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 over-expression 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-
CA-9 in invasive ductal breast carcinoma

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

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

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 tumorstatic 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
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 Laboratories 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
CA-9 in invasive ductal breast carcinoma

The relationships between CA-9 expression and clinicopathological features of breast cell. The correlations between CA-9 expression and clinicopathological features of breast cancer 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 chemotherapeutic 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

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