Pathol. Oncol. Res. (2016) 22:277–286
DOI 10.1007/s12253-015-9935-6

RESEARCH

Roles of Carbonic Anhydrase IX in Development of Pancreatic Cancer

Yuji Li[1] · **Ming Dong**[1] · **Weiwei Sheng**[1] · **Longping Huang**[1]

Received: 22 October 2014 / Accepted: 18 March 2015 / Published online: 30 July 2015

Abstract The aim of this study was to study the effects of carbonic anhydrase IX (CA IX) towards the invasion and metastasis of pancreatic cancer. The expressions of CA IX in 58 cases of pancreatic cancer and paired paracancerous normal tissues, obtained from 2005 to 2012 in the first Affiliated Hospital of China Medical University, were detected, as well as its expressions in different pancreatic cancer cell lines, aiming to detect the impacts of CA IX silencing towards the invasion and metastasis of pancreatic cancer cells. The CA IX expressions in 58 pancreatic cancer cases were higher than those in the paired paracancerous normal tissues ($P<0.01$), and positively correlated with the tumor size and the UICC staging UICC ($P<0.05$), the multivariate analysis showed that the high expression of CA IX was the independent risk factor towards the prognosis of pancreatic cancer ($P<0.05$). The CA IX was highly expressed in AxPC–1 and Miapaca–2, and the interference effects were significant. CA IX silencing could significantly inhibit the invasion and metastasis of AxPC-1 and Miapaca. We support a pro-tumor role of CA IX in the development and progression of pancreatic cancer.

Keywords Carbonic anhydrase IX · Pancreatic cancer · Invasion · Migration

✉ Ming Dong
mingdongcn@163.com

[1] Department of General Surgery, The First Affiliated Hospital, China Medical University, Shenyang 110001, China

Introduction

The pancreatic cancer was one of the malignant digestive system tumors that had the worst prognosis. In 2010, USA was estimated to add 43,140 cases of pancreatic cancer, and in the same year, 36,800 patients died of this disease, ranking the 4th place of tumor-caused death, and its 5-year survival rate was less than 5 % [1]. In the past 20 years, Chinese pancreatic cancer patients were also increased by four times [2]. The delay of tumor diagnosis timing and its malignant biological behaviors were the keys towards the poor prognosis of pancreatic cancer, and made it crucial to investigate the molecular markers that could help towards the early diagnosis and prognosis judgment of pancreatic cancer. Through detecting and intervening the target proteins, thus exploring its effects on the biological behaviors of tumors and describing the pathogenesis of cancers had become a hot spot of research in recent years.

CA was a family of zinc-containing enzymes, widely presenting inside the mammals. CA had at least 14 kinds of isozymes, among which CA IX was located at the downstream of VHL tumor suppressor gene, and could be activated by the HIF-1pathway [3], under the hypoxic conditions, it could maintain the normal pH value of tumor cells, make the tumor to adapt the hypoxic microenvironment, thus ensuring the continued proliferation of tumor cells in the hypoxic regions. It would be largely expressed in the hypoxic tumors, and related to the proliferation, adhesion and progression of tumor cells [4, 5]. Currently, it was found that its expression was significantly enhanced in a variety of tumors such as bladder cancer, cervical squamous cell carcinoma, non-small cell carcinoma and renal cell carcinoma [4, 6–9], while under the hypoxic conditions, the CA IX interference could delay the proliferation of monolayer-cultured breast cancer cells, and the cell clone survival rate would be reduced [10],

suggesting that it was closely related to the tumors' occurrence and development. As for the pancreatic cancer, Juhász only detected the expression of CA IX in the pancreatic cancer [11], while its clinicopathological parameters and impacts on the prognosis of pancreatic cancer were not reported. This study previously explored the clinicopathological significance of CAI and CAII expressions in the pancreatic cancer, and found that CAI and CAII were negatively correlated with the clinical parameters of pancreatic cancer, such as the differentiation degree, tumor size, and poor prognosis [12], while they were not the cancer-promoting factors towards the occurrence and development of pancreatic cancer, and proposed that CA IX might just be opposite to CAI and CAII. Combined with the above literatures, we believed that CA IX might be the potential cancer-promoting gene in the pancreatic cancer. In this study, the immunohistochemistry, Western blot, qRT-PCR and immunofluorescence were performed to explore its effects in the occurrence and development of pancreatic cancer.

Materials and Methods

Specimens

58 pancreatic cancer specimens resected in the First Affiliated Hospital of China Medical University from January 2005 to December 2012 were collected, among which 9 and 18 cases were the freshly resected specimens for Western blot and Real-time PCR detecting, respectively. All patients in our study did not accept postoperative therapy because of the side effect and indefinite therapeutic effectiveness of chemoradiation. The liquid nitrogen was used to store the specimens. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of China Medical University. Written informed consent was obtained from all participants.

All the specimens were pathologically confirmed as the pancreatic ductal adenocarcinoma, as shown in Table 1. The pancreatic AxPC-1, BxPC-3 and PANC-1 cell lines were purchased from Shanghai Institute of Cell Bank, Chinese Academy of Sciences, Miapaca was gifted from the biological teaching and researching department of Chinese Medical University. The cells were wall-adherently monolayer-cultured in 10 % FBS-containing RPMI-1640 medium (Gibco, USA), in which the concentrations of NaHCO3, penicillin G and streptomycin were 2 g/L, 100 U/L and 100 μg/L, respectively, at 37 °C and 5 % CO_2, the medium was changed every other day, and the cells in the logarithmic growth phase were taken for the experiment.

Table 1 Clinicopathological significance of CA IX expression in the pancreatic cancer

Parameters	No. of patients	CAIX		*P*
		Negative	Positive	
Cases	58	19	39	
Age (years)				
≤65	43	15	28	0.559
>65	15	4	11	
Gender				
Male	40	14	26	0.588
Female	18	5	13	
Tumor location				
Head	42	14	28	0.880
Body-tail	16	5	11	
Tumor size (cm)				
<2.5	19	10	9	0.024
≥2.5	39	9	30	
Differentiation				
Well	19	8	11	0.290
Moderate and poor	39	11	28	
T stage				
T1+T2	13	6	7	0.243
T3+T4	45	13	32	
Lymph nodes metastasis				
N0 (negative)	43	17	26	0.063
N1 (positive)	15	2	13	
TNM stage				
I+IIA	40	17	23	0.018
IIB+III	18	2	16	
Perineural invasion				
Absent	43	15	28	0.559
Present	15	4	11	
Vascular permeation				
Absent	31	8	23	0.227
Present	27	11	16	
Pre-therapeutic CA19-9 level				
<37 U/ml	13	3	10	0.398
≥37 U/ml	45	16	29	
Liver metastasis				
Negative	40	14	26	0.588
Positive	18	5	13	

Immunohistochemical Staining

The immunohistochemistry used the SP staining method to detect the expressions of CA IX in the pancreatic cancer tissues and paracancerous normal tissues. For each patient, serial sections (4 μm thick) were cut from paraffin blocks, mounted on acid-cleaned glass lides and heated at 60 °C. Sections were deparaffinized in graded alcohol and rehydrated, and the

endogenous peroxidase activity was inhibited by incubation with 3 % H_2O_2 in ethanol. Slides were incubated with 5 % goat serum (30 minate room temperature) to reduce non-specific background staining and then incubated with primary antibodies CA IX (Abcam, Cambridge, UK) at 1:1000 in a moist chamber overnight at 4 °C PBS replaced primary antibody for the negative contrl, whereas colonic tissues were used for the positive control as the antibody related protocol suggested. The avidin–biotin peroxidase complex (DAKO, Denmark) procedure was then performed, and peroxidase activity was detected with diaminobenzidine as substrate. Finally, sections were counter-stained with haematoxylin and cover-slipped with a synthetic mounting medium. Each slice was randomly selected five fields (200-fold) for the photographing, and two pathologists assessed the results with the double-blind method. Referring to the scoring method of Masunaga [13], each slice was randomly selected five fields under the 400-fold microscope: 1) counted 100 cells per field, and calculated the percentage of positive cells within these five fields: 0–9 % 0 point; 10 %–24 % 1 point; 25 %–49 % 2 points; 50 %–74 % 3 points; ≥75 % 4 point. 2) Staining intensity: no staining 0 point, light yellow 1 point, yellow or dark yellow 2 points, brown or dark brown 3 points. The sum of these two was used for the grading, and >2 was considered as the positive expression.

Western Blot Detection

The tissue samples (9 cases) and whole-cell lysates were homogenized in lysis buffer and the insoluble material was removed by centrifugation at 4 °C for 15 min at 12000×g. The protein concentrations of the soluble fractions were determined using the bicinchoninic acid (BCA) assay by the BCA quantification kit. The samples were all quantified as 5 μg/μl. Each lane was loaded 30–60 μg. Equal quantities of lysates were separated by 10 % sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were then electroblotted onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5 % skim milk for 2 h, the membrane was incubated with CA IX (1:1000) and GAPDH antibody (1:1000, Santa Cruz, CA, UK) overnight at 4 °C. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated anti-mouse (1:10000, Santa Cruz, CA, UK). Protein bands were detected with enhanced ECL reagents (Thermo scientific, Rockford, IL, USA) and visualized using a chemoluminescence device (MF-chemibis 3.2 DNR). We calculated the relative protein level by comparing the amount of CA IX versus GAPDH. The Quantity one software was used, and according to the areas and densities of electrophoretic bands after the ECL illumination, the gray value of each band was calculated.

Real-time PCR

Total RNA was extracted from tissue samples and PC cells with TRIZOL reagent, as described by the manufacturer (Takara Bio, Otsu, Japan). We keep RNA level of all samples at the same level via nucleotide quantification (all RNA samples were diluted to 1 μg/μl) before real-time PCR detection. cDNA was synthesized from total RNA by using the Expand Reverse Transcriptase Kit (Thermo scientific, Rockford, IL, USA). The expression of target genes was analyzed in a Light Cycler 2.0 with the Light Cycler kit (Takara). The fluorescent real-time PCR reaction system was 25 μl (SYBR green 12.5 μl, DEPC-treated water 8.5 μl, cDNA template 2 μl and 10 μM upstream and downstream primers 1 μl, respectively), the reaction conditions were: denatured at 94 °C for 5 min, 94 °C for 5 s, 60 °C for 30 s, 40 cycles. All the reactions were set the duplicates, and the DEPC water was used to replace the template cDNA as the negative control. CA IX upstream primer 5-'GAAGAAATCGCTGAGGAAGG-3', downstream primer 5'-AGGGCGGTGTAGTCAGAGAC-3'; GAPDH upstream primer 5 '-CATGAGAAGTATGACA ACAGCCT-3', downstream primer 5'-AGTCCTTCCACG ATACCAAAGT-3'. The threshold cycles (CTs) of CA IX and GAPDH obtained were subtracted by the corresponding CT of GAPDH to generate the corrected CT value. The normal tissues were set as the control, and the formula when compared with the cancer tissues: $2^{-\Delta\Delta}$ Ct=2-[(△ Ct cancer target gene-△ Ct cancer reference gene)-(△ Ct paracancerous tissue target gene-△ Ct paracancerous tissue reference gene)].

Transient Transfection

One day before the transfection, the cells that were cultured near the confluency status were digested into the single cell suspensions by 0.25 % trypsin, then seeded into the 24-well plates with the concentration of 2×104 and 5×104/well, respectively, when the confluency degree reached 70 %–80 %, the transfection was performed. The experiment also set the blank control group, the empty vector transfection group and the plasmid transfection group; the transfection was performed according to the instructions of LipofectamineTM 2000 transfection kit (Invitrogen Corporation). 4–6 h later, transfection complexes were discarded, and the 10 % FBS-containing complete medium was used for the continuous culture, the correlated detections were performed 48 h later.

Transwell and Migration Assay

The prepared 24-well chamber, with 8 μm-pore-size semipermeable membrane, together with the autoclaved EP tube and tips, were precooled on ice, the 1 % FBS-containing RPMI1640 was precooled at 4 °C for the future use. The concentration of BD Matrigel was then adjusted as 1:10 by

1 % FBS-containing RPMI1640, mixed evenly, then 50 μl was placed into the upper layer of pre-cooled chamber, when it became evenly flat, it was then placed in the room temperature for 5 min, followed by the drying at 37 °C, 8 h later, when the Matrigel was completely solidified, it could be used. The prepared pancreatic cancer cell lines were routinely trypsin-digested, centrifuged, adjusted the density, volumized into 200 μl 1 % FBS-containing RPMI1640 culture medium and seeded into the upper layer of the chamber. The lower chamber was added 500 μl 20 % FBS-containing RPMI1640 medium, then cultured in the incubator for 48 h, the cells that did not penetrate the membranes in the upper chamber were erased by a cotton swab, then added in the preconfigured fixative (methanol: glacial acetic acid=3:1) for 30 min, dried and performed the Giemsa staining for 8 min, then the tap water was used for the gent rinsing, and dried for the photography under the microscope. Each well was randomly chosen three fields for the 200-fold photography, and the invasion cells were counted and the mean value was calculated. The cell migration experiment was carried out when the chamber was not added the matrigel, and the rest steps were the same.

MTT, Flow Cytometry and Annexin V/PI Dual-Staining Method

The pancreatic cancer cell lines were all evenly added into the 96-well plates with the density as 4–6×104/ml and the volume as 100 μl for 6–8 h culture. After the cells were wall-adherent, the AZ solution with different concentrations was added (the concentration gradients were shown in Results). 48 h later, each well was added 10 % MTT solution (5 mg/ml) and cultured at 37 °C for 4 h. The supernatant was discarded, and then added 100 μl DMSO and shock for 20 min. the quantitative ELISA test instrument was used to detect the optical density values with the experimental wavelength as 570 nm and the reference wavelength as 450 nm. Cell survival rate=(OD value of the experimental group-OD value of the zeroing well)/(OD value of the control group- OD value of the zeroing well)×100 %. The AZ-treated (100 μM) cells (1–5×106/ml) were collected 48 h later, and placed in a centrifuge tube, FITC and PI were added, respectively, and incubated for 15 min in the darkness, then the flow cytometry was performed for the detection. Through the image analysis, the apoptotic cells in the lower right quadrant were used for the apoptosis rate calculation. The experiments were repeated three times.

Statistical Analysis

All the statistical processing used SPSS17.0. The results were expressed as mean±standard error, the intergroup comparison used the *t* test, the multi-group comparison used the analysis of variance, the pairwise comparison used the LSD method, with $P<0.05$ considered as the statistical significance.

Results

Expressions of CA IX in Pancreatic Cancer and Paracancerous Tissues

The IHC results showed that: the expressions of CA IX in 58 cases of pancreatic cancer tissues were significantly higher than those in the paracancerous tissues (t=2.872; $P=0.006$). CA IX was mainly expressed in the cytoplasm and cell membrane of pancreatic tissues, and only expressed in the pancreatic ductal epithelial cells of normal pancreatic tissues, while not in the acinar and islet cells (Fig. 1). The Western blot results showed that: the expressions of CA IX protein in 9 cases of pancreatic cancer were significantly higher than those in the paracancerous tissues (t=3.278; $P=0.004$). Realtime-PCR results showed that: the expressions of CA IX mRNA in 18 cases of pancreatic cancer were significantly higher than those in the paracancerous tissues (t=2.449; $P=0.025$) (Fig. 2).

Clinicopathological Significance of CA IX Expression in the Pancreatic Cancer and Paracancerous Tissues

In the pancreatic cancer, the high expression of CA IX was positively correlated with the tumor size ($X^2=5.066$; $P=0.024$) and UICC staging ($X^2=5.553$; $P=0.018$), while exhibited no correlation with the patient's age, sex, tumor location, differentiation degree, lymph node metastasis, perineural invasion and preoperative CA19-9 level (Table 1). The univariate analysis found that the patients with high CA IX expressions had poor prognosis ($P=0.016$), meanwhile, the tumor UICC staging ($P=0.005$), lymph node metastasis ($P=0.005$), vascular invasion ($P=0.044$) and postoperative liver metastasis ($P=0.001$) were also the factors that would affect the prognosis of pancreatic cancer. The multivariate analysis found that, the CA IX expression ($P=0.019$) and postoperative liver metastasis of pancreatic cancer ($P=0.006$) were the independent risk factors that would affect the prognosis of pancreatic cancer (Fig. 3, Table 2).

Expressions of CA IX in Different Pancreatic Cancer Cell Lines

The Western blot and qRT-PCR results showed that: the expressions of CA IX protein and mRNA were lower in PANC-1 and BxPC-3, while higher in AsPC-1 and Miapaca-2, IF also showed that the CA IX fluorescence was weaker in PANC-1 and BxPC-3, while stronger in AsPC-1 and Miapaca-2. It's mainly expressed in the cytoplasm of pancreatic cancer cells (Fig. 4). So we chose AsPC-1 and Miapaca-2 cells for the further CA IX interference experiments.

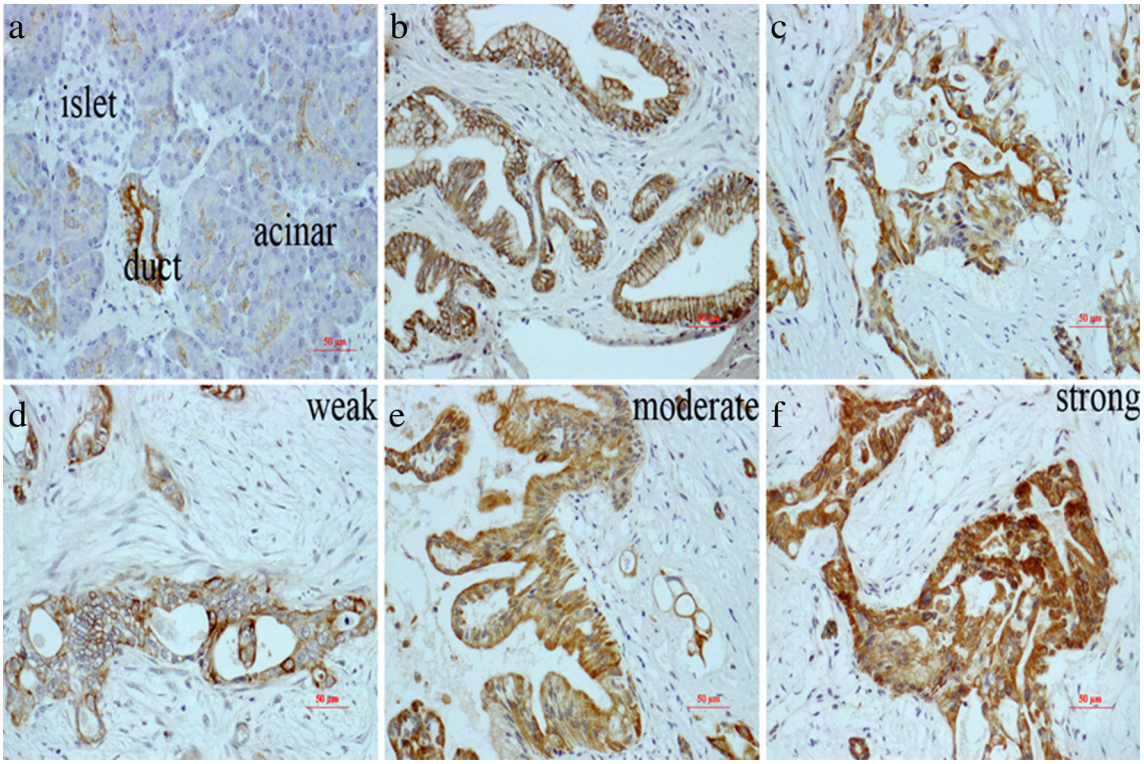

Fig. 1 Expression of CA IX in the pancreatic cancer and paracancerous tissues. a, in the normal pancreatic tissues, only expressed in the epithelial ductal cells, while not expressed in the pancreatic islets and acinar cells; b, CA IX was expressed on the membrane in the pancreatic cancer; c, CA IX was expressed in the cytoplasm in the pancreatic cancer; d, e, f the weak, medium and strong expressions of CA IX was in the pancreatic cancer

Impacts of CA IX on Invasion and Migration of Pancreatic Cancer Cells

CA IX exhibited significant interference effects in AsPC-1 and Miapaca-2 cells, the performance was that the protein and mRNA levels were significantly lower than the control group (AsPC-1: CA IX was reduced than Mock and siRNAcontrol by 77 % and 71 %, respectively; Miapaca −2: CA IX was reduced than Mock and siRNAcontrol by 81 % and 73 %, respectively). But the CA IX silencing did not affect the expressions of invasion and metastasis-related indicators (E-cad and vimentin, Fig. 5).

2.5 impacts of CA IX Silencing on Invasion and Migration of Pancreatic Cancer Cells

The CA IX silencing significantly inhibited the invasion and metastasis of AsPC-1 and Miapaca-2 cells (Fig. 6). The invasive power of AsPC-1 cells (cells that penetrated Matrigel) was, when compared with: CAIsiRNA: 31.33±3.512; siRNA control: 54.67±5.895. Metastatic power was, when compared with: CAIsiRNA: 49.33±5.132; siRNA control: 88±3. The invasive power and metastatic power of the 2 groups exhibited the significantly statistical difference. The invasive power of Miapaca-2 cells was, when compared with: CAIsiRNA: 71.67

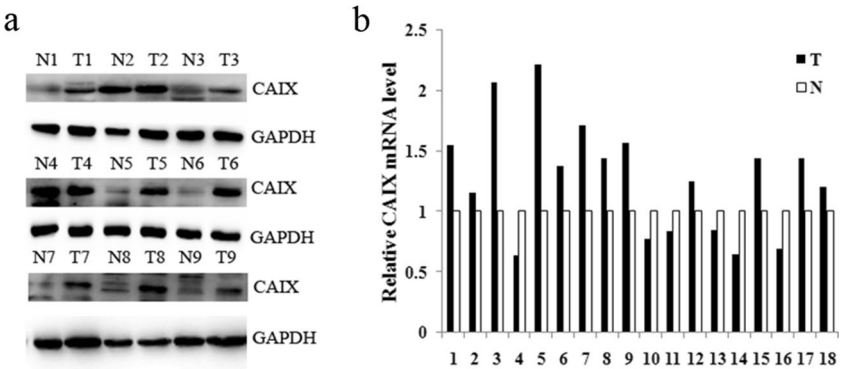

Fig. 2 Expressions of CA IX protein (**a**) and mRNA (**b**) in the pancreatic cancer and paracancerous tissues. T: pancreatic cancer; N: paracancerous normal pancreatic tissue

Table 2 Univariate and multivariate analysis of prognosis

Parameters	Median survival (days)	Univariate analysis *P* (log rank)	Multivariate analysis hazard ratio (95 % CI)	*P*
Age (<65/ ≥65 years)	468/321	0.401	–	
Gender (male/female)	421/317	0.643	–	
Tumor location (Head/Body-tail)	468/355	0.289	–	
Tumor size (<2.5/ ≥2.5 cm)	565/321	0.059	–	
well /poor and moderate differentiation	520/315	0.428	–	
Invasion depth (T1+T2/ T3+T4)	468/291	0.068	–	
Lymph nodes metastasis (N0/N1)	468/195	0.020	2.855 (0.723–11.268)	0.134
TNM stage (I+IIA /IIB+III)	468/180	0.005	0.665 (0.175–2.252)	0.549
Perineural invasion (absent/present)	520/418	0.360	–	
Vascular permeation (absent/present)	565/256	0.044	2.147 (0.965–4.529)	0.065
CA19-9 level (<37 U/ml/ ≥37 U/ml)	468/418	0.490	–	
Liver metastasis (Negative /Positive)	629/255	0.001	2.962 (1.367–6.417)	0.006
CAIX (positive/negative)	615/317	0.016	2.551 (1.170–5.562)	0.019

±6.658; siRNA control: 112.7±5.686. The metastatic power was, when compared with: CAIsiRNA: 99.33±4.041; siRNA control: 144.7±6.506, the invasive power and metastatic power of the 2 groups exhibited the significantly statistical difference.

Discussion

The survival rate of pancreatic cancer was low, and the survival time was short after the diagnosis. The main reason was that the peripancreatic tissues and/or vessels invasion and distant metastasis would appear earlier [14]. How to effectively reduce the invasion and metastasis of pancreatic cancer was the key to improve the therapeutic effects. The related researches about inhibiting the metastasis of pancreatic cancer cells had also become a hot spot of comprehensive treatments towards the pancreatic cancer. So, whether CA IX could be used as the target gene and applied into the treatment of pancreatic cancer?

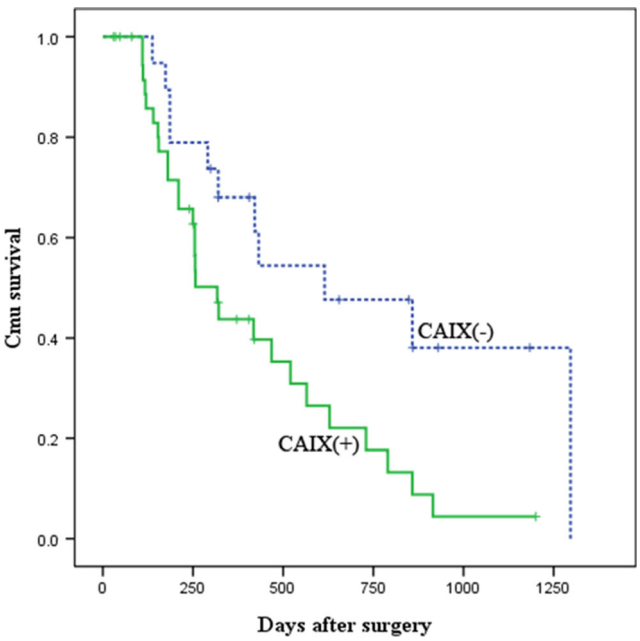

Fig. 3 Relationships of CA IX expression and prognosis of patients with pancreatic cancer. (+) positive expression; (−) Negative expression

CA IX was originally identified as a tumor-associated molecule. Its expression was recently reported in several normal human digestive tissues, but rarely reported in cancers [15]. Interestingly, as a membrane binding protein, CA IX had both membranous and cytoplasmic staining in pancreatic cancer tissues. As the previous study showed [11], CA IX exhibit was not only confined to the plasma membrane of pancreatic cancer, carcinoma cells with increased CA IX expression exhibit both membranous and cytoplasmic. In addition, stronger basolateral membranous and cytoplasmic immunoreaction for CA IX was also found in the hyperplastic epithelium of pancreas [15]. Meanwhile, Kivela also showed that the cytoplasmic staining intensity for CA IX was clearly higher in adenomas and carcinomas (more so in the superficial part) compared to the normal mucosa and hyperplastic lesions [16]. Moreover, the cytoplasmatic positivity of CA IX in pancreatic cancer cell lines strongly in our study verified that CA IX really had a specific membranous and cytoplasmatic expression in both pancreatic cancers.

In various tumor tissues, such as kidney cancer, bladder cancer, lung cancer and colorectal cancer, etc., CA IX expression would be significantly increased [7–9, 17]. Juhász [11] found through the early IHC detection that: there was no significant difference in the expressions of CA IX in the

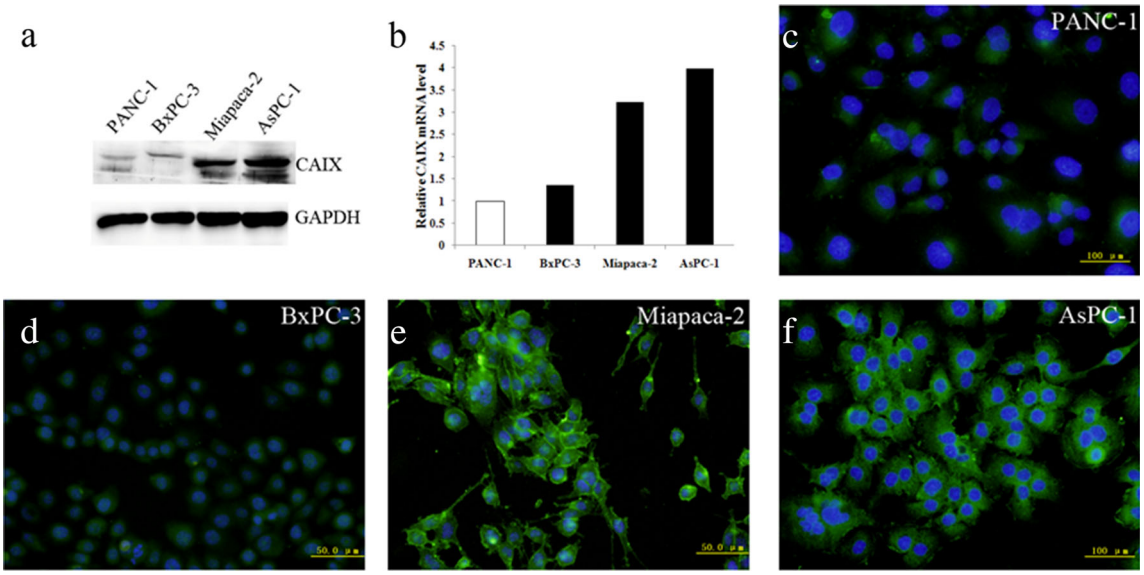

Fig. 4 Expression and localization of CA IX in the pancreatic cancer cells

pancreatic cancer and paracancerous tissues, while our IHC results showed that: the expression of CA IX in the pancreatic cancer was significantly higher than that in the paracancerous normal tissues. This might be related with the differences of reagents, experimental methods and scoring methods. Meanwhile, Juhász randomly selected four pairs of pancreatic cancer and paracancerous tissues, and through Western blot, he found that the expressions of CA IX showed no difference in the pancreatic cancer and paracancerous tissues. But when we expanded the sample size to 9 cases for Western blot and 18 cases for Real-time PCR, we further verified that CA IX expression in the pancreatic cancer was significantly higher than that in the paracancerous normal tissues. CA IX was highly expressed in the pancreatic cancer suggested that it might be involved in the malignancy process of pancreatic cancer.

Meanwhile, the chi-square test showed that: the CA IX expression was positively correlated with the tumor size and UICC staging. In a variety of tumor tissues, the reports about the relationships of CA IX and the clinicopathological parameters were not consistent. For example, in the ovarian cancer, Choschzick et al. [18] found that the overexpression of CA IX was not significantly correlated with the tumor staging and histological grading ($P<0.05$). Similarly, Juhász et al. [11] found that CA IX was not related with the tumor size, staging, differentiation degree and distant metastasis of pancreatic cancer. In breast cancer, Schutze et al. [19] examined the expressions of 169 cases of breast cancer, and found that the high expression of CA IX was positively correlated with the age (</≥50 year; $P=0.040$) and tumor-positive nodes ($P=0.001$); in China, Li et al. [20] performed IHC to detect the CA IX expressions in 117 cases of breast cancer, and found its positive correlation with the tumor size and histological grading. Ba et al. [21] further confirmed that: the CA IX expression was closely related to the lymph node metastasis ($P=0.015$) and clinical staging ($P=0.018$); but not related with the age ($P=0.375$), tumor size ($P=0.288$) and tumor pathology grading ($P=0.526$). In bladder cancer, the high CA IX expression was positively correlated with the tumor T stage (Ta vs T1-T4; $P<0.001$), histologic grading (low vs high; $P<0.001$) and distant metastasis ($P=0.032$) [7]. Because there was controversy about the clinical parameters of CA IX in various cancer studies, there still needed a large-sample experiment to much more objectively evaluate the relationships between the CA IX expression and the pathological parameters in future to further clarify whether it would affect the malignancy process of tumors.

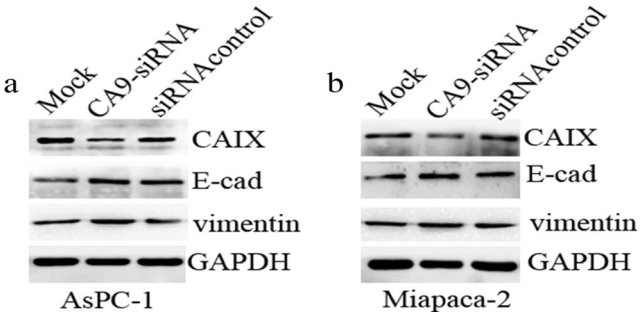

Fig. 5 The silencing effects of CA IX in AsPC-1 (**a**) and Miapaca-2 (**b**) and its influence towards the expression of cell invasion and metastasis-related indicators (E-cad and vimentin)

In most tumors, CA IX was closely associated with the poor prognosis of cancer patients. As in the non-small cell carcinoma, Kim et al. [11] studied the postoperative resected specimens of early non-small cell carcinoma patients and found: CA IX was related with the shorter disease-free survival period, and was an independent factor of poor prognosis.

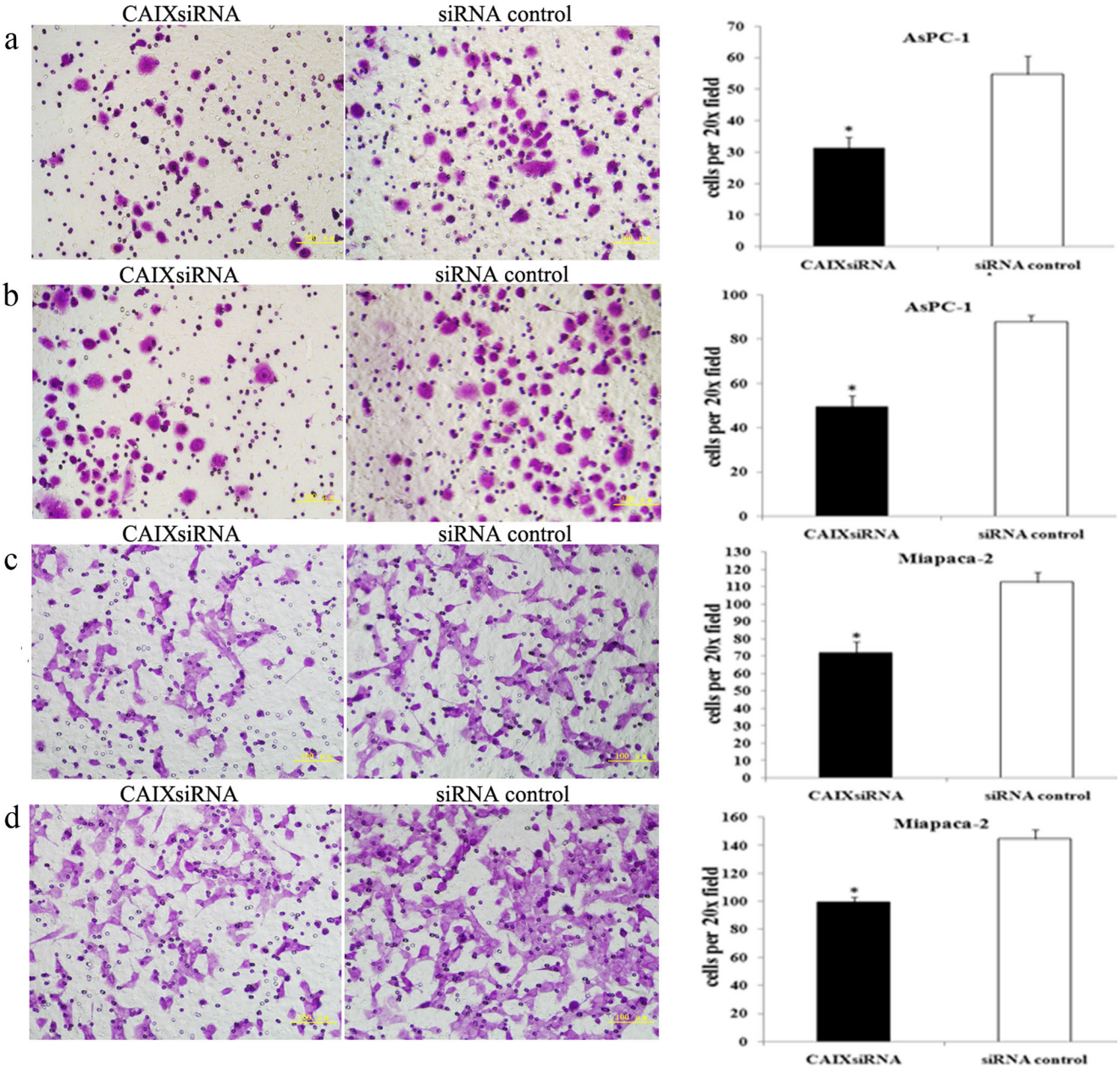

Fig. 6 Impacts of CA IX silencing on the invasion and metastasis of pancreatic cancer cells. The CA IX silencing could significantly inhibit the invasion of (**a**) and metastasis (**b**) of AsPC-1; the CA IX silencing could significantly inhibit the invasion of (**a**) and metastasis (**b**) of Miapaca-2 cells

Simi et al. [22] analyzed the relations of CA IX expressions with the prognosis of different pathological types of non-small cell carcinoma, the results displayed that the high expression of CA IX mRNA could predict the poor clinical consequences (P=0.001) and shorter disease-free survival period (P=0.004); the multivariate research also displayed that the CA IX expression was an independent risk factor towards NSCLC (P<0.001). Also, in the ovarian cancer and oral squamous cell carcinoma, the univariate and multivariate analysis showed that: CA IX was an independent risk factor towards the cancer prognosis [16, 23]. It was also found in the kidney cancer that the 5-year disease-free survival rate of the patients with positive expression of CA IX was significantly lower than the negative group, and the difference was statistically significant [9]. One invasive bladder cancer study found that the higher CA IX expression was an independent predictor of reduced overall survival [7]. The study of tongue squamous cell carcinoma found that the high expression of CA IX was associated with the poorer overall survival rate [24]. Our study also showed that: the CA IX expression was an independent risk factor towards the prognosis of pancreatic cancer. Combined with the high expression of CA IX in the pancreatic cancer, and positively correlated

with the tumor size and UICC staging, we hypothesized that the high expression of CA IX promoted the malignant progression of pancreatic cancer.

We selected AsPC-1 and Miapaca-2 that had the high expression of CA IX for the experiments, the results showed that the CA IX silencing significantly inhibited the invasion and metastasis of AsPC-1 and Miapaca-2 pancreatic cancer cell lines. This conclusion further validated the conclusions of tissue specimens, indicating CA IX might possibly impact the malignant progress of pancreatic cancer (UICC staging) through promoting the invasion and migration of pancreatic cancer, thus ultimately promoted the occurrence of pancreatic cancer, as well as resulted in the poor prognosis of pancreatic cancer patients.

It had been confirmed that CA IX played the role of oncogene in a variety of tumors. In the cervical cancer, the CA IX overexpression inhibited the cells-cell adhesion, promoted the cell-matrix adhesion, dissociation of adhesive plaques and cellular metastatic power [25]; while in the breast cancer, the CA IX silencing could significantly inhibit the in situ tumor formation of breast cancer cells in 4 T1 mouse and lung metastasis thus caused (whether in the spontaneous in situ tumor formation or the established metastasis model) [26]. So, what was the mechanism that mediated the regulation of CA IX towards the invasion and metastasis of pancreatic cancer? Studies showed that the CA IX isozyme CAII achieved the promotion towards the invasion and metastasis of pancreatic cancer through affecting the epithelial cell-matrix transition (EMT) [12]. Therefore, we speculated that whether CA IX could regulate the EMT occurrence of pancreatic cancer, thus ultimately affected the invasion and metastasis of pancreatic cancer?

It was known that the occurrence of EMT was accompanied by the expression changes of multiple genes, such as the downregulation of epithelial phenotype E- cadherin protein (E-cad) and cytokeratin, as well as the expression upregulation of mesenchymal phenotype proteins such as the waveform protein Vimentin, N-caderin (N-cad) and fibronectin. Presently, certain study confirmed: the CA IX silencing or CA IX inhibitors' interference could significantly inhibit the fibroblast-induced enhanced invasiveness of prostate cancer cells and EMT occurrence [27].

To test this idea, we examined whether the CA IX silencing could firstly affect the expressions of EMT marking proteins, namely E-cad and vimentin. However, we found that in the AsPC-1 and BxPC-3 cells, the CA IX silencing did not affect the expressions of E-cad and vimentin. So we thought that the CA IX-regulated invasion and migration of pancreatic cancer cells was independent from the EMT occurrence.

Through this experiment, it was proved that CA IX played an important role in the occurrence and development of pancreatic cancer, and as one of the factors that could affect the prognosis of pancreatic cancer, it provided a new way of thinking for improving the situation of pancreatic cancer treatment, as well as the prognosis, although its mechanism of action was not clear yet, it could be broken through the continuous explorations and researches.

Conflict of interest All authors have no conflict of interest regarding this paper.

References

1. Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics. CA Cancer J Clin 60:277–300. doi:10.3322/caac.20073
2. Philip PA, Mooney M, Jaffe D, Eckhardt G, Moore M, Meropol N, Emens L, O'Reilly E, Korc M, Ellis L, Benedetti J, Rothenberg M, Willett C, Tempero M, Lowy A, Abbruzzese J, Simeone D, Hingorani S, Berlin J, Tepper J (2009) Consensus report of the national cancer institute clinical trials planning meeting on pancreas cancer treatment. J Clin Oncol 27:5660–5669. doi:10.1200/JCO.2009.21.9022
3. Ivanov S, Liao SY, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merrill MJ, Proescholdt MA, Oldfield EH, Lee J, Zavada J, Waheed A, Sly W, Lerman MI, Stanbridge EJ (2001) Expression of hypoxia-induc-ible cell-surface transmembrane carbonic anhydrase in human cancer. Am J Pathol 158:905–919. doi:10.1016/S0002-9440(10)64038-2
4. Sundfor K, LyngH REK (1998) Tumour hypoxia and vascular density as predictors of metastasis in squamouscell carcinoma of the uterine cervix. Br J Cancer 78:822–827
5. Brizel DM, ScullySP HJM, Layfield LJ, Bean JM, Prosnitz LR, Dewhirst MW (1996) Tumor oxygenation predicts for the likelihood of distant metastases in humans of tissue sarcoma. Cancer Res 56:941–943
6. Kim SJ, Rabbani ZN, Dewhirst MW, Vujaskovic Z, Vollmer RT, Schreiber EG, Oosterwijk E, Kelley MJ (2005) Expression of HIF-1, CA IX, VEGF, and MMP-9 in surgically resected non-small cell lung cancer. Lung Cancer 49:325–335. doi:10.1016/j.lungcan.2005.03.036
7. Klatte T, Seligson DB, Rao JY, Yu H, de Martino M, Kawaoka K, Wong SG, Belldegrun AS, Pantuck AJ (2009) Carbonic anhydrase IX in bladder cancer: a diagnostic, prognostic, and the rapeutic moleeular marker. Cancer 115:1448–1458. doi:10.1002/cncr.24163
8. Rasheed S, Harris AL, Tekkis PP, Turley H, Silver A, McDonald PJ, Talbot IC, Glynne-Jones R, Northover JM, Guenther T (2009) Assessment of microvessel density and carbonic anhydrase-9 (CA-9) expression in rectal cancer. Pathol Res Pract 205:1–9. doi:10.1016/j.prp.2008.08.008
9. Choueiri TK, Regan MM, Rosenberg JE, Oh WK, Clement J, Amato AM, McDermott D, Cho DC, Atkins MB, Signoretti S (2010) Carbonic anhydrase IX and pathological features as predictors of outcome in patients with metastatic clear- cell renal cell carcinoma receiving vascular endothelia growth factor- targeted therapy. BJU Int 106:772–778. doi:10.1111/j.1464-410X.2010.09218.x
10. Robertson N, Potter C, Harris AL (2004) Role of carbonic anhydrase IX in human tumor cell growth, survival, and invasion. Cancer Res 64:6160–6165. doi:10.1158/0008-5472.CAN-03-2224
11. Juhász M, Chen J, Lendeckel U, Kellner U, Kasper HU, Tulassay Z, Pastorekova S, Malfertheiner P, Ebert MP (2003) Expression of carbonic anhydrase IX in human pancreatic cancer. Aliment Pharmacol Ther 18:837–846, **pii:1738**
12. Sheng W, Dong M, Zhou J, Li X, Dong Q (2013) Down regulation of CAII is associated with tumor differentiation and poor prognosis

in patients with pancreatic cancer. J Surg Oncol 107:536–543. doi:10.1002/jso.23282
13. Masunaga R, Kohno H, Dhar DK, Ohno S, Shibakita M, Kinugasa S, Yoshimura H, Tachibana M, Kubota H, Nagasue N (2000) Cyclooxygenase-2 expression correlates with tumor neovascularization and prognosis in human colorectal carcinoma patients. Clin Cancer Res 6:4064–4068
14. Stathis A, Moore MJ (2010) Advanced pancreatic carcinoma: current treatment and future challenges. Nat Rev Clin Oncol 7:163–172. doi:10.1038/nrclinonc.2009.236
15. Kivelä AJ, Parkkila S, Saarnio J, Karttunen TJ, Kivelä J, Parkkila AK, Pastoreková S, Pastorek J, Waheed A, Sly WS, Rajaniemi H (2000) Expression of transmembrane carbonic anhydrase isoenzymes IX and XII in normal human pancreas and pancreatic tumours. Histochem Cell Biol 114:197–204
16. Kivela AJ, Saarnio J, Karttunen TJ, Kivelä J, Parkkila AK, Pastorekova S, Pastorek J, Waheed A, Sly WS, Parkkila TS, Rajaniemi H (2001) Differential expression of cytoplasmic carbonic anhydrases, CA I and II, and membrane-associated isozymes, CA IX and XII, in normal mucosa of large intestine and in colorectal tumors. Dig Dis Sci 46:2179–2186
17. Kim SJ, Rabbani ZN, Vollmer RT, Schreiber EG, Oosterwijk E, Dewhirst MW, Vujaskovic Z, Kelley MJ (2004) Carbonic anhydrase IX in early-stage non small cell lung cancer. Clin Cancer Res 10:7925–7933. doi:10.1158/1078-0432.CCR-04-0636
18. Choschzick M, Oosterwijk E, Müller V, Woelber L, Simon R, Moch H, Tennstedt P (2011) Overexpression of carbonic anhydrase IX (CA IX) is an independent unfavorable prognostic marker in endometrioid ovarian cancer. Virchows Arch 459:193–200. doi:10.1007/s00428-011-1105-y
19. Schutze D, Milde-Langosch K, Witzel I, Rody A, Karn T, Schmidt M, Choschzick M, Jänicke F, Müller V (2013) Relevance of cellular and serum carbonic anhydrase IX in primary breast cancer. J Cancer Res Clin Oncol 139:747–754. doi:10.1007/s00432-013-1378-4
20. Li MP, Ren LF, Cai HG, Yang HY, Lu B, Zhang P, Bao L (2013) Significance of carbonic anhydrase IX protein expression in molecular subtyping of breast cancers. Zhonghua Bing Li Xue Za Zhi 42:182–185. doi:10.3760/cma.j.issn. 0529-5807.2013.03.009
21. En-ping BA, Ya-li LV, Lin LIU, Bo ZHAO (2011) Expression of carbonic anhydrase-9 correlates with malignant phenotypes of invasive breast ductal carcinoma. Chin J Clinicians (Electronic Edition) 5:4656–4660. doi:10.3877/cma.j.issn. 1674-0785.2011.16.083
22. Simi L, Venturini G, Malentacchi F, Gelmini S, Andreani M, Janni A, Pastorekova S, Supuran CT, Pazzagli M, Orlando C (2006) Quantitative analysis of carbonic anhydrase IXmRNA in human non-small cell lung cancer. Lung Cancer 52:59–66. doi:10.1016/j.lungcan.2005.11.017
23. Klimowicz AC, Bose P, Petrillo SK, Magliocco AM, Dort JC, Brockton NT (2013) The prognostic impact of a combined carbonic anhydrase IX and Ki67 signature in oral squamous cell carcinoma. Br J Cancer 109:1859–1866. doi:10.1038/bjc.2013.533
24. Kim SJ, Shin HJ, Jung KY, Baek SK, Shin BK, Choi J, Kim BS, Shin SW, Kim YH, Kim JS, Oosterwijk E (2007) Prognostic value of carbonic anhydrase IX and Ki-67 exression in squamous cell carcinoma of the tongue. J Clin Onco 37:812–819. doi:10.1093/jjco/hym121
25. Shin HJ, Rho SB, Jung DC, Han IO, Oh ES, Kim JY (2011) Carbonic anhydrase IX (CA9) modulates tumor-associated cell migration and invasion. J Cell Sci 124:1077–1087. doi:10.1242/jcs.072207
26. Lou Y, McDonald PC, Oloumi A, Chia S, Ostlund C, Ahmadi A, Kyle A, Auf dem Keller U, Leung S, Huntsman D, Clarke B, Sutherland BW, Waterhouse D, Bally M, Roskelley C, Overall CM, Minchinton A, Pacchiano F, Carta F, Scozzafava A, Touisni N, Winum JY, Supuran CT, Dedhar S (2011) Targeting tumor hypoxia: suppression of breast tumor growth and metastasis by novel carbonic anhydrase IX inhibitors. Cancer Res 71:3364–3376. doi:10.1158/0008-5472.CAN-10-4261
27. De Craene B, Berx G (2013) Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer 13:97–110. doi:10.1038/nrc3447