CA IX is upregulated in $CoCl_2$-induced hypoxia and associated with cell invasive potential and a poor prognosis of breast cancer

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Received September 16, 2015; Accepted October 23, 2015

DOI: 10.3892/ijo.2015.3253

Abstract. Hypoxia, a common phenomenon during the development of malignant solid tumors including breast cancer, serves to propagate a cascade of molecular pathways triggered by hypoxia-inducible factor-1α (HIF-1α). Carbonic anhydrase IX (CA IX), one of the target genes of HIF-1α, has been reported to be involved in progression of malignant tumors. The objective of this study was to investigate the expression of HIF-1α and CA IX in hypoxia, involvement of CA IX in the regulation of migration and invasion/metastasis and its prognostic significance in breast cancer. We used cobalt chloride ($CoCl_2$) as a hypoxia-mimetic agent and found that the expression of HIF-1α protein, CA IX mRNA and protein, is effectively upregulated, except for HIF-1α mRNA. Data showed that the elevated CA IX expression is closely related to *in vitro* cell migration and invasion, and the underlying mechanism of this process may be associated with epithelial-mesenchymal transition (EMT). The study of clinical tissue samples also demonstrated that CA IX is an independent prognostic marker that may serve as a useful clinical biomarker for predicting tumor progression and the invasion/metastasis of breast cancer. These results provide further insight into the role of CA IX in tumor progression and put forward further strong evidence as well as new consideration for CA IX target therapy.

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Key words: carbonic anhydrase IX, breast cancer, hypoxia, invasion/metastasis, prognosis

Introduction

Breast cancer is one of the most common causes of cancer incidence worldwide and is also the second leading cause of cancer death among women after lung cancer in the United States alone (1). Therapeutic failure and death for breast cancer patients are mainly caused from invasiveness and metastasis (2,3). Hypoxia is one of the most common conditions encountered within the tumor microenvironment that drives cancer development (4,5). Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that can be activated by hypoxia. This basic-helix-loop-helix-PAS heterodimer composed of a HIF-1α subunit and a HIF-1β subunit is overexpressed in many neoplastic tissues and functions as a key regulator of the adaptive responses to the hypoxic microenvironment (4). HIF-1α is stabilized during periods of hypoxia resulting in its translocation to the nucleus where HIF-1α heterodimerizes with HIF-1β (6,7), and then triggers the expression of an array of adaptive genes to support tumor survival, including carbonic anhydrase IX (CA IX).

CA IX, with an extracellular active site and an NH_2-terminal proteoglycan-like region (8), is a transmembrane glycoprotein belonging to a large family of zinc metallic-enzymes that catalyze the rapid reversible hydration of carbon dioxide (CO_2) to bicarbonate and protons (9-11). CA IX is observed in very restricted normal tissues (9,12), but overexpressed in many solid tumors, including breast cancer, which may be attributed to the hypoxic microenvironments caused by oxygen deprivation as a result of the uncontrollable proliferation of cancer cells relative to the inadequate amount of available oxygen supplied by the blood vessels (13,14). CA IX has drawn great research concerns because it plays an important role in the regulation of intracellular pH favoring tumor cell growth and survival (8,15), and also contributes to cancer development by increasing cancer cell motility, invasiveness and metastasis (16,17). Mechanism involved in the influences of CA IX on such diverse biological events is very complicated and still remains unclear, making further mechanism validation necessary (9).

There are two commonly used methods which enable *in vitro* cell assays under hypoxia. One is to induce gas at specific concentration into an air-tight chamber, and the other is to biochemically make a hypoxic condition within the

cell (18). The latter approach relies upon chemical treatments to induce signaling events associated with hypoxia. A number of chemicals have been used as chemical inducer of hypoxia, and cobalt chloride ($CoCl_2$) is most widely used among them. The role of such inducers is to stabilize the transcription factor HIF-1α and mimic hypoxia (18,19). $CoCl_2$ not only acts as iron chelator and replaces iron by cobalt at the iron-binding center of prolyl hydroxylase to reduce the hydroxylation activity, but also blocks the von Hippel-Lindau tumor suppressor protein (pVHL) binding to the PAS domain of HIF-1α, hence, escaping degradation by a ubiquitin-proteasomal pathway (20). The mechanism of $CoCl_2$ simulating hypoxia is similar with hypoxic microenvironment *in vivo*, because they have identical signal transduction and transcription regulation (21). Therefore in this study $CoCl_2$ was used as chemical inducer of hypoxia.

In this study, we explored the effect of $CoCl_2$ induced hypoxia on the CA IX protein and mRNA expression, and the invasive potential in breast cancer cell lines. Our data indicated that elevated CA IX in hypoxia induced by $CoCl_2$ promotes breast cancer migration/invasion *in vitro*. The expression of E-cadherin, vimentin, matrix metalloproteinase 2 (MMP2) and MMP9 using western blot analysis were examined to explore the underlying mechanism. In addition, a study of 149 clinical cases demonstrated that high level of CA IX expression is associated with invasive breast cancer progression and predicts poor patient prognosis.

Materials and methods

Cell culture and hypoxia treatment. Two different breast cancer cell lines, possessing different invasion abilities, were applied in our experiment: MCF7 cell lines with low invasion abilities and MDA-MB-231 with high invasion abilities (22,23). The cell lines were obtained from the cell bank of the Chinese Academy of Sciences and were grown in DMEM (high glucose) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Cultured cells were maintained in a humidified, 5% CO_2 atmosphere at 37˚C. For hypoxia experiments, cells were treated with 200 μmol/l $CoCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) for 24, 48 and 72 h, and maintained in a humidified, 5% CO_2 atmosphere at 37˚C (12).

Western blot analysis. Total protein was extracted from cells and fresh tissues using RIPA lysis buffer. For western blot analyses, equal amounts of protein from different samples were electrophoresed, transferred to PVDF membranes (Millipore, Bedford, MA, USA), blocked in 5% non-fat milk for 2 h, and incubated overnight with antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), CA IX (Abcam, Cambridge, UK), HIF-1α (Novus Biologicals, Littleton, CO, USA), MMP2, MMP9 (Sigma-Aldrich), E-cadherin, vimentin (Cell Signaling Technology, Beverly, MA, USA), respectively. Then membranes were incubated for 1 h at room temperature with the appropriate HRP-conjugated secondary antibodies (ProteinTech Group, Chicago, IL, USA). Detection by the LAS-3000 Luminescent Imaging Analyzer (Li-Cor Biosciences, Lincoln, NE, USA) was performed according to the manufacturer's instructions (Fuji Photo Film Co., Ltd., Tokyo, Japan). GAPDH was used as a loading control.

Quantitative RT-PCR (RT-qPCR) analysis. TRIzol® reagent (Invitrogen, San Diego, CA, USA) was used to extract total RNA from cells following the manufacturer's instructions. RT-PCR was performed using the one-step qRT-PCR kit (Takara, Otsu, Japan). RT-qPCR was carried out in 20 ml solution with 1 mg cDNA and 1 mmol/l of each forward and reverse primer and 2X SYBR-Green mix (Takara). GAPDH was used for relative quantification. The primers used in the real-time PCR reactions were designed based on information from the human genomic database. The following primers were used for the specific amplification of GAPDH, CA IX, and HIF-1α: GAPDH forward, 5'-CAT CAA GAA GGT GGT GAA GC-3' and reverse primer, 5'-GGA AAT TGT GAG GGA GAT GC-3'; CA IX forward, 5'-TGG AGA ATG TGA GAA GCC A-3' and reverse primer, 5'-ATA ATG AGC AGG ACA GGA C-3'; HIF-1α forward, 5'-ATG CTT ACA CAC AGA AAT G-3' and reverse primer, 5'-ACT GAG GTT GGT TAC TGT T-3'. A $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative mRNA expression level for each target gene.

Cell migration and invasion assays. For migration assay, Boyden dual chamber assay was performed using Transwell chambers with 8 μm pore size membranes (Corning Costar, Cambridge, MA, USA). A total of $5x10^4$ cells were suspended in serum-free media and added to the upper chamber, media containing 20% FBS were used as chemoattractant in the lower chamber. After 48-72 h incubation at 37˚C with 5% CO_2, the media and cells remaining in the upper chamber were removed using a cotton bud. The insert was fixed in methanol and detected by crystal violet staining. The cells attached to the lower side of the insert were photographed using an inverted microscope (Nikon, Tokyo, Japan). The number of migrating cells was counted in five random fields with 10x20 power and calculating the mean number of migrating cells. All experiments were performed in triplicate. The invasive capability of cells was assessed by using Boyden dual chamber assay as described previously with some modifications. The Transwell chamber membranes were coated with 40 μl of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) for 3 h at 37˚C, and the assays were subsequently performed similarly to those of the cell migration assays.

Patients and tissue samples. All tissues were obtained from Huashan Hospital of Fudan University (Shanghai, China). The study was approved by the ethics committee of Huashan Hospital, Fudan University (Shanghai, China), and all patients provided written informed consent. Eleven pairs of fresh breast cancer and normal breast tissues were obtained in 2012, and specimens were instantly acquired in liquid nitrogen and stored at -80˚C until analyzed. Paraffin-embedded tissue samples from 149 primary breast cancer patients (mean age, 46.36 years; range, 24-84 years) between January 1999 and December 2002 were collected. None of the 149 invasive breast cancer patients received chemotherapy or radiation therapy prior to surgery. After successful radical mastectomy, all 149 patients received chemotherapy of cyclophosphamide, methotrexate and 5-fluorouracil (CMF). All patients were followed after surgery (until December 31, 2008), and detailed and complete clinicopathological data were collected on each patient. The clinical tumor lymph node metastasis (TNM) stage

of each cancer was based on the World Health Organization guidelines (24) and the histological grade was classified according to Scarff-Bloom-Richardson grading (25). The follow-up time ranged from 2.6 months to 119.6 months, with a median time of 83.1 months. At the end of the follow-up period, 123 patients were still alive and 26 had died of the disease. The overall survival rates were calculated from the date of resection to the follow-up deadline or date of mortality.

Immunohistochemistry. Specimens of tumor tissue were fixed in 10% formalin and embedded in paraffin wax. Three-micrometer sections were then cut from the paraffin blocks for immunohistochemical (IHC) analysis. Slides were dehydrated in xylene and graded alcohols. Antigen retrieval was performed with 0.01 mol/l citrate buffer at pH 6.0 at 95˚C for 10 min. Then slides were incubated with a diluted CA IX mouse monoclonal antibody in 1:200 dilution for 12 h followed by incubations with biotinylated secondary antibody for 1 h, and diaminobenzidine (Dako Corp., Glostrup, Denmark) for 10 min. Slides were again counterstained with Mayer's hematoxylin. The saturation and intensity of the immunostained cells were evaluated over three visual fields, at a power of x200 under a light microscope (Carl Zeiss, Oberkochen, Germany). CA IX immunoreactivity was detected mainly in the cytomembrane. For the statistical analysis, with reference to a previous study, only tumors showing a strong membranous staining in ≥10% or more cells were considered positive for CA IX (26). An immunoglobulin-negative control was used to rule out non-specific binding. These procedures were performed by two independent investigators and one pathologist who were blinded to the model/treatment type for the series of specimens.

Statistical analysis. Statistics were calculated by SPSS software. All experiments were repeated at least three times and the results are presented with mean ± standard errors (SEM). ANOVA and the Student's t-test were used to determine statistically significant differences between experimental groups. The Kaplan-Meier method was used to calculate the overall survival rate, and the prognostic significance was evaluated by the log-rank test. The correlation of CA IX immunoreactivity with the patients' clinicopathological variables was analyzed using Chi-square test and Fisher's exact test. Differences were considered significant at $P<0.05$.

Results

Effect of $CoCl_2$-induced hypoxia on protein and mRNA expression of HIF-1α and CA IX. Western blot analysis was performed to examine the expression of HIF-1α and CA IX under normoxia and $CoCl_2$-induced hypoxia at 24, 48 and 72 h in MDA-MB-231 and MCF7 cell lines (Fig. 1A). HIF-1α protein expression was induced in both cell lines after treatment with 200 μmol/l $CoCl_2$ for 24, 48 and 72 h. The HIF-1α expression level was increased by 156, 110 and 86% in MDA-MB-231, and 272, 173 and 113% in MCF7, compared to control cells at 24, 48 and 72 h, respectively. CA IX protein expression was significantly increased after $CoCl_2$-induced hypoxia treatment for 72 h, but not for 24 and 48 h. After cultured in $CoCl_2$ for 72 h, the CA IX expression level in MDA-MB-231 and MCF7 showed an increase of 85 and 36%. Subsequently, we measured the mRNA expression of HIF-1α and CA IX with RT-qPCR to further investigate expression of HIF-1α and CA IX at the mRNA level in $CoCl_2$-induced hypoxia (Fig. 1B). After the hypoxic treatment by $CoCl_2$ in MDA-MB-231 and MCF7 cells, we detected remarkable increase of the CA IX mRNA expression level at 24, 48 and 72 h. However, the HIF-1α mRNA expression was reduced by hypoxic stimulation in both cell lines. These results suggested that hypoxic stimulation increased both protein and mRNA expression of CA IX and the protein expression of HIF-1α, whereas HIF-1α mRNA expression was downregulated by hypoxic stimulation.

Promotion of migration and invasion by upregulated CA IX under $CoCl_2$-induced hypoxia. In order to verify whether upregulated CA IX under $CoCl_2$-induced hypoxia modulate the migration and invasion ability, Transwell assay without and with Matrigel was performed. Hypoxic MDA-MB-231 and MCF7 cells showed significant increase in migration and invasion ability compared to the normoxia groups after $CoCl_2$-induced hypoxia treatment for 72 h (Fig. 2B), while the changes of migration and invasion ability for 24 h were quite the opposite (Fig. 2A).

Change of MMP2, MMP9, E-cadherin and vimentin expression level is accompanied with promoted migration and invasion. MMP2 and MMP9 proteins have been reported to be important factors involved in promoting cancer cell migration and invasion. Cells undergoing epithelial-mesenchymal transition (EMT) show enhanced invasion. E-cadherin and vimentin proteins are principal EMT markers. MMP2 and MMP9 levels were significantly higher in cells exposed to $CoCl_2$ for 72 h than in control groups (Fig. 3). E-cadherin was not detected in MDA-MB-231 cells, neither was vimentin in MCF7. We also observed the upregulation of vimentin in MDA-MB-231 cells and decreased expression of E-cadherin in MCF7 cells after cultured with 200 μmol/l $CoCl_2$ for 72 h. On the contrary, cells exposed to $CoCl_2$ for 24 h showed the opposite results. All these changing trends were in agreement with those of CA IX protein expression. This correlation suggests that CA IX upregulation under $CoCl_2$-induced hypoxia may have a causal role in the promotion of *in vitro* cell migration and invasion in both MDA-MB-231 and MCF7 cells.

Expression of CA IX in breast cancer and normal breast tissues. To investigate the expression pattern of CA IX in human breast cancer and normal breast tissues, western blot analysis was used to detect the CA IX expression in 11 pairs of fresh breast cancer and normal breast tissues (Fig. 4). There was almost no CA IX protein that can be detected in normal breast tissues. High expression of CA IX was observed in some breast cancer tissues. Compared to normal breast tissues, several breast cancer tissues showed significantly elevated expression levels of CA IX ($P<0.001$).

Correlation of CA IX expression and poor prognosis in 149 breast cancer patients. To investigate CA IX expression in human breast cancer, we performed IHC staining on 149 breast cancer cases. CA IX immunoreactivity was readily detected in the cytomembrane (Fig. 5A). Only tumors showing a strong

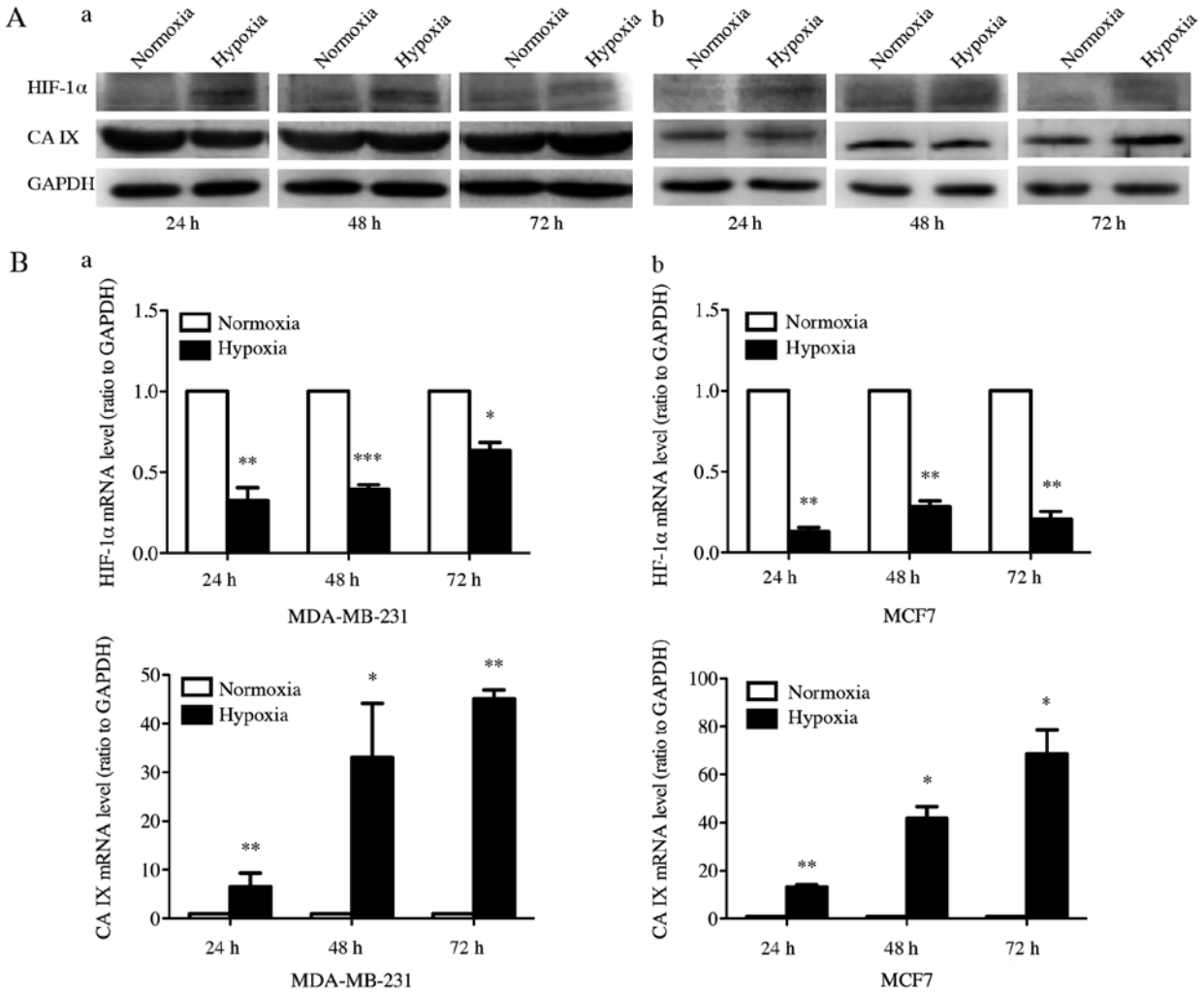

Figure 1. HIF-1α and CA IX expression in MDA-MB-231 (a) and MCF7 (b) breast cancer cells exposed to normoxia or $CoCl_2$-induced hypoxia. (A) HIF-1α and CA IX protein levels assessed by western blot analysis. GAPDH levels were shown as loading control. (B) HIF-1α and CA IX mRNA levels analyzed by RT-qPCR. GAPDH was used as the internal control. The relative gene expression is expressed as $2^{-\Delta\Delta Cq}$. Results are the average ± SD of three independent experiments in triplicate. *P<0.05, **P<0.01 and ***P<0.001.

membranous staining in ≥10% or more cells are considered positive for CA IX. CA IX was positive in 22/139 tumors (15%). Table I shows the patient clinicopathological characteristics and the correlations of CA IX expression to clinical factors. We did observe significant correlations of CA IX with tumor size (P=0.019), lymph node metastasis (P=0.006), metastatic status (P<0.001), clinical TNM stage (P=0.003) and ER status (P=0.018). However, no significant differences were identified between the expression levels of CA IX and age (P=0.614), menopause (P=0.328), histological grade (P=0.261), PR status (P=0.131) or HER-2 expression (P=0.557). The prognostic value of CA IX expression levels was evaluated in 149 breast cancer cases using Kaplan-Meier analysis and the log-rank test. Among the 149 breast cancer cases, 9/22 (41%) patients with positive CA IX expression survived, whereas 114/127 (90%) patients with negative CA IX expression survived. The overall survival rate was 83%. Among these specimens, a Kaplan-Meier survival analysis of 149 cancer specimens revealed a correlation between higher CA IX expression levels and shorter survival times (P<0.001) (Fig. 5B). Using univariate survival analysis, the clinical TNM stage (P=0.001), CA IX expression level (P=0.002) and distant metastasis (P=0.005) were found to be significantly associated with prognosis, however, no significant differences were identified between prognosis and age (P=0.582), menopause (P=0.839), chemotherapy (P=0.948), tumor size (P=0.215), lymph node metastasis (P=0.105), histological grade (P=0.667), ER expression (P=0.331), PR expression (P=0.078) or HER-2 expression (P=0.068) (Table II). Multivariate analyses were then used to determine whether CA IX expression levels were an independent prognostic predictor of clinical outcomes. The results revealed that CA IX expression (HR=5.758; 95% CI, 2.286-14.502; P<0.001) and clinical TNM stage (HR=7.256; 95% CI, 3.293-15.987; P<0.001) (Table II) showed significant prognostic effects on overall survival. These results suggested that CA IX is an independent prognostic factor for breast cancer.

Discussion

Hypoxia is a common consequence of solid tumor growth in breast cancer and other cancers, which serves to propagate a cascade of molecular pathways known as hypoxic response, including angiogenesis, glycolysis, and alterations in microen-

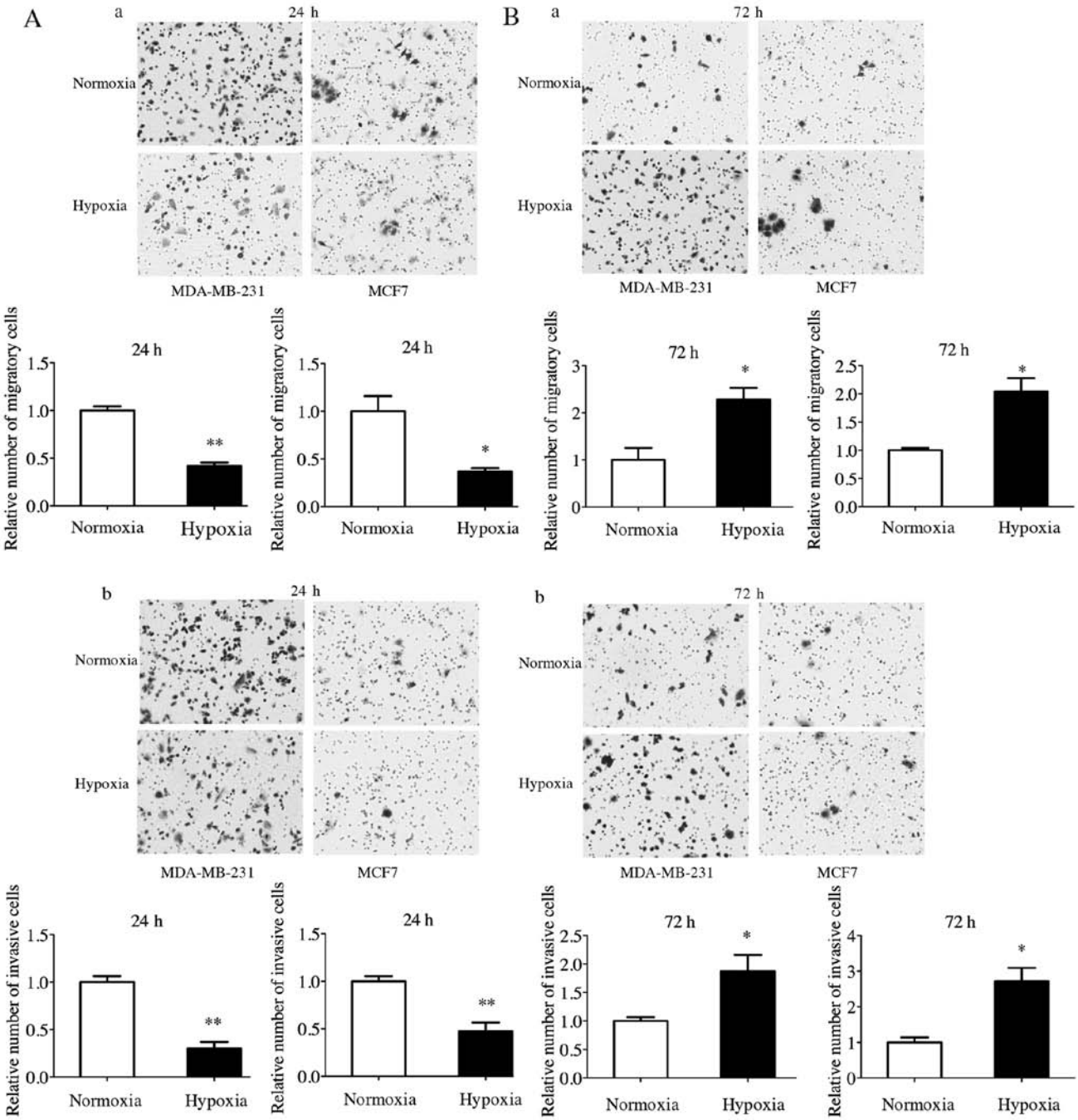

Figure 2. Promotion of cell migration and invasion of breast cancer *in vitro* by upregulated CA IX under $CoCl_2$-induced hypoxia. (A) Normoxia and hypoxia groups, after the treatment of $CoCl_2$ for 24 h. (B) Normoxia and hypoxia groups, after the treatment of $CoCl_2$ for 72 h. Migration (a) and invasion (b) were evaluated by using Transwell chamber assays. The bar graphs show the relative number of cells migrated or invaded in five fields for each experimental group as an average, corresponding to the upper figures. $^{*}P<0.05$, $^{**}P<0.01$.

vironmental pH (27). Recent experimental and clinical studies demonstrated that intra-tumor hypoxia may be a key factor in tumor microenvironment promoting invasive growth and metastasis (21).

The present study utilized $CoCl_2$ to mimic hypoxia, which provides an easily performed platform to investigate the hypoxic response. Hypoxia-inducible factors provide critical regulation of this response (27). HIF-1 is a transcription factor found in mammalian cells cultured under reduced oxygen tension, and plays an essential role in cellular and systemic homeostatic responses to hypoxic microenvironments. HIF-1α is rapidly degraded by the proteasome under normal conditions but is stabilized by hypoxic conditions (28). In the present study, $CoCl_2$-induced hypoxia caused increases in expressions of HIF-1α protein, CA IX mRNA, and CA IX protein in both breast cancer cell lines. However, in contrast to the elevation of HIF-1α protein expression after cultured in $CoCl_2$ for 24, 48 and 72 h, downregulations of HIF-1α mRNA were observed for the same periods. This phenomenon may be due to the tight regulation at the post-transcriptional level of HIF-1α by a mechanism that ensures the capacity for brisk induction and decay of this key central regulator of the hypoxia response (29).

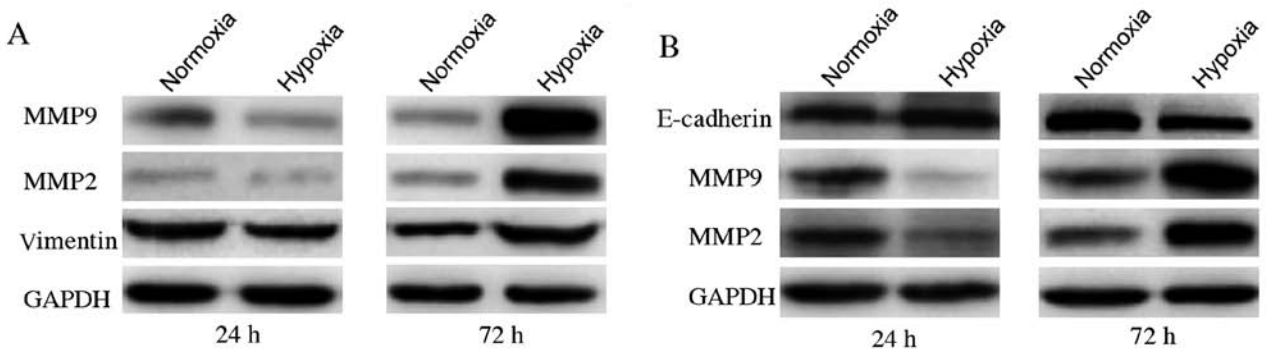

Figure 3. Expression of MMP2, MMP9, E-cadherin and vimentin in MDA-MB-231 and MCF7 breast cancer cells exposed to normoxia or $CoCl_2$-induced hypoxia. (A) MMP2, MMP9 and vimentin expression in MDA-MB-231 assessed by western blot analysis. (B) MMP2, MMP9 and E-cadherin expression in MCF7 assessed by western blot analysis. GAPDH levels were shown as loading control.

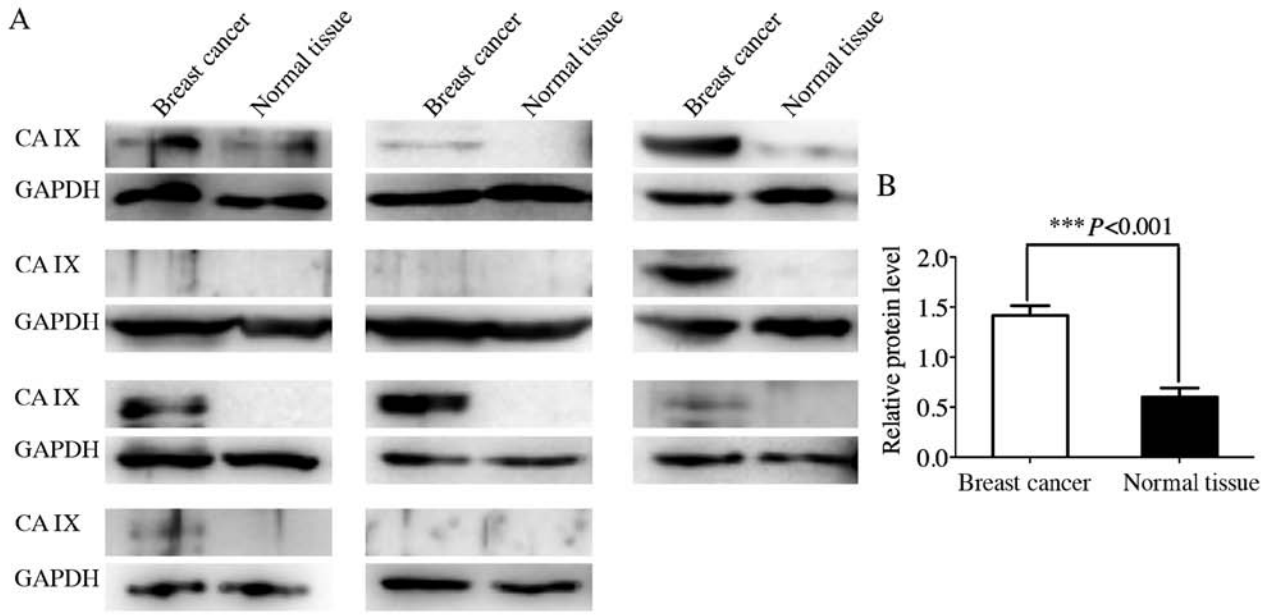

Figure 4. Expression analysis of CA IX protein in breast cancer and normal breast tissues. (A) CA IX expression assessed by western blot analysis. GAPDH levels were shown as loading control. (B) CA IX expression levels were compared between breast cancer and normal breast tissues ($^{***}P<0.001$). The bar graphs show the relative protein levels to GAPDH.

Chen *et al* (12) have reported that HIF-1α expression is responsive to proliferation-induced hypoxia in the initial stage and then modulates tumor progression in later stages. According to the western blot analysis at different incubation times with $CoCl_2$, the elevation of CA IX tends to lag behind HIF-1α elevation, which is more obvious in earlier hypoxic period. We speculate that the initial downregulated CA IX expression may be attributed to the toxicity of $CoCl_2$. Cobalt itself is cytotoxic, and $CoCl_2$ affects cell division and morphology, while in some cases, inducing mitochondrial DNA damage and apoptosis (18,21,30). The asynchronous changes between HIF-1α and CA IX expression in our study are probably because of their different half-lives (31). Previous findings have found that HIF-1α was undetectable within minutes after re-oxygenation, whereas CA IX protein was relatively stable and persisted longer than HIF-1α (12,29,32).

CA IX is a target gene of HIF-1α (33), and transcription of the CA IX gene is tightly regulated by a hypoxic-responsive element (HRE) in the 5' promoter region (4,34), which is the binding site of HIF-1. Although previous studies showed that the expression of CA IX could also be regulated by mechanisms other than HIF-1α, for instance, the AP-1 transcription factor (29), the phosphatidylinositol-3-kinase (PI3K) pathway, and the mitogen-activated protein kinase (MAPK) pathway (13), an accumulation of evidence revealed that HIF-1α is the exclusive regulator of CA IX activity, others are likely secondary to the modulation of HIF-1α. Moreover, additional transcription factor binding sites present in the CA IX promoter appear to coordinate with the HRE to promote or amplify the HIF-1 response (15). However, this point of view still remains to be elucidated in future.

We observed that the migration and invasion of MDA-MB-231 and MCF7 cells was inhibited after treatment with $CoCl_2$ for 24 h, but when the incubation time increased to 72 h, cell migration and invasion are significantly enhanced compared to control cells. The activities of migration and invasion was coinciding with the expression level of CA IX. It's well known that hypoxic state inhibits tumor cells from

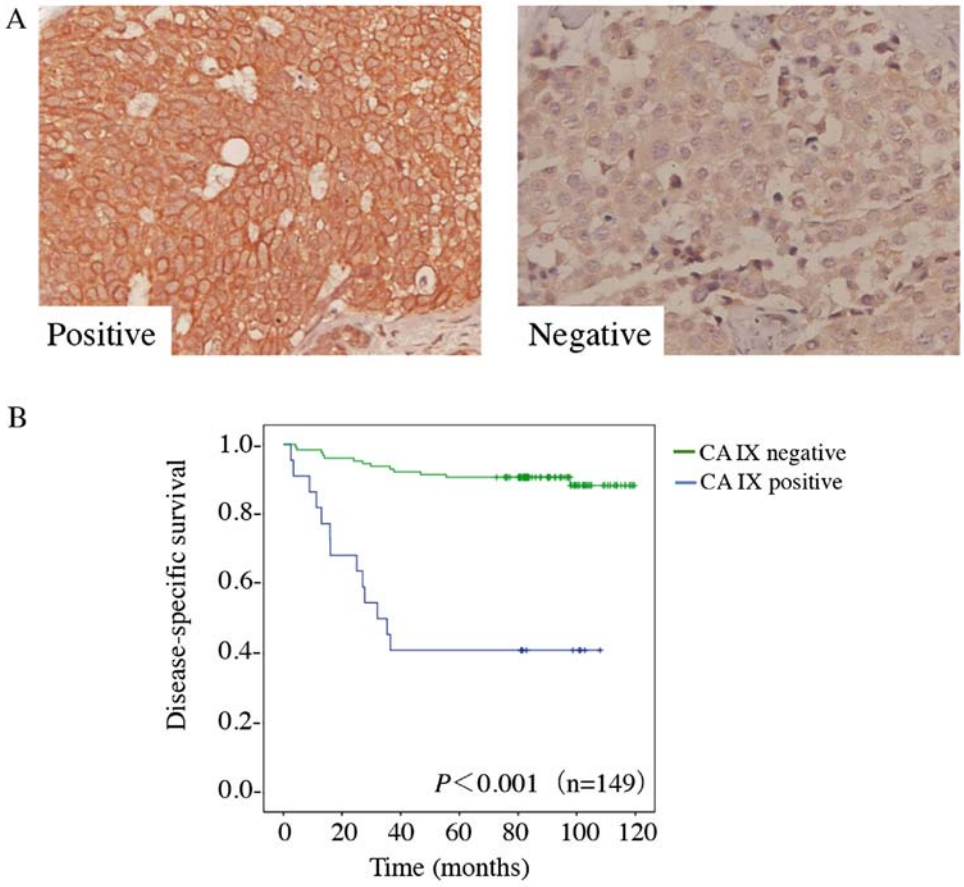

Figure 5. CA IX expression is correlated with poor survival in invasive breast cancer patients. (A) Representative immunohistochemical staining of CA IX evaluated as positive and negative is shown. All representative images were taken at x200. (B) Kaplan-Meier plot of disease-specific survival of 149 patients with breast cancer stratified by CA IX expression is demonstrated. A log-rank test showed significant differences between groups (P<0.001).

gaining energy by mitochondrial respiratory chain. In order to adapt to the hypoxic microenvironment and satisfy the demand for energy, tumor cells employ the mechanism that consumes the least oxygen, aerobic glycolysis (13), leading to increased glucose consumption, and excessive lactic acid production and secretion by highly upregulated lactate and ATP-driven proton pumps (35). This metabolic alteration results in the intracellular acidosis, and the expression of CA IX increases to prevent intracellular acidosis by catalyzing the reversible hydration of CO_2 to bicarbonate and protons with the extracellular active domain. Then the bicarbonate is shuttled into the cytoplasm to buffer intracellular pH, while the proton remains in the extracellular space, thus generating an increasingly acidic extracellular space (15). In addition, CO_2 production by CA IX also causes acidosis in the tumor environment, which may be a significant source of acidity in tumors (4,36). Moellering *et al* have hypothesized that microenvironmental acidosis leads to more aggressive invasive behavior during carcinogenesis (37). Estrella *et al* have reported that the acidity generated by the tumor microenvironment drives local invasion (38). The extracellular acidification by CA IX was suggested to be related to the induced cell growth factor, tumorigenic transformation, extracellular matrix breakdown, and protease activation in hypoxia (33), hence, facilitating tumor invasion/metastasis. In fact, the action of CA IX in hypoxic tumors extends further beyond the control of intra-tumor pH. There is evidence that CA IX also contributes to cell processes such as cell adhesion, which is vital for metastatic progression in human cancer (9,15). It was reported that by interaction with β-catenin, CA IX could reduce E-cadherin-mediated cell adhesion in MDCK cells, which is associated with tumorigenesis and invasion (11).

One of our important observations was that the hypoxia induced by $CoCl_2$ led to a significant reduction in the expression of the epithelial marker (E-cadherin) and an increase in the expression of the mesenchymal marker (vimentin), suggesting that cells with elevation of CA IX expression under hypoxia showed overt EMT, leading to the formation of more aggressive and metastatic tumors. EMT is a critical step in cancer cell migration and invasion (3). During the process, cell adhesions were weakened, cell motility was augmented, and then cell metastatic potential was enhanced. It is reasonable to assume that one mechanism by which upregulated CA IX in hypoxic microenvironment contributes to cancer progression may be through induction of EMT. In human cervical carcinoma cell line C33A, Shin *et al* showed that CA IX overexpression strongly activated the EMT process through inhibition of Rho-GTPase activity (10). They further reported CA IX-DKK1 interaction and transcriptional regulation of the β-catenin signaling pathway may be involved in the effect of CA IX overexpression on the enhanced metastatic potential (39).

The MMP family (e.g. MMP2 and MMP9) could degrade most of the components of the extracellular matrix (3). Several studies have described the presence and role of MMPs in many

Table I. Correlations of CA IX and major clinicopathological factors (n=149).

		CA IX expression		
Factors	Total	Positive	Negative	P-value[a]
Age (year)				
Mean (SD)	55.34 (11.318)	22	127	0.614
Menopause				
No	91	16	75	0.328
Yes	58	6	52	
Tumor size				
≤3	124	14	110	0.019
>3	25	8	17	
Lymph node metastasis				
0-3	131	15	116	0.006
>3	18	7	11	
Metastatic status				
No	138	15	123	<0.001
Yes	11	7	4	
Histological grade				
I-II	126	17	109	0.480
III	23	5	18	
Clinical TNM stage				
I-II	115	11	104	0.003
III-IV	34	11	23	
ER				
Negative	64	15	49	0.018
Positive	85	7	78	
PR				
Negative	83	16	67	0.131
Positive	66	6	60	
HER2				
Negative	56	10	46	0.557
Positive	93	12	81	
TNBC				
No	126	13	113	0.001
Yes	23	9	14	

[a]P<0.05 was considered statistically significant. TNBC, triple negative breast cancer.

Table II. Univariable and multivariate survival analysis of the influencing factors (n=149).

	Univariate	Multivariate		
Variable	P-value[a]	P-value[a]	HR	95% CI
Age (years)	0.582			
Menopause	0.839			
Chemotherapy	0.948			
T	0.215			
N	0.105			
Histological grading	0.667			
Clinical TNM stage	0.001	<0.001	7.256	3.293-15.987
CA IX	0.002	<0.001	5.758	2.286-14.502
ER	0.331			
PR	0.078			
HER2	0.068			
Distant metastasis	0.005	0.279	1.806	0.619-5.267

[a]P<0.05 was considered statistically significant. HR, hazard ratio; CI, confidence interval; T, tumor size; N, lymph node metastasis.

types of cancers including breast cancer and MMPs have been found to be involved in breast cancer progression and metastasis (40). Mehner *et al* showed that MMP9 is specifically needed for breast cancer invasion and pulmonary metastasis, while MMP2 knockdown can reduce cellular invasion (41). We found that in both MDA-MB-231 and MCF7 cells, $CoCl_2$ treatment for 72 h induced a significant increase in the expression of MMP2 and MMP9. Additionally, the study showed that the changing trend of MMP2/MMP9 was correlated with CA IX expression, indicating the participation of MMP2/MMP9 in the enhancement of cell migration and invasion associated with upregulated CA IX expression in hypoxia.

Here it was verified using western blot analysis that CA IX is not present in normal breast tissues, but is upregulated in invasive tumors. There was almost no CA IX protein that can be detected in normal breast tissues, while the incidence and level of CA IX in some breast cancer tissues were considerably higher (42,43). Additionally, we studied the expression of CA IX in 149 clinical primary breast cancer cases using IHC staining. In agreement with previous reports that overexpression of CA IX is an important predictor of breast cancer (31,44-46), our IHC examination of 149 breast cancer patients showed that the increase in CA IX expression is correlated with tumor size, lymph node metastasis, metastatic status and clinical TNM stage, which are known indicators of a relatively poor prognosis in breast cancer patients. Following the analysis of CA IX expression in 149 invasive breast cancers, the correlation between CA IX and patient prognosis was determined. The results showed that CA IX could potentially be used as a factor in predicting poor outcomes for invasive breast cancer patients. This result was confirmed using univariate and multivariate survival analyses, whereby high CA IX expression was found to be an independent prognostic predictor of poor survival. The data from the clinical cases were consistent with the results from the cell lines *in vitro*.

In conclusion, this study suggested that $CoCl_2$-induced hypoxia can effectively upregulate CA IX expression. We showed that the elevated CA IX expression is closely related to *in vitro* cell migration and invasion, and the underlying mechanism of this process may be associated with EMT. The study of clinical tissue samples also demonstrated that CA IX

is an independent prognostic marker that may serve as a useful clinical biomarker for predicting tumor progression and the invasion/metastasis of breast cancer. These results provide further insight into the role of CA IX in tumor development and put forward further strong evidence and aspects for CA IX target therapy.

Acknowledgements

This study was supported by grants from the National Nature Science Foundation of China (nos. 81272387, 81470857 and 81502272), and the Shanghai Natural Science Foundation (no. 134119b1100). We thank members of our laboratory for helpful discussions.

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