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Original Article

Expression of carbonic anhydrase-9 correlates with metastasis and prognosis of Chinese patients with invasive breast ductal carcinoma

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Abstract: Background: Invasive breast ductal carcinoma is characterized by a heterogeneously hypoxic environment. Hypoxia might stimulate the malignant potential of cancer cells. The purpose of our study was to firstly clarify the significance of hypoxia in Chinese patients with invasive breast ductal carcinoma by evaluating the expression of a hypoxic marker, namely carbonic anhydrase-9 (CA-9). Methods: The expression of CA-9 in the 100 samples was detected by non-biotin immunohistochemical method, the expression of positive cells for CA-9 was evaluated, and its association with histological grade, lymphatic metastasis, TNM stage and prognosis was assessed. Results: The CA-9 expression was positive in 29 (29.0%) of 100 invasive breast ductal carcinomas. CA-9 positive expression was significantly corresponding to lymph node metastasis ($P = 0.015$), TNM stage ($P = 0.018$) and overall survival rate ($P = 0.0001$) or disease-free survival rate ($P = 0.0001$), but not to age ($P = 0.375$), tumor size ($P = 0.288$) and histological grade ($P = 0.526$). CA-9 was an independent prognostic factor ($P = 0.002$). Conclusions: It is concluded that expression of CA-9 is strongly associated with neoplastic metastasis which suggests hypoxic microenvironment may play an important role in invasive breast ductal carcinoma. Hypoxia might be associated with aggressive tumor phenotype of invasive breast ductal carcinoma. The hypoxic marker CA-9 may be a useful prognostic indicator.

Keywords: Breast neoplasms, immunohistochemistry, carbonic anhydrase-9 (CA-9), Hypoxia, prognosis

Introduction

Breast cancer has become the second most frequent cause of female deaths, threatening women all over the world. In China, the incidence of breast cancer increases very rapidly and breast cancer has become the most common female malignant tumor. In spite of advances in diagnosis and treatment, almost one-fourth of women with this neoplasm will die. The major causes of treatment failure and/or death for breast cancer patients are tumor recurrence and metastasis. The use of adjuvant and palliative therapies in patients with breast carcinoma rely primarily on prognostic factors, such as tumor grade and size, axillary nodal status, distant metastasis, and candidate biomarkers, such as hormone receptor [nuclear estrogen receptor (nER) and progesterone receptor (PR)] expression, and C-erbB-2/Her-2/neu amplification/overexpression. Further, expression of hormone receptors and overexpression of C-erbB-2 help in guiding therapeutic strategies and predict response to chemotherapy, endocrine therapy, and specific immunotherapy with the antibody, trastuzumab. Therefore, such biomarkers in breast neoplasms provide information regarding the outcome of patients. A study in search of additional biomarkers is necessary for patients with breast cancer.

Increased metabolic demands in neoplasms require adequate oxygenation. As tumors enlarge, they outgrow their local blood supply, resulting in a relatively hypoxic tumor cell microenvironment. It has been demonstrated that tumor cell hypoxia is one of the key stimuli for the release of angiogenic factors necessary for angiogenesis and tumor growth [1]. Carbonic anhydrases (CAs) are zinc metalloenzymes which play an important role in pH homeosta-

Table 1. Correlation between CA-9 expression and clinicopathologic features in 100 patients with invasive ductal breast carcinoma.

Clinicopathological features	CA-9		*P* value
	positive (%)	negative	
Age			0.375
≥50	13 (34.2)	25	
<50	16 (25.8)	46	
Menopause			0.114
Yes	14 (38.9)	22	
No	15 (23.4)	49	
Tumor size			
≤2 cm	3 (14.3)	17	
>2~≤5 cm	23 (31.9)	49	
>5 cm	3 (27.3)	5	
Axillary lymph Node Metastasis			0.015
Negative	8 (16.7)	40	
positive	21 (40.4)	31	
Grade			0.526
I	5 (29.4)	12	
II	14 (25.0)	42	
III	10 (37.0)	17	
Stage			0.018
I~II	17 (22.4)	59	
III~IV	12 (50.0)	12	

sis, water and electrolyte regulation, oxygen balance and the catalysis of the reversible conversion of carbon dioxide and water to hydrogen ions and bicarbonate, and are considered vital to normal cellular function [2-4]. The carbonic anhydrase-9 (CA-9) gene contains a hypoxia response component within its promoter region which is activated by hypoxia-inducible factors; overlap has been demonstrated in tumors between hypoxic regions and areas of CA-9 expression [3, 5, 6]. CA-9 has also been linked to cellular proliferation and malignant transformation [3, 5]. CA-9 protein has been shown to be elevated in the presence of hypoxia in human renal carcinoma [7, 8], head and neck cancer [9], cervical squamous cell carcinoma [10], non-small cell lung cancer [11], and ovarian cystadenocarcinomas [12]. CA-9 expression has been correlated with recognized markers of hypoxia and angiogenesis [9, 11, 13-15]. CA-9 expression and poor prognosis have been correlated in various malignant tumors [11, 14, 16, 17]. However, the converse of this has also been demonstrated in some malignancies [18-20]. It is not clear whether CA-9 is simply a marker of hypoxia, a component of tumor pH stabilization, or contributing factor to tumor growth and dissemination.

A number of novel anti-angiogenic drugs have been shown to be effective for the treatment of malignancies, including colorectal cancer. In animal models, the CA inhibitor, acetazolamide, augmented the tumoristatic effect of other chemotherapeutic agents [21] and, small molecule CA-9 specific inhibitors have been proposed as potential therapeutic agents [22]. The understanding of the role of hypoxic factors in breast cancer will be important in determining groups at increased risk of developing recurrences and might identify patients who would most benefit from specific anti-angiogenic therapies. Up to date, there is no report of CA-9 expression in Chinese patients with breast cancer in literature. In this study, we investigated CA-9 expression in invasive ductal breast cancers and their correlation with histopathological variables and outcome.

Materials and methods

Patients and specimens

One hundred patients who had undergone modified radical mastectomy for treatment of invasive ductal breast carcinomas during 1998-2000 at Chinese People's Liberation Army Hospital, Beijing, China were confirmed histologically and were enrolled in this study. Ethical approval for this study was not required by our institution as the experiments carried out did not relate to patient's privacy, impairment or treatment. Paraffin tissue of tumor specimens were retrieved from the archives of the Department of Pathology. Clinical information, such as tumor size, grade, stage, and axillary lymph node status were obtained from medical records and the pathology reports (**Table 1**).

Immunohistochemical analysis

Immunohistochemical staining was done on 3-4 μm slides from formalin-fixed, paraffin-embedded tissues. Paraffin slides were then deparaffinized in xylene and rehydrated. The antigen retrieval was performed with slides heated in 0.01 M citrate buffer (pH 6.0) in a microwave oven for 5 minutes at 100°C. After antigen retrieval, the slides were then cooled in running tap water. The slides were rinsed with

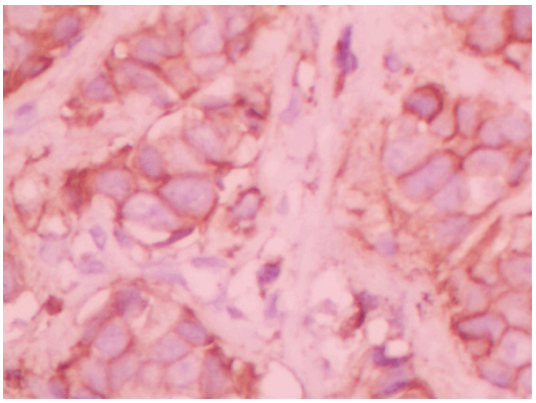

Figure 1. Expression of CA-9 protein in invasive ductal breast carcinoma. CA-9 was expressed positive in the membrane of cancer cells (CA-9 × 400).

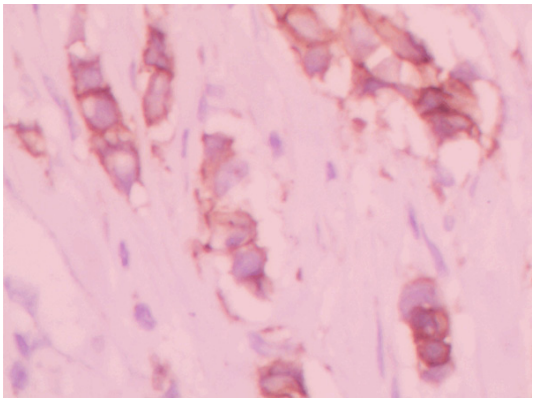

Figure 2. Expression of CA-9 protein in invasive ductal breast carcinoma. CA-9 was expressed positive in the membrane of cancer cells at the infiltrating cords. (CA-9 × 400).

PBS and the endogenous peroxidase was inactivated with 3% hydrogen peroxide. After blocking with 10% goat serum, the slides were incubated with primary polyclonal rabbit antibody to human CA-9 (Santa Cruz Biotec, USA) diluted 1:100 in blocking solution overnight at 4°C. The sections were rinsed in PBS and incubated for 20 minutes with polyperoxidase-anti-mouse/rabbit IgG (Zymed Laboratories Inc.) and then peroxidase reactivity was visualized using a DAB substrate kit (Zymed Labratories Inc.). Finally, the sections were counterstained with hematoxylin and mounted. Negative control sections were incubated with normal rabbit serum instead of the primary antibody. Positive and negative controls were included in each run.

Evaluation of immunohistochemistry

Scores were applied as follows: score 0, negative staining in all cells; score 1+, weakly positive or focally positive staining in <10% of the cells; score 2+, moderately positive staining covering 10% to 50% of the cells; and score 3+, strongly positive staining, including >50% of the cells. For statistical analysis, as well as to reduce intraobserver variability, the immunohistochemical scores were further grouped into two categories: negative (0 and 1+) and positive (2+ and 3+) [23].

Statistical analysis

Fisher's exact test (two sided), Pearson Chi's square test for trends in proportions, Spearman's correlation coefficient test, and Kaplan-Meier's method with log rank test or Cox Regression method for univariate or multivariate overall survival analysis were used to assess the associations between expression of CA-9 and clinicopathological indices by SPSS 15.0 for Windows (Chicago, IL). A P <0.05 was considered statistically significant.

Results

Clinicopathological characteristics of the patients and tumors

The age of the patients ranged from 28-92 years, with an average of 49 years. 17 were at grade 1, 56 at grade 2 and 27 at grade 3, according to histological grading. 16 were at stage I, 60 at stage II, 21 at stage III and 3 at stage IV, according to clinical staging of TNM, respectively. Lymphatic metastasis in regional nodes at operation was confirmed in 52 cancers of this study. All 100 women were followed after surgical treatment for a mean period of 45.3 months (range, 8-131 months); 33 cases were recurred and 17 cases dead. The details of patient characteristics and descriptive statistics for the tumors are shown in **Table 1**.

Correlation between CA-9 expression and clinicopathological features

CA-9 expression was positive in 29 (29.0%) of 100 invasive ductal breast carcinomas. CA-9 was expressed in the tumor cell membrane (**Figures 1** and **2**). CA-9 was negative in normal

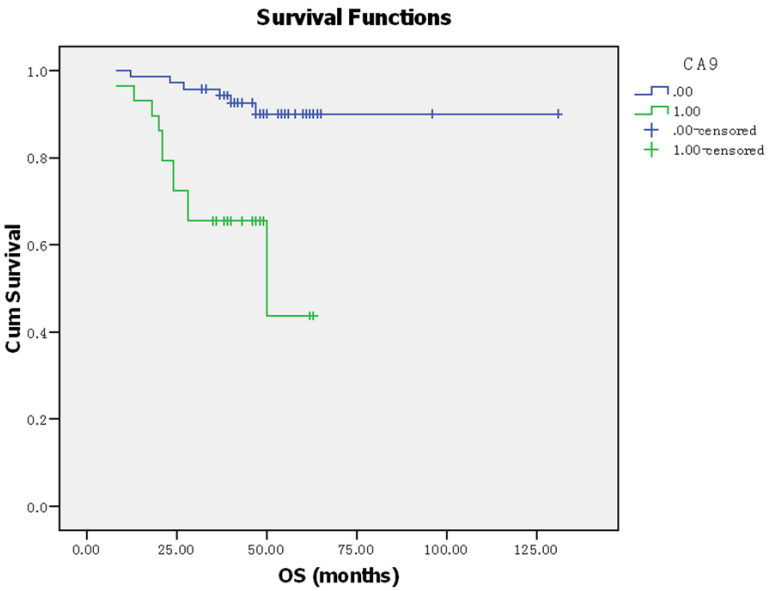

Figure 3. Kaplan-Meier survival analysis by CA-9 status (n = 100). The y-axis represents the percentage of patients; the x-axis, their survival in months ("censored" means living). The green line represents CA-9-positive patients with a trend of worse survival than the blue line representing CA-9-negative patients (Log rank = 15.64; *P* = 0.0001). Mean overall survival (OS) time was 45.5 months for the CA-9-positive group and 121.3 months for the CA-9-negative group.

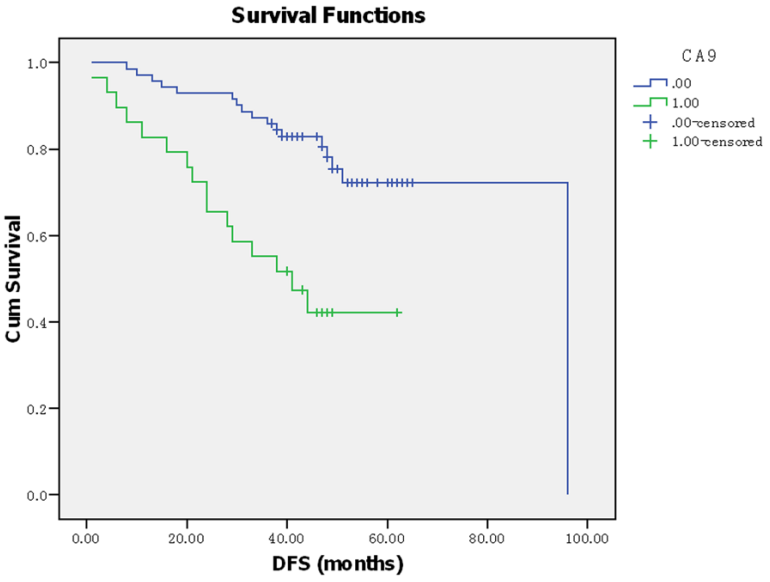

Figure 4. Kaplan-Meier survival analysis by CA-9 status (n = 100). The y-axis represents the percentage of patients; the x-axis, their survival in months ("censored" means living). The green line represents CA-9-positive patients with a trend of worse survival than the blue line representing CA-9-negative patients (Log rank = 14.68; *P* = 0.0001). Mean disease-free survival (DFS) time was 39.3 months for the CA-9-positive group and 78.9 months for the CA-9-negative group.

breast cell. The relationships between CA-9 expression and clinicopathological features of the tumors were shown in **Table 1**. CA-9 expression level was high in tumors with metastasis in axillary lymph nodes (40.4%), stage III~IV (50.0%) and significantly correlated with metastasis of axillary lymph nodes (*P* = 0.015) and clinical stage (*P* = 0.018). There was no statistically significant association between CA-9 expression and age, tumor size and grade (**Table 1**).

Correlation between CA-9 expression and prognosis

By Kaplan-Meier's method with log rank test for univariate overall survival (OS) analysis and disease-free survival (DFS) analysis, It was showed that in the 100 invasive ductal breast cancer patients with a modified radical mastectomy, the overall survival rate and the disease-free survival rate for CA-9-positive patients was significantly poorer than that of CA-9-negative patients (*P* = 0.0001, *P* = 0.0001, **Figures 3**, **4**). By Cox Regression method for multivariate survival analysis, CA-9 was an independent prognostic factor (*P* = 0.002).

Discussion

Recent studies have shown that many human tumors are hypoxic, probably due to compromised micro-circulation within a tumor. The tumor hypoxia is associated with a more aggressive malignant phenotype, increased risk of metastasis, and resistance to chemo- and radiotherapy [24-28]. Carbonic anhydrase 9 (CA-9) is induced by hypoxia in a range of tumor cell lines in an HIF-1-dependent manner [6], its role being to regulate tissue pH [29]. It has been directly and indirectly validated the use of

CA-9 as an intrinsic surrogate marker of hypoxia by some studies [14, 30, 31]. This study investigated for CA-9 expression in relation to clinicopathological characteristics and prognosis in 100 cases of Chinese patients with invasive ductal breast carcinoma. We have demonstrated a correlation between increasing clinical stage or lymphatic metastasis and CA-9 expression, and have shown a relationship between CA-9 expression and poor prognosis, a finding consistent with previous study by Hussain et al. In this study, we report that 29 of 100 cases of invasive ductal breast cancer express CA-9 but CA-9 expression was not detected in normal breast tissue. Disagreement exists with if CA-9 expression was associated with a worse prognosis as an independent prognostic factor [16, 32-34] in Western countries. Our results from China are in general agreement with those published by Chia and Hussain et al [16, 32], but not with that by Span et al [33, 34]. In those series of 103 and 144 women with breast cancer studied by Chia and Hussain et al, CA-9 was expressed in 48% and 26% of cases, different from that of our series. The percentage of CA-9 positive tumors (29%) in our series was between the results of Chia and Hussain et al. It may be due, in part, to heterogeneity in CA-9 staining both within and between individual tumors, which might lead to inaccuracy in estimating the number of positive and negative tumors. Additionally, this may be related to differences in technique and interpretation in nonstandardised immunohistochemistry assays. Hypoxia is reported to be an adverse prognostic factor in most human tumors. However, the converse of this has also been demonstrated in some malignancies [18-20, 33, 34]. The differences between tumor types need further explore. This study demonstrates that 29% of breast cancers are positive for CA-9 expression. Multivariate analysis in our study showed CA-9 to be an independent predictor of overall survival. This information may have prognostic value in that CA-9 expression is a predictor of poorer survival independently of other prognostic factors. This information may, therefore, facilitate a more refined selection of patients for adjuvant treatment. By adding to established prognostic factors, CA-9 expression may contribute to the identification of patients at greater risk of relapse who should be offered adjuvant treatment while sparing those whose prognosis is already good. Furthermore, as hypoxia is related to resistance to chemotherapy and radiotherapy, CA-9 expression may serve as a predictive factor to guide the selection of the most appropriate adjuvant treatment modality. Finally, the expression of CA-9 in a number of breast tumors examined in this study, compared to the absence of CA-9 in normal breast tissue, indicates that hypoxia and hypoxia-related gene expression may present a useful target for novel targeted therapies, for example drugs or gene therapy vectors that are specifically activated under hypoxic conditions. This study provides a rationale basis for the further study of these approaches in breast cancer. Randomized studies with translational end points are required to further elucidate the prognostic and predictive value of CA-9. Prospective study within the context of an adjuvant chemotherapy trial is underway to investigate and explore this correlation in clinical trial setting.

Disclosure of conflict of interest

None.

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References

[1] Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 2003; 9: 677-84.

[2] Kivela AJ, Parkkila S, Saarnio J, Karttunen TJ, Kivela J, Parkkila AK, Bartosova M, Mucha V, Novak M, Waheed A, Sly WS, Rajaniemi H, Pastor ekova S, Pastorek J. Expression of von Hippel-Lindau tumor suppressor and tumor-associated carbonic anhy- drases IX and XII in normal and neoplastic colorectal mucosa. World J Gastroenterol 2005; 11: 2616-2625.

[3] Robertson N, Potter C, Harris AL. Role of carbonic anhydrase IX in human tumor cell growth, survival, and invasion. Cancer Res 2004; 64: 6160-6165.

[4] Saarnio J, Parkkila S, Parkkila AK, Haukipuro K, Pastorekova S, Pastorek J, Kairaluoma MI, Karttunen TJ. Immunohistochemical study of colorectal tumors for expression of a novel transmembrane carbonic anhydrase, MN/CA IX, with potential value as a marker of cell proliferation. Am J Pathol 1998; 153: 279-285.

[5] Pastorek J, Pastorekova S, Callebaut I, Mornon JP, Zelnik V, Opavsky R, Zat'ovicova M, Liao S, Portetelle D, Stanbridge EJ, et al. Cloning and

characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment. Oncogene 1994; 9: 2877-88.

[6] Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL. Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 2000; 60: 7075-7083.

[7] Liao SY, Aurelio ON, Jan K, Zavada J, Stanbridge EJ. Identification of the MN/CA9 protein as a reliable diagnostic biomarker of clear cell carcinoma of the kidney. Cancer Res 1997; 57: 2827-2831.

[8] Tomisaki S, Ohno S, Ichiyoshi Y, Kuwano H, Maehara Y, Sugimachi K. Microvessel quantification and its possible relation with liver metastasis in colorectal cancer. Cancer 1996; 77: 1722-1728.

[9] Beasley NJ, Wykoff CC, Watson PH, Leek R, Turley H, Gatter K, Pastorek J, Cox GJ, Ratcliffe P, Harris AL. Carbonic anhydrase IX, an endogenous hypoxia marker, expression in head and neck squamous cell carcinoma and its relationship to hypoxia, necrosis, and microvessel density. Cancer Res 2001; 61: 5262-5267.

[10] Brewer CA, Liao SY, Wilczynski SP, Pastorekova S, Pastorek J, Zavada J, Kurosaki T, Manetta A, Berman ML, DiSaia PJ, Stanbridge EJ. A study of biomarkers in cervical carcinoma and clinical correlation of the novel biomarker MN. Gynecol Oncol 1996; 63: 337-344.

[11] Giatromanolaki A, Koukourakis MI, Sivridis E, Pastorek J, Wykoff CC, Gatter KC, Harris AL. Expression of hypoxia-inducible carbonic anhydrase-9 relates to angiogenic pathways and independently to poor outcome in non-small cell lung cancer. Cancer Res 2001; 61: 7992-7998.

[12] Hynninen P, Vaskivuo L, Saarnio J, Haapasalo H, Kivela J, Pastorekova S, Pastorek J, Waheed A, Sly WS, Puistola U, Parkkila S. Expression of transmembrane carbonic anhydrases IX and XII in ovarian tumours. Histopathology 2006; 49: 594-602.

[13] Koukourakis MI, Giatromanolaki A, Sivridis E, Simopoulos K, Pastorek J, Wykoff CC, Gatter KC, Harris AL. Hypoxia-regulated carbonic anhydrase-9 (CA9) relates to poor vascularization and resistance of squamous cell head and neck cancer to chemoradiotherapy. Clin Cancer Res 2001; 7: 3399-3403.

[14] Loncaster JA, Harris AL, Davidson SE, Logue JP, Hunter RD, Wycoff CC, Pastorek J, Ratcliffe PJ, Stratford IJ, West CM. Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer Res 2001; 61: 6394-6399.

[15] Swinson DE, Jones JL, Richardson D, Wykoff C, Turley H, Pastorek J, Taub N, Harris AL, O'Byrne KJ. Carbonic anhydrase IX expression, a novel surrogate marker of tumor hypoxia, is associated with a poor prognosis in non-small-cell lung cancer. J Clin Oncol 2003; 21: 473-482.

[16] Chia SK, Wykoff CC, Watson PH, Han C, Leek RD, Pastorek J, Gatter KC, Ratcliffe P, Harris AL. Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. J Clin Oncol 2001; 19: 3660-3668.

[17] Couvelard A, O'Toole D, Turley H, Leek R, Sauvanet A, Degott C, Ruszniewski P, Belghiti J, Harris AL, Gatter K, Pezzella F. Microvascular density and hypoxia-inducible factor pathway in pancreatic endocrine tumours: negative correlation of microvascular density and VEGF expression with tumour progression. Br J Cancer 2005; 92: 94-101.

[18] Bui MH, Seligson D, Han KR, Pantuck AJ, Dorey FJ, Huang Y, Horvath S, Leibovich BC, Chopra S, Liao SY, Stanbridge E, Lerman MI, Palotie A, Figlin RA, Belldegrun AS. Carbonic anhydrase IX is an independent predictor of survival in advanced renal clear cell carcinoma: implications for prognosis and therapy. Clin Cancer Res 2003; 9: 802-811.

[19] Murakami Y, Kanda K, Tsuji M, Kanayama H, Kagawa S. MN/CA9 gene expression as a potential biomarker in renal cell carcinoma. BJU Int 1999; 83: 743-747.

[20] Rasheed S, Harris AL, Tekkis PP, Turley H, Silver A, McDonald PJ, Talbot IC, Glynne-Jones R, Northover JM, Guenther T. Assessment of microvessel density and carbonic anhydrase-9 (CA-9) expression in rectal cancer. Pathol Res Pract 2009; 205: 1-9.

[21] Teicher BA, Liu SD, Liu JT, Holden SA, Herman TS. A carbonic anhydrase inhibitor as a potential modulator of cancer therapies. Anticancer Res 1993; 13: 1549-1556.

[22] Vullo D, Franchi M, Gallori E, Pastorek J, Scozzafava A, Pastorekova S, Supuran CT. Carbonic anhydrase inhibitors: inhibition of the tumor-associated isozyme IX with aromatic and heterocyclic sulfonamides. Bioorg Med Chem Lett 2003; 13: 1005-1009.

[23] Liu Q, Li J, Zheng X, Jin F, Dong H. Expression of CD133, PAX2, ESA, and GPR30 in invasive ductal breast carcinomas. Chin Med J 2009; 122: 2763-2769.

[24] Brizel DM, Scully SP, Harrelson JM, Layfield LJ, Bean JM, Prosnitz LR, Dewhirst MW. Tumour oxygenation predicts for likelihood of distant metastases in human soft tissue sarcoma. Cancer Res 1996; 56: 941-943.

[25] Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ. Hypoxia mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 1996; 379: 88-91.

[26] Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumour hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 1996; 56: 4509-4515.

[27] Reynolds TY, Rockwell S, Glazer PM. Genetic instability induced by the tumour microenvironment. Cancer Res 1996; 56: 5754-5757.

[28] Kim CY, Tsai MH, Osmanian C, Graeber TG, Lee JE, Giffard RG, Di Paulo JA, Peehl DM, Giaccia AJ. Selection of human cervical epithelial cells that possess reduced apoptotic potential to low-oxygen conditions. Cancer Res 1997; 57: 4200-4204.

[29] Svastova E, Hulikova A, Rafajova M, Zat'ovicova M, Gibadulinova A, Casini A, Cecchi A, Scozzafava A, Supuran CT, Pastorek J, Pastorekova S. Hypoxia activates the capacity of tumour-associated carbonic anhydrase IX to acidify extracellular pH. FEBS Lett 2004; 577: 439-445.

[30] Turner KJ, Crew JP, Wykoff CC, Watson PH, Poulsom R, Pastorek J, Ratcliffe PJ, Cranston D, Harris AL. The hypoxia-inducible genes VEGF and CA9 are differentially regulated in superficial vs invasive bladder cancer. Br J Cancer 2002; 86: 1276-1282.

[31] Hoskin PJ, Sibtain A, Daley FM, Wilson GD. GLUT1 and CA IX as intrinsic markers of hypoxia in bladder cancer: relationship with vascularity and proliferation as predictors of outcome of ARCON. Br J Cancer 2003; 89: 1290-1297.

[32] Hussain SA, Ganesan R, Reynolds G, Gross L, Stevens A, Pastorek J, Murray PG, Perunovic B, Anwar MS, Billingham L, James ND, Spooner D, Poole CJ, Rea DW, Palmer DH. Hypoxia-regulated carbonic anhydrase IX expression is associated with poor survival in patients with invasive breast cancer. Br J Cancer 2007; 96: 104-109.

[33] Span PN, Bussink J, Manders P, Beex LVAM, Sweep CGJ. Carbonic anhydrase-9 expression levels and prognosis in human breast cancer: association with treatment outcome. Br J Cancer 2003; 89: 271-276.

[34] Span PN, Bussink J, Manders P, Beex LVAM, Sweep CGJ. Carbonic anhydrase IX expression is more predictive than prognostic in breast cancer. Br J Cancer 2007; 96: 1309.