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Abundant tumor promoting stromal cells in lung adenocarcinoma with hypoxic regions

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ABSTRACT

Objectives: Carbonic anhydrase IX (CAIX) is a marker of hypoxia and its expression by cancer associated fibroblasts (CAFs) was reportedly associated with the poor prognosis of lung adenocarcinoma. This study aimed to characterize the hypoxic microenvironment containing CAIX (+) CAFs.

Materials and methods: First, we evaluated the clinicopathological significance of CAIX expression by CAFs in 3 cm and above lung adenocarcinoma (n = 188). We then compared the expressions of E-cadherin, ezrin, ALDH-1, CD44, EGFR, HSF-1, Glut-1, and PD-L1 in cancer cells, as well as those of CD204 and podoplanin in stromal cells between CAIX (+) CAFs and CAIX (−) CAFs cases (n = 25, each).

Results: In total, 48 patients had CAIX (+) CAFs (26%). Multivariate analysis revealed that CAIX expression by CAFs could serve as an independent unfavorable prognostic factor for recurrence-free survival ($p < 0.05$). The staining score of hypoxia marker Glut-1 in cancer cells was significantly higher in cases with CAIX (+) CAFs than in those with CAIX (−) CAFs (median: 20 vs. 0, $p < 0.01$). In addition, the numbers of CD204 (+) tumor-associated macrophages (TAMs) and podoplanin (+) CAFs were significantly higher in the CAIX (+) CAFs group than in the CAIX (−) CAFs group (TAMs: 31.5 vs. 17.0: $p < 0.01$, CAFs: 20 vs. 0: $p < 0.05$). The staining score of the other markers did not differ between the groups.

Conclusion: Our results indicate that the presence of abundant tumor promoting stromal cells, CD204 (+) TAMs, and podoplanin (+) CAFs is characteristic of the tumor microenvironment containing CAIX (+) CAFs, which contributes to an increase in aggressive behavior in lung adenocarcinoma with hypoxic regions.

1. Introduction

Hypoxia triggers biological changes in cancer tissue, including angiogenesis, glycolysis, pH regulation, growth factor signaling, genetic instability, invasion, and metastasis [1]. Under hypoxic conditions, the expression of carbonic anhydrase IX (CAIX), a member of the carbonic anhydrase (CA) family, is induced by hypoxia-inducible factor 1 (HIF–1) protein binding to the hypoxia-responsive element of the CAIX promoter [2–6]. Therefore, CAIX expression acts as an endogenous marker of hypoxia. The expression of CAIX has been observed in a wide variety of tumors, including lung cancer, and it has been reported to be associated with poor outcomes in patients [7–14]. We have previously determined that CAIX expression by cancer cells of lung adenocarcinoma was associated with poor outcome [15]. Thereafter, Nakao reported that CAIX was also expressed by cancer associated fibroblasts (CAFs) in 24.7% of the lung adenocarcinoma cases, and that CAIX expression by CAFs was significantly correlated with the presence of conventional prognostic factors [16]. Multivariate analysis revealed that a statistically significant association was observed between CAIX expression by CAFs, but not cancer cells, and a lower survival rate. These findings suggested the possibility that adenocarcinomas with CAIX (+) CAFs displayed high malignant potential, and that CAIX

Abbreviations: CA IX, Carbonic anhydrase IX; CAFs, Cancer associated fibroblasts; TAMs, Tumor-associated macrophages; HIF–1, Hypoxia-inducible factor 1; EGFR, Epidermal growth factor receptor; ALDH1, Aldehyde dehydrogenase 1; HSF–1, Heat shock factor-1; PD–L1, Programmed cell death-1

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expression by CAFs could serve as a more accurate indicator of the hypoxic tumor microenvironment. However, the reason behind the association between the presence of CAIX (+) CAFs and poor prognosis remains unclear.

CAFs and tumor-associated macrophages (TAMs) are the major cellular components of the tumor microenvironment. Many studies have shown that certain types of CAFs and TAMs have tumor promoting functions. It has previously been reported that podoplanin and CD204 are markers of tumor promoting CAFs and TAMs, respectively [17–24]. Understanding how microenvironmental factors modulate the recruitment of these CAFs and TAMs will be essential in the development of novel therapies that target stromal cells.

It is generally accepted that hypoxia is linked to cellular changes, including stemness [25–27], metabolic change [28], heat shock pathway [29], and immune tolerance [30] via several pathways. However, most of the studies conducted in this area in this area have only focused on the effect of hypoxia on cancer cells themselves. In this study, we hypothesized that the action of hypoxia on stromal cells also acts as a major driving force of hypoxia-mediated tumor aggressiveness. To this end, we examined whether the hypoxic microenvironment characterized by the presence of CAIX (+) CAFs is correlated with the number of tumor promoting stromal cells, CD204 (+) TAMs, and podoplanin (+) CAFs in lung adenocarcinoma.

2. Materials and methods

2.1. Patients

Since hypoxic zone is likely smaller in adenocarcinoma smaller than 3 cm, we selected tumors measuring 3 cm and above. A total of 208 consecutive patients with lung adenocarcinoma measuring 3 cm and above underwent complete resections by lobectomy, or by more extensive procedures, and systematic node dissections at the National Cancer Center Hospital East between August 1999 and July 2003. Complete resection was defined by the presence of cancer-free surgical margins, both on gross and histological examination. We excluded 20 patients from our study because they underwent incomplete resection, radiation therapy, or both; the remaining 188 patients were enrolled. None of the enrolled patients were treated by neo-adjuvants, adjuvants, or radiation before recurrence.

All specimens were collected after obtaining written comprehensive informed consent from the patients. This study was approved by the Institutional Review Board of the National Cancer Center (IRB number 2016-390).

2.2. Pathologic studies

All surgical specimens were fixed with methanol and embedded in paraffin. The tumors were cut at approximately 5-mm intervals, and serial 4-μm sections were stained with hematoxylin and eosin (H & E), and by the Alcian blue-periodic acid–Schiff method to visualize cytoplasmic mucin production, and by the Verhoeff–Van Gieson method to observe elastic fibers. Vascular and pleural invasion were evaluated in the sections stained by the Verhoeff–Van Gieson method. Two observers (H.N. and G.I.) who had no knowledge of the clinical data independently reviewed all slides. Histological diagnosis was based on the fourth edition of World Health Organization histological classification and the disease stages were based on the seventh edition of the UICC TNM classification [31,32]. Pathologic stage was determined according to the classification of the International Union Against Cancer.

2.3. Immunohistochemical staining

After pathologic assessment of the H & E-stained slides, the block containing the maximal cut surface of the primary tumor was selected from each resected lung specimen. Sections with 4-μm thickness were cut from the selected paraffin blocks and mounted on silanized slides. The sections were then deparaffinized in xylene and dehydrated in a graded ethanol series. After washing with distilled water, they were placed in 0.1 M citric acid buffer. Antigen retrieval was performed by placing and heating the slides at 95 °C for 20 min in a microwave oven and allowing them to cool for 1 h at room temperature. Next, the slides were washed three times in phosphate-buffered saline (PBS) and immersed in a 0.3% hydrogen peroxide solution in methanol for 15 min to inhibit endogenous peroxidase activity. They were washed three times in PBS, and nonspecific binding was blocked by preincubation with 2% normal swine serum in PBS (blocking buffer) for 30 min at room temperature. Individual slides were then incubated overnight at 4 °C with rabbit polyclonal antibody against human carbonic anhydrase IX (11071–1AP; Proteintech, Chicago, IL, USA) in the blocking buffer. The slides were again washed three times in PBS and then incubated with EnVision+ System– HRP Labelled Polymer (Dako, Glostrup, Denmark) for 1 h at room temperature. After extensive washing in PBS, a color reaction was developed for 3 min in 2% 3,3′–Diaminobenzidine Tetrahydrochloride (DAB) in 50 mM of Tris buffer (pH 7.6) containing 0.3% hydrogen peroxidase. Finally, the slides were counterstained with Mayer's hematoxylin, dehydrated, and mounted. Two observers (H.N. and G.I.) independently evaluated the staining in a blinded manner, and a consensus was reached by evaluating the tissue together using a conference microscope whenever their evaluation differed. Human renal clear cell carcinoma was used as a positive control for CAIX staining. If cell membranous and cytoplasmic staining was present in > 10% of the CAFs and > 10% of the cancer cells, the sample was considered positive for CAIX expression by CAFs and cancer cells, respectively.

Information on the other antibodies used in this study is summarized in Supplementary Table 2. The immunostaining scores of E-cadherin, ezrin, ALDH1, EGFR, CD44, PD–L1, and HSF–1 were evaluated based on the staining intensity and the percentage of cancer cells that were stained. The following scoring system was used: 0 (negative staining, defined as no immunoreactivity); 1+ (weak staining intensity); and 2+ (strong staining intensity).

We also evaluated the extent of staining in a lesion corresponding to every 10 percentages (0–100%). The staining scores were calculated by multiplying the percentage values by the staining intensity, with the scores ranging from 0 to 200. The number of CD204 (+) TAMs in the stroma was counted in three high-power microscopic fields in the invasive area, and the averages was determined [20]. Staining scores for podoplanin (+) cancer-associated fibroblasts (podoplanin-CAFs) were calculated by multiplying the percentages of podoplanin-CAFs per stromal area (0–100%) by the immunohistochemical staining intensity [33].

2.4. Statistical analysis

The correlation between CAIX expression and various clinicopathologic factors was analyzed by the chi-square test or Fisher's exact test.

Recurrence-free survival was calculated using the Kaplan–Meier method, and the difference between the groups was analyzed using a log-rank test. Statistical-significance was defined as a p-value of $p < 0.05$. Differences in immunohistochemical scores were calculated using the Wilcoxon signed rank test. Analyses were performed using the statistical software EZR version 1.32 of R version 3.2.2 [34].

3. Results

3.1. CAIX expression by cancer cells and CAFs

Fig. 1A shows a representative expression of CAIX by cancer cells and CAFs. CAIX-positive cells were heterogeneously present in different