry (IHC). TMAs (cat. no. 140317A; AlenaBio Biotechnology Ltd., Xi'an, China) with samples from healthy and breast cancer tissue, with stage and grade information, were purchased from US Biomax, Inc. (Rockville, MD, USA). For IHC analysis, TMA sections were deparaffinized in 100% xylene and rehydrated in graded ethanol solutions. The sections were then boiled under pressure in citrate buffer (pH 6.0) for 5 min for antigen retrieval. TMA sections were incubated at 37˚C for 1 h with EpCAM (1:200 dilution; cat no. 21050-1-AP; Wuhan Sanying Biotechnology, Wuhan, China,) and COX-2 (1:100 dilution; cat. no. 12375-1-AP; Wuhan Sanying Biotechnology) antibodies in TBS containing 1% bovine serum albumin (Sangong Pharmaceutical Co., Ltd., Shanghai, China). After washing with PBS, the sections were incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1:500 dilution; cat. no. SA00001-2; Wuhan Sanying Biotechnology). Signal development was performed by adding 250 μl 3,3'diaminobenzidine (Sangong Pharmaceutical Co., Ltd.) substrate solution to each slide and incubating for 3 min in the dark. Finally, slides were washed 3 times in water and drained. Images were captured using an Aperio ScanScope® CS system (Nikon Instruments Inc., Vista, CA, USA). EpCAM or COX-2 positive staining on TMA sections was semi-quantitatively analyzed by two independent investigators using the following criteria: 0, background staining; 1, weakly positive; 2, moderately positive; 3, strongly positive staining.

Cell culture and transfection. The MCF-7 and MDA-MB-231 human breast adenocarcinoma cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were maintained in DMEM-F12 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% calf serum (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate, and 10 mM HEPES. MDA-MB-231 cells were cultured in Leibovitz's L-15 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% calf serum, 1% penicillin/streptomycin and 10 mM HEPES. All cells were incubated in a 5% CO_2 humidified atmosphere at 37˚C.

Cells that were in the logarithmic growth phase were transfected with complementary DNAs encoding human EpCAM and control empty plasmid (cat. no. BC014785; Wuhan Sanying Biotechnology), or small interfering (si)RNA targeting EpCAM (si-EpCAM) and control scrambled siRNA (sequences 5'-UGCUCUGAGCGAGUGAGAATT-3' and 5'-UUCUCA CUCGCUCAGAGCATT-3', respectively; GenePharma Co., Ltd., Shanghai, China), using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as the delivery agent, according to the manufacturer's protocol. Subsequent experiments were performed 48 h post-transfection.

Western blot analysis. To prepare whole cell extracts, cells at 90% confluence were washed in PBS prior to incubation with lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA (pH 8.0), 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride and 0.5% Nonidet P-40 on ice for 10 min. Debris was removed from the lysates by centrifugation at 9,000 x *g* for 10 min at 4˚C and the supernatants were collected. Protein concentration was determined with the Coomassie Protein Assay Reagent using bovine serum albumin as a standard. Equal amounts of protein (50 μg) were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes, which were blocked with TBS containing 0.5% Tween-20 and 5% fat-free dry milk for 2 h at 37˚C. The membranes were then incubated for 3 h at 37˚C with EpCAM (1:1,000 dilution; cat. no. 21020-1-AP; Wuhan Sanying Biotechnology), COX-2 (1:800 dilution; cat. no. 12375-1-AP; Wuhan Sanying Biotechnology) and GAPDH (1:2,000 dilution; cat. no. 10494-1-AP; Wuhan Sanying Biotechnology) primary antibodies. Following incubation with a HRP-conjugated anti-goat secondary antibody (1:1,000 dilution; cat. no. SA00001-2; Wuhan Sanying Biotechnology) for 40 min at 37˚C, the protein bands were visualized using an enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Chalfont, UK). Western blots presented are representative of at least 3 independent experiments. Protein expression was quantified using densitometry analysis with Labworks software version 4.6 (Labworks LLC, Lehi, UT, USA). Band intensity is expressed as the mean ± standard error of 3 experiments for each group. GAPDH was used as the loading control.

Statistical analysis. Immunohistochemical scores for EpCAM and COX-2 were tabulated, and the χ^2 test for trend analysis was performed to investigate the relationship between EpCAM and COX-2 expression and pathological diagnostic criteria for breast cancer. Spearman's correlation coefficient analysis was performed to test for positive or negative correlations between EpCAM and COX-2 expression across breast cancer subtypes and diagnostic parameters. Statistical significance was analyzed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Correlation between EpCAM and COX-2 expression and clinicopathological parameters in breast cancer. To investigate the expression of EpCAM and COX-2 in breast cancer tissue, immunohistochemistry was performed on a series of 134 human breast cancer samples within TMAs. Representative EpCAM and COX-2 staining is presented in Fig. 1. A total of 92 (68.66%) samples highly expressed EpCAM and COX-2,

whereas 19 (4.17%) samples exhibited low EpCAM and COX-2 expression; a total of 18 (13.43%) samples exhibited high EpCAM and low COX-2 expression, whereas 5 (3.73%) samples exhibited low EpCAM and high COX-2 expression. A positive correlation was revealed between EpCAM and COX-2 expression in breast cancer (r=0.63, P=0.009; Table I).

To examine the relationship between EpCAM and COX-2 expression and clinicopathological characteristics of the disease, the correlation between EpCAM and COX-2 expression and the following parameters was investigated: Age at the time of diagnosis, tumor differentiation, lymph node metastasis, as well as the expression of ER, PR, HER2, p53 and the proliferation marker Ki-67 (Table II). The results suggested that EpCAM and COX-2 expression were significantly correlated with the histological grade of the tumor (P<0.05). High expression of EpCAM and COX-2 was more frequently observed in higher grade (poorly differentiated) tumors compared with in lower grade tumors. Furthermore, a significant correlation was revealed between EpCAM and COX-2 expression and the expression of ER, PR and Ki-67 (P<0.05); however, no correlation was apparent with lymph node metastasis and p53 expression. Notably, the expression of EpCAM was positively correlated with the expression of HER2 (P<0.05), whereas no correlation was revealed between COX-2 and HER2 expression (Table II).

Correlation between EpCAM and COX-2 expression in breast cancer cell lines. The aforementioned results demonstrated that EpCAM and COX-2 expression were positively correlated in breast cancer tissue samples. The expression levels of EpCAM and COX-2 were also detected in two breast cancer cell lines. Western blot analysis demonstrated that EpCAM and COX-2 protein expression levels varied between these two cell lines. The expression of EpCAM and COX-2 appeared higher in MDA-MB-231 cells compared with MCF-7 cells (Fig. 2).

Regulation of COX-2 by EpCAM. To investigate whether EpCAM was involved in the regulation of COX-2 in breast cancer, MCF-7 and MDA-MB-231 breast cancer cells were transfected with EpCAM overexpression plasmid or control. The results demonstrated that overexpression of EpCAM promoted the expression of COX-2 (Fig. 3). Furthermore, MCF-7 and MDA-MB-231 cells were transfected with siEpCAM or control in order to silence the expression of EpCAM. Western blot analysis revealed that EpCAM silencing decreased the expression of COX-2 in both cell lines (Fig. 4). These results suggested that EpCAM may be involved in the regulation of COX-2 expression.

Discussion

EpCAM expression is frequently increased in breast cancer. It has previously been demonstrated that EpCAM may be involved in breast cancer cell growth and metastasis (11). The present study revealed a positive correlation between the expression of EpCAM and COX-2 in breast cancer tissue samples. High EpCAM and high COX-2 expression were more commonly detected in poorly differentiated tumors compared with well and moderately differentiated tumors. The correlation between EpCAM and COX-2 expression was also observed in breast cancer cell lines. These results suggested that EpCAM and COX-2 may have underlying biological connections in breast cancer.

Table I. Correlation between EpCAM and COX-2 expression in breast cancer.

	EpCAM			
	Low	High	r	P-value
COX-2				
Low	19	18	0.63	0.009
High	5	92		

EpCAM, epithelial cell adhesion molecule; COX, cyclooxygenase.

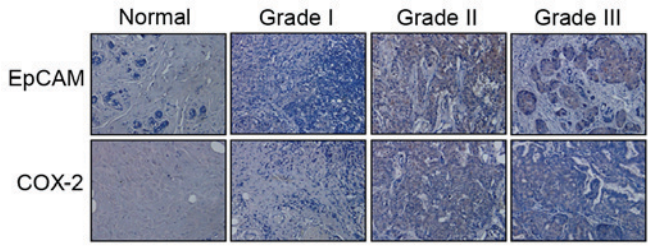

Figure 1. Expression of EpCAM and COX-2 in breast cancer. Immunohistochemical staining was performed using anti-EpCAM and anti-COX-2 antibodies. Representative photomicrographs of immunohistochemical staining for EpCAM and COX-2 in breast cancer samples of different grades are presented. EpCAM, epithelial cell adhesion molecule; COX, cyclooxygenase (original magnification, x100).

The results of the present study, combined with results from our previous preclinical studies (10,19), suggested a potential for EpCAM as a therapeutic target in breast cancer. The regulatory role of EpCAM in gene expression has previously been reported, including MMP-9 expression (20), thus suggesting that inhibition of the regulatory functions of EpCAM may suppress various tumor cell processes that drive carcinogenesis. In addition, EpCAM has previously been suggested as a potential protein marker for cells undergoing enhanced EMT or for cancer cells with aggressive phenotypes (11,21), and the transcription factor activator protein 1 has been reported to be involved in the transcriptional activation of the EpCAM gene (22). Furthermore, important roles for EpCAM have been suggested in the promotion of tumorigenic or metastatic behavior of breast cancer cells. Specifically, EpCAM was demonstrated to serve a role in mediating the effects of epidermal growth factor in human ovarian cancer cell migration (23) and was associated with prostate cancer metastasis via the phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt/mechanistic target of rapamycin signaling pathway (24). High EpCAM expression was also associated with gastric cancer cell proliferation and disease progression (25).

COX-2 has previously been used as a prognostic factor for malignancy and has been associated with carcinogenesis. The COX-2 pathway has been implicated in various processes associated with tumor progression, such as angiogenesis,

Table II. Relationship between the expression of EpCAM and COX-2 and clinicopathological parameters of breast cancer.

	EpCAM			COX-2		
Characteristic	Low	High	P-value	Low	High	P-value
Age (years)						0.278
≤50	14	76	0.324	21	62	
>50	10	34		16	35	
Histological grade						0.014
G1	13	13	0.024	18	6	
G2	8	33		12	32	
G3	3	64		7	59	
Lymph node status						0.428
Negative	14	42	0.358	20	38	
Positive	10	68		17	59	
AJCC stage						0.019
I	11	19	0.015	5	25	
II	9	63		12	60	
III	4	28		7	25	
ER						0.001
Negative	18	24	0.008	19	15	
Positive	6	86		18	82	
PR						0.015
Negative	17	42	0.025	23	23	
Positive	7	68		14	74	
HER2						0.591
Negative	14	35	0.035	17	49	
Positive	10	75		20	48	
p53						0.628
Negative	12	62	0.349	20	45	
Positive	12	48		17	52	
Ki-67						0.024
Negative	18	28	0.019	26	15	
Positive	6	82		11	82	
Total	24	110		37	97	

EpCAM, epithelial cell adhesion molecule; COX, cyclooxygenase; AJCC, American Joint Committee on Cancer; ER, estrogen receptor; PR, progesterone receptor; HER, human epidermal growth factor receptor.

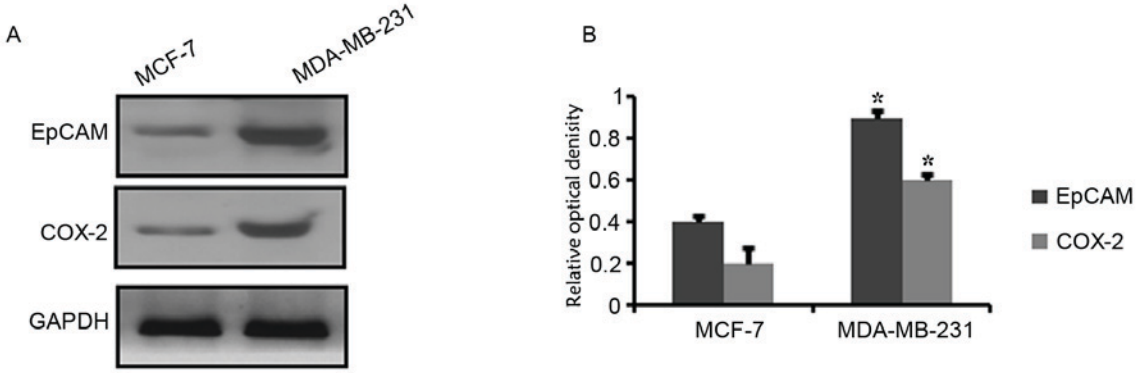

Figure 2. Expression of EpCAM and COX-2 in breast cancer cell lines. (A) Representative western blots of EpCAM and COX-2 expression in MCF-7 and MDA-MB-231 cells. GAPDH was used as a loading control. (B) EpCAM and COX-2 protein expression levels were quantified relative to GAPDH and expressed as the mean ± standard error of the mean. *P<0.05 vs. MCF7 for each gene. EpCAM, epithelial cell adhesion molecule; COX, cyclooxygenase.

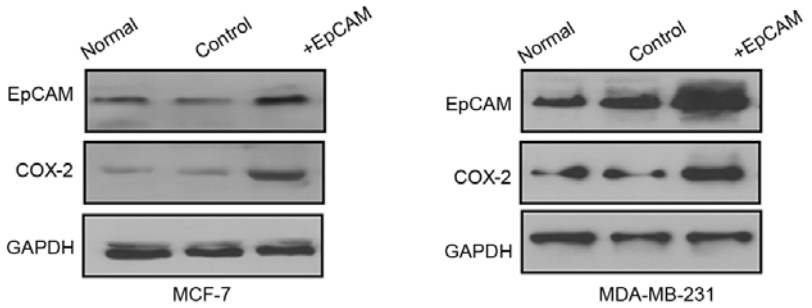

Figure 3. Following transfection with EpCAM plasmid or control for 48 h, western blot analysis was used to detect EpCAM and COX-2 expression in MCF-7 and MDA-MB-231 cells. EpCAM, epithelial cell adhesion molecule; COX, cyclooxygenase.

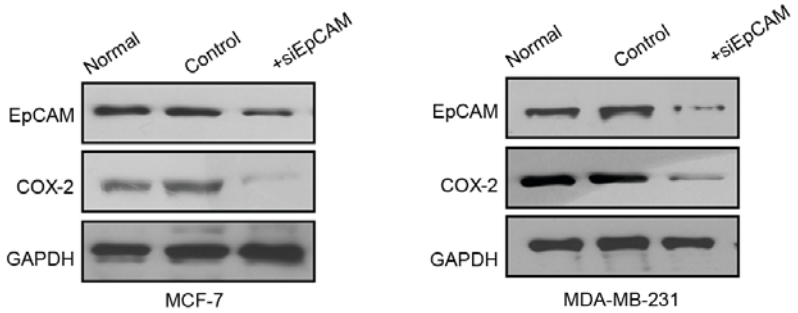

Figure 4. Following transfection with siEpCAM or control for 48 h, western blot analysis was used to detect EpCAM and COX-2 expression in MCF-7 and MDA-MB-231 cells. si, small interfering; EpCAM, epithelial cell adhesion molecule; COX, cyclooxygenase.

proliferation and invasion (26). Therefore, it may be hypothesized that COX-2 has potential as a prognostic biomarker for breast cancer. Previous studies have reported that COX-2 was upregulated and associated with tumor invasiveness and clinical outcome in numerous types of human cancer (27-30). In the present study, a correlation was revealed between high COX-2 expression and poor differentiation status ($P<0.05$). COX-2 expression was also correlated with factors of poor prognosis, such as high Ki-67 proliferative rate and poor differentiation. In relation with the aforementioned findings regarding the involvement of EpCAM in the regulation of COX-2 expression in breast cancer cells, these results suggested that EpCAM expression may modulate COX-2 expression in human breast cancer, and that various subtypes of COX-2-positive carcinomas may respond to therapeutic strategies that target EpCAM.

In conclusion, the present study identified a positive correlation between EpCAM and COX-2 expression in breast cancer cell lines and tissue specimens. EpCAM and COX-2 were associated with the prognosis of breast cancer patients, with a high EpCAM/COX-2 ratio being indicative of poor prognosis. In addition, EpCAM was reported to potentially regulate COX-2 expression in breast cancer cells. These results demonstrated that EpCAM may serve an important role in COX-2 regulation, and suggested that the inhibition of these proteins may hold potential as a multi-target therapeutic approach for the treatment of patients with breast cancer.

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