Title:

Abundant tumor promoting stromal cells in lung adenocarcinoma with hypoxic regions

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Abbreviations:

Carbonic Anhydrase IX; CA IX

Cancer associated fibroblasts; CAFs

Tumor-associated macrophages; TAMs

Hypoxia-inducible factor 1; HIF-1

Epidermal growth factor receptor; EGFR

Aldehyde dehydrogenase 1; ALDH1

ABSTRACT

Objectives

Carbonic Anhydrase IX (CAIX) is a marker of hypoxia and its expression by cancer associated fibroblasts (CAFs) was reportedly associated with poor prognosis of lung adenocarcinoma. The aim of this study was to characterize the hypoxic microenvironment containing CAIX (+) CAFs.

Materials and methods

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

Result

There were 48 cases with 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 CAIX (+) CAFs cases than in CAIX (-) CAFs cases (median: 20 vs 0, $p<0.05$). In addition, the numbers of CD204 (+)

tumor-associated macrophages (TAMs) and podoplanin (+) CAFs were significantly higher in the CAIX (+) CAFs group than that in the CAIX (-) CAF group (TAMs: 31.5 vs 17.0, CAFs: 20 vs 0: $p<0.05$). The staining score of the other markers did not differ between both 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 contribute 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 its expression has also been reported to be associated with poor outcome in patients. [7-14] We have previously determined that CAIX expression by cancer cells of lung adenocarcinoma was associated with poor outcome. [15] After that, 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 expression by CAFs could serve as a more accurate indicator of the hypoxic tumor microenvironment. However, how the reason why the presence of CAIX (+) CAFs is associated with 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 been previously 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 tumor progression. However, most of the studies that have been carried out 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

A total of 208 consecutive patients with lung adenocarcinoma underwent complete resections by lobectomy, or 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 observed both grossly and histologically. We excluded 20 patients from our study because they underwent incomplete resection, radiation therapy, or both; the remaining 188 patients were enrolled in this study.

All specimens were collected after the patients provided written comprehensive informed consent. 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 4um sections were stained with hematoxylin and eosin (H & E) and by the Alcian blue-periodic acid–Schiff method to

visualize cytoplasmic mucin production and 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. The histologic diagnoses were based on the revised 4th World Health Organization (WHO) histologic classification. [25] 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 measuring 4-um thick were cut from the paraffin blocks selected as above and mounted on silanized slides. The sections were then deparaffinized in xylene and dehydrated in a graded ethanol series. After washing them with distilled water, they were placed in 0.1 M of citric acid buffer. Antigen retrieval was performed by placing and heating the slides to 95◦C for 20 minutes in a microwave oven and allowing them to cool for 1 hour at room temperature. Next, the slides were washed 3 times in phosphate-buffered saline (PBS) and immersed in a 0.3% hydrogen peroxide solution in

methanol for 15 minutes to inhibit endogenous peroxidase activity. They were washed 3 times in PBS, and nonspecific binding was blocked by preincubation with 2% normal swine serum in PBS (blocking buffer) for 30 minutes at room temperature. Individual slides were then incubated overnight at 4C with rabbit polyclonal antibody against human Carbonic anhydrase IX (11071-1AP, Proteintech, Chicago, IL) in the blocking buffer. The slides were again washed 3 times in PBS and then incubated with EnVision for 1 hour at room temperature. After extensive washing in PBS, a color reaction was developed for 3 minutes in 2% 3, 30-diaminobenzidine in 50 mM of Tris buffer (pH 7.6) containing 0.3% hydrogen peroxidase. Finally, the slides were counterstained with Meyer 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 by 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 ten 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. Scoring of podoplanin (+) CAFs was also carried out based on the staining intensity and the percentage of stained area as mentioned above.

2.4. Statistical Analysis

The correlation between CAIX expression and various clinicopathologic factors were analyzed by the chi-square test or Fisher 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 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.

3. RESULTS

3.1. CAIX expression by cancer cells and CAFs

Figure 1A shows a representative expression of CAIX by cancer cells and CAFs. CAIX-positive cells were heterogeneously present in different areas. CAIX was expressed mainly on the cell membrane in cancer cells (Figure 1B). Its expression was also found in stromal spindle cells that were morphologically identified as fibroblasts (Figure 1C). The frequency of the cases with CAIX positive cancer cells and CAFs was 31.9% (60/188) and 25.5% (48/188), respectively.

3.2. Prognostic significance of CAIX expression by cancer cells and CAFs

The clinicopathological characteristics of all the patients are listed in Supplemental Table 1. Univariate analysis demonstrated that CAIX expression by cancer cells and CAFs was significantly associated with a poor outcome ($p < 0.01$ and $p < 0.01$, respectively). Lymph node involvement ($p < 0.01$), vascular invasion ($p < 0.01$), lymphatic permeation ($p < 0.01$), and pleural invasion ($p < 0.01$) were also associated with worse

prognosis. Multivariate analysis of these 7 factors revealed that CAIX expression by CAFs, but not cancer cells, could serve as an independent prognostic factor (Table 2).

Correlation between CAIX expression by CAFs and clinicopathological factors

The correlation between CAIX expression by CAFs and clinicopathological factors is summarized in Supplemental Table 1. Smoking history ($p<0.05$), vascular invasion ($p<0.01$), and plural invasion ($p<0.05$) were observed in the CAIX (+) CAF group at a significantly higher frequency.

3.3. Correlation between CAIX expression by CAFs and recurrence-free survival

The Kaplan-Meier curve for recurrence-free survival rate based on the expression status of CAIX expression in the CAFs is shown in Figures 2A. The 5-year recurrence-free survival rate of the patients with CAIX (+) CAFs was 34.1%, whereas that of patients with CAIX (-) CAFs was 53.2%. Patients with CAIX (+) CAFs had a significantly shorter interval before recurrence as compared to those with CAIX (-) CAFs ($p<0.01$).

We selected cases where the tumor size was limited within 3-5 cm in diameter and the lymph node showed negative for metastasis. (n=107). In this cohort also, the CAIX (+) CAFs group (n=25) also showed worse prognosis in recurrence-free survival ($p<0.01$) (Figure2B).

3.4. Comparison of immunostaining scores of cancer cells and stromal cells between CAIX (+) CAFs and CAIX (-) CAFs cases

The results above suggest that the tumor microenvironment containing CAIX (+) CAFs displayed more aggressive behavior. To better understand this phenomenon, we compared the immunophenotype of both cancer ~~cells~~ and stromal cells in CAIX (+) CAFs cases and CAIX (-) CAFs cases whose tumor size was 3-5 cm in diameter and without lymph node metastasis (n=25, each). The samples from each group were selected by Matched-pair analysis.

3.4.1. Cancer cells (Figure3)

We examined the expression levels of stem cell-related molecules; ALDH1 and CD44, however, we did not observe any significant difference in both groups. There was no significant difference in epidermal growth factor receptor (EGFR) expression in both groups. We also investigated the expression of EMT-related molecule (E-cadherin) and invasion related molecule (ezrin) in cancer cells and found that the expression levels of these molecules were similar. There was also no significant difference in the expression level of the immune checkpoint marker PD-L1.

CAIX is a well-known as a marker of hypoxia. Thus, we expected the

microenvironment containing CAIX (+) CAFs to be hypoxic. To confirm this, we evaluated Glut-1, another hypoxic marker. The median staining score for Glut-1 (+) was 0 (0–60) for the CAIX (-) CAF group and 20 (0–50) for the CAIX (+) CAF group. Glut-1 expression in cancer cells was significantly higher in the CAIX (+) CAF group ($p<0.01$), suggesting the presence of a more hypoxic environment.

Lastly, we examined the expression level of the stress-related molecule, HSF-1, and observed that its expression was not significantly different in both groups.

3.4.2. Stromal cells (Figure 4)

The median staining score for podoplanin (+) CAFs was 0 (0–80) for the CAIX (-) CAF group and 20 (0–90) for the CAIX (+) CAF group. The median number of CD204 (+) TAMs was 17.0 (2.25–49.5) for the CAIX (-) CAF group and 31.5 (6.0–57.5) for the CAIX (+) CAF group. The number of podoplanin (+) CAFs and CD204 (+) TAMs in the stroma of CAIX (+) CAF tumors were significantly higher than in CAIX (-) CAF tumors ($p<0.01$ and $p< 0.05$, respectively).

4. DISCUSSION

In the current study, we found that CAIX expression by CAFs, not by cancer cells, was

an independent worse prognostic factor for recurrence-free survival in lung adenocarcinoma with over 3cm in size. Moreover, the numbers of CD204 (+) TAMs and podoplanin (+) CAFs, which reportedly function as tumor-promoting stromal cells, were significantly higher in CAIX (+) CAFs group. Our results suggest that the increased number of tumor-promoting stromal cells present in hypoxic tumor environments contributes to increased aggressive behavior in lung adenocarcinoma with hypoxic regions. This is the first clinicopathological report that extensively analyzes the composition of the hypoxic microenvironment.

Hypoxia has been proved to play important roles in tumor progression. We observed a significantly increased number of CD204 (+) macrophages (M2 macrophages) in CAIX (+) CAF samples, suggesting that hypoxic conditions might recruit or convert more M2 macrophages. Zhang et al. reported that hypoxic condition significantly promotes the metastasis of lung carcinoma in an animal hypoxia model, accompanied by an increased infiltration of M2 macrophages. [26] They also demonstrated that skewing of macrophage M2 polarization by hypoxia relies substantially on the activation of ERK signaling. Collectively, our current results also support their finding that tumor hypoxia promotes tumor progression by selectively promoting macrophage M2 polarization.

We have previously demonstrated that CAFs expressing podoplanin enhance cancer

cell invasion and tumor formation using *in vitro* and *in vivo* models. [21,22,24] Moreover podoplanin expression in CAFs also predicts a poorer outcome in patients with lung adenocarcinoma. [23] These results strongly suggest that podoplanin (+) CAFs have tumor-promoting function. In this study, we observed an increase in the number of podoplanin (+) CAFs and CD204 (+) TAMs in CAIX (+) CAF samples as compared to CAIX (-) CAF samples. In human glioma cell lines LN308 and U87MG cells, 72 h of hypoxia resulted in a robust increase in podoplanin mRNA as compared to normoxic expression levels. [27] Therefore, our results can be explained by the possibility that hypoxia directly increases the transcription of podoplanin in CAFs. Alternatively, certain soluble factors secreted from cancer cells under hypoxic condition might increase the expression of podoplanin in CAFs. Further *in vitro* analysis would be required to understand the molecular mechanisms underlying this phenomenon.

Hypoxia has recently emerged as a major factor that influences tumor proliferation and malignant progression. The hypoxia-inducible transcription factors HIF-1 and HIF-2 play a role in the adaptive cellular response to low oxygen tensions. The HIF heterodimer binds to and induces the expression of a panel of genes that lead to modification of a vast range of cellular functions. This allows cancer cells to not only survive but also undergo epithelial to mesenchymal transition (EMT), a process by

which epithelial cells lose their polarity and are converted to a mesenchymal phenotype E-cadherin, [28] a cell adhesion molecule, is frequently downregulated in cancer cells and has been proposed as an important mediator in EMT. [29] In the current study, we observed that E-cadherin expression in cancer cells was not correlated with the presence of CAIX CAFs. The expression of Ezrin, another invasion-related marker, [30] was also not correlated with the expression status of CAIX in CAFs. Taken together, our current results raised the possibility that under hypoxic tumor microenvironment, changes in cancer cell phenotype might not have a great influence on the tumor progression. Rather, more abundant tumor promoting stromal cells may play major roles in tumor progression.

In conclusion, we observed that tumor microenvironments containing CAIX (+) CAFs are characterized by the presence of abundant tumor promoting stromal cells, CD204 (+) TAMs, and podoplanin (+) CAFs, which contribute to the aggressive behavior of lung adenocarcinoma with hypoxic regions. Our findings provide important insight into the biological role of tumor-promoting stromal cells within the hypoxic microenvironment of lung adenocarcinoma. They also demonstrate the importance of assessing the biologic behavior of both cancer cells and stroma cells to better understand the hypoxic cancer microenvironment.

Conflict of interest: The authors have no conflict of interests to disclose.

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Comprehensive informed consent was obtained in the study.

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FIGURE LEGENDS

Figure.1 *Immunohistochemical expression of CAIX in lung adenocarcinoma*

A. CAIX expression in cancer cells and stromal cells

B. Higher magnification of the (*) area. CAIX expression was observed in the cell membrane of cancer cells.

C. Higher magnification of the (**) area. CAIX expression was observed in the cell membrane of CAFs.

Figure.2 *Relapse free survival (RFS) curve of CAIX expression by CAFs*

A. RFS of all cases in which CAFs expressed CAIX (n=188).

B. RFS of selected cases (3–5 cm in diameter and negative lymph node metastasis) in which (CAFs expressed CA IX (n=107).

Figure.3 *Comparison of immunostaining scores of cancer cells between CAIX (+) CAFs cases and CAIX (-) CAFs cases*

A. Comparison of immunohistochemical staining scores of CD44, ALDH-1, EGFR, Ezrin, E-cadherin, PD-L1, Glut-1 and HSF-1.

B. Representative figures of Glut-1 positive (left) and negative (right) cases.

Figure.4 *Comparison of immunostaining scores of stromal cells between CAIX (+) CAFs cases and CAIX (-) CAFs cases*

A. Comparison of immunohistochemical staining scores of podoplanin (+) CAFs and CD204 (+) TAMs.

B. Representative figures of podoplanin positive (upper left) and negative (upper right), as well as CD204 positive (lower left) and negative (lower right) cases.

Fig. 1

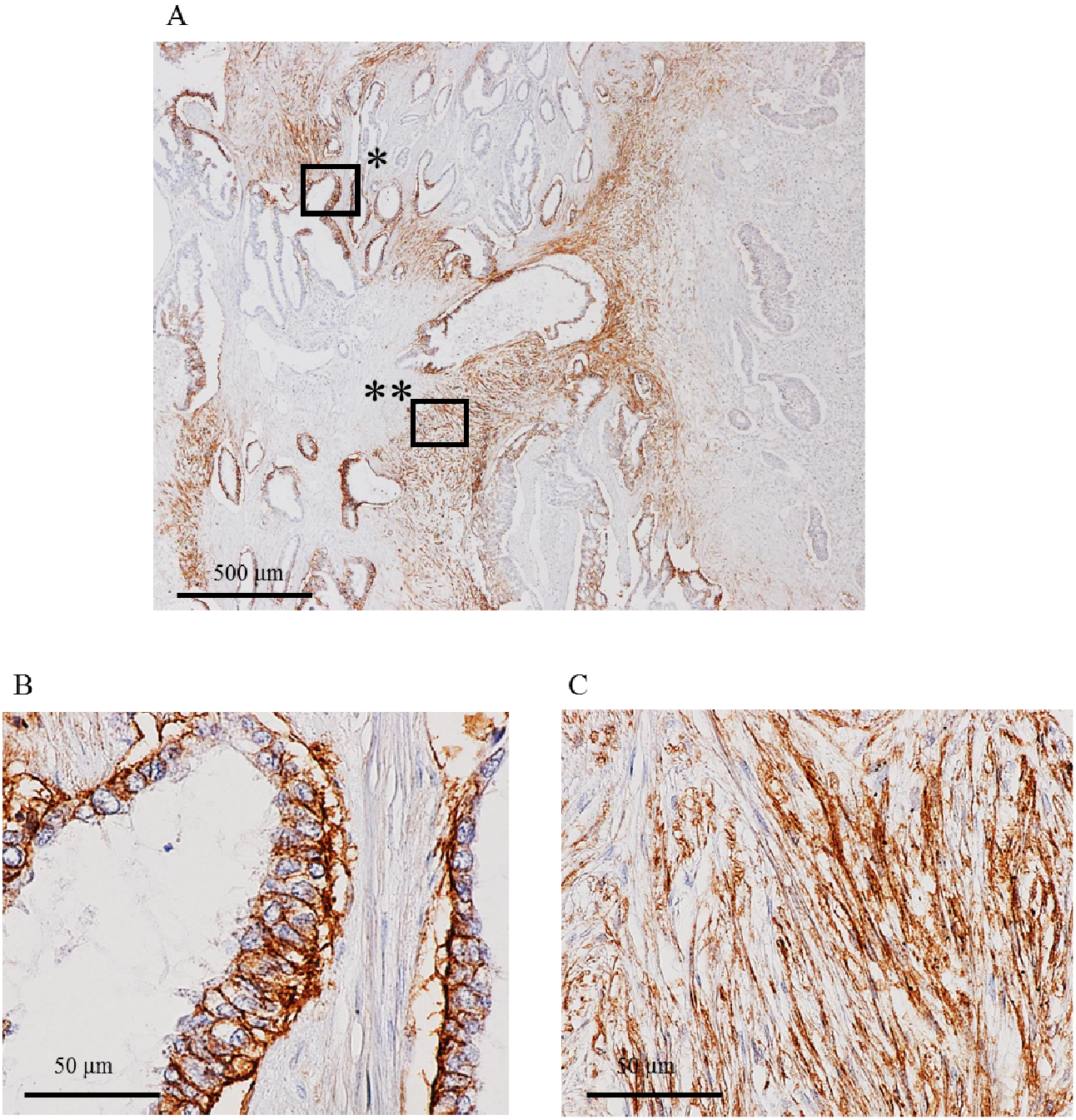
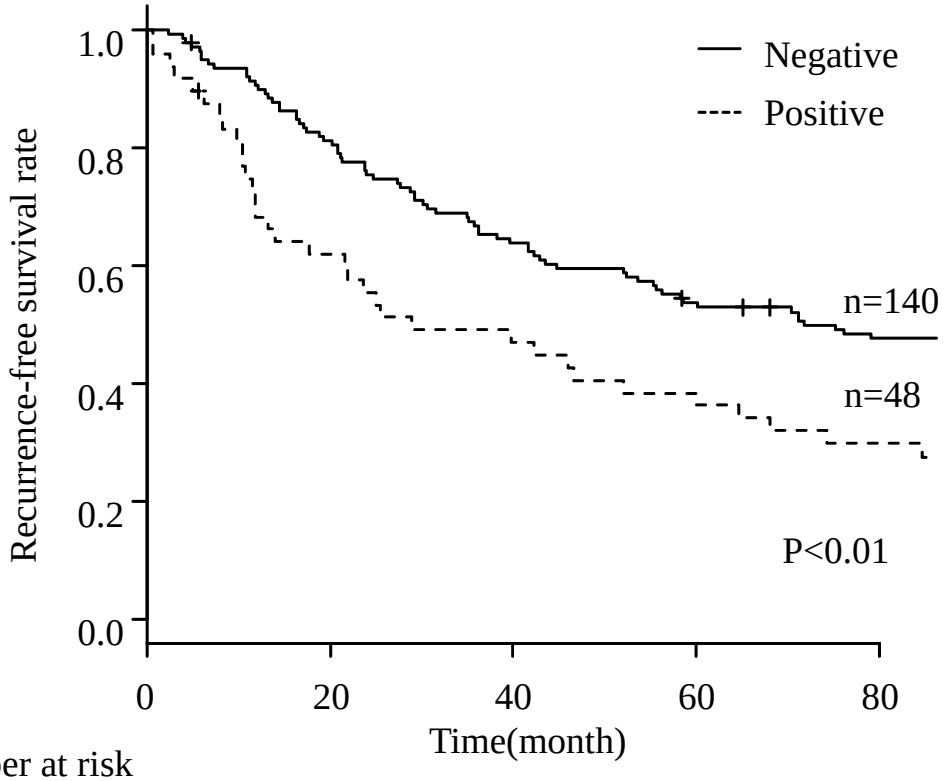


Fig. 2

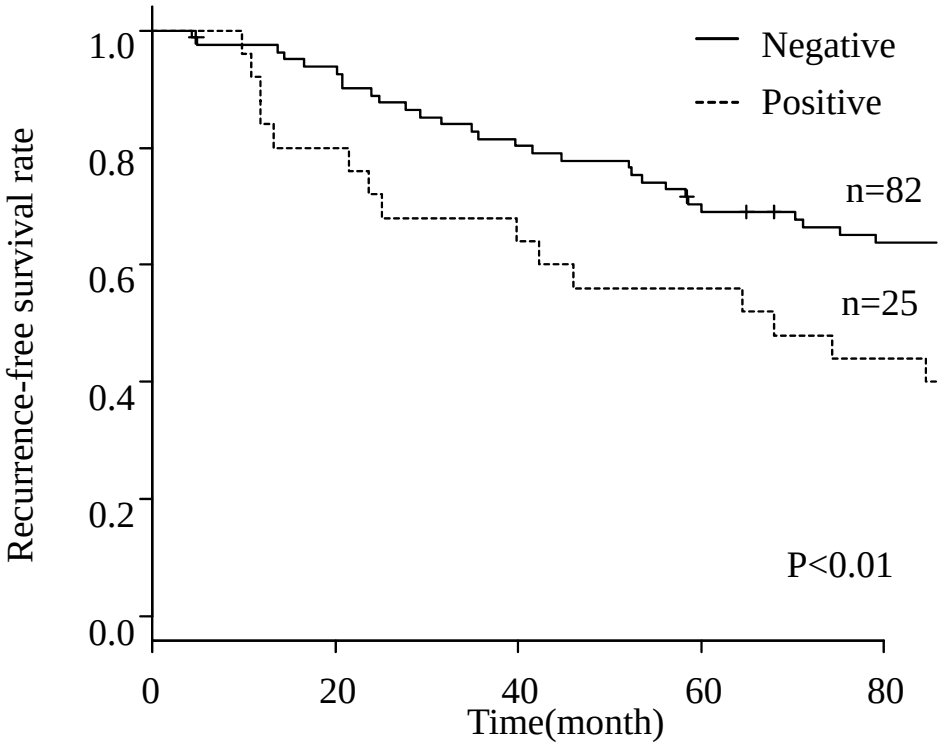
A



Number at risk

Negative	140	113	89	74	64
Positive	48	29	22	17	14

B

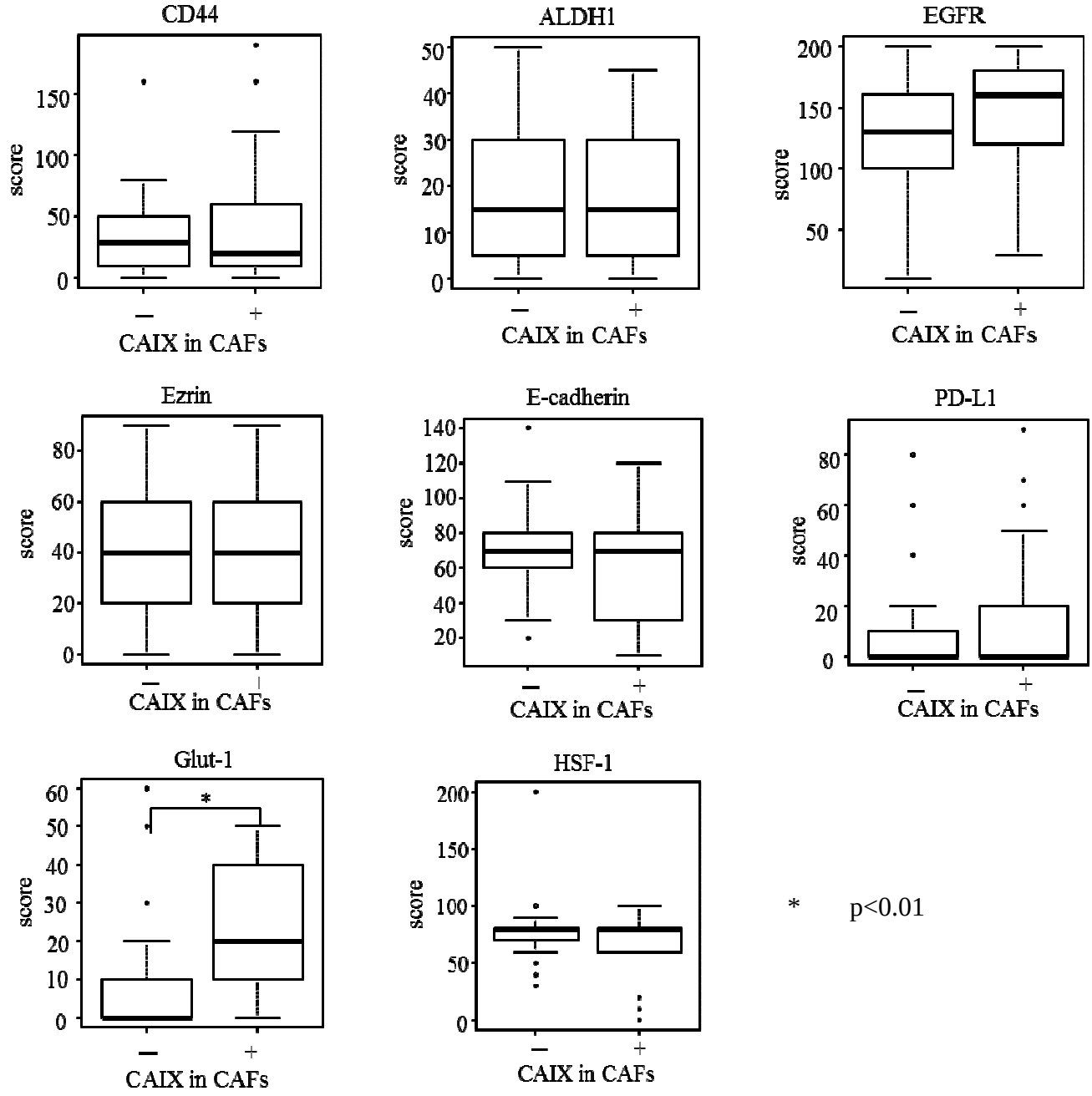


Number at risk

Negative	82	76	65	56	49
Positive	25	20	16	14	11

Fig. 3

A


CD44
ALDH1
EGFR
Ezrin
E-cadherin
PD-L1
Glut-1
HSF-1
score
CAIX in CAFs
–
+
*
* p<0.01

B

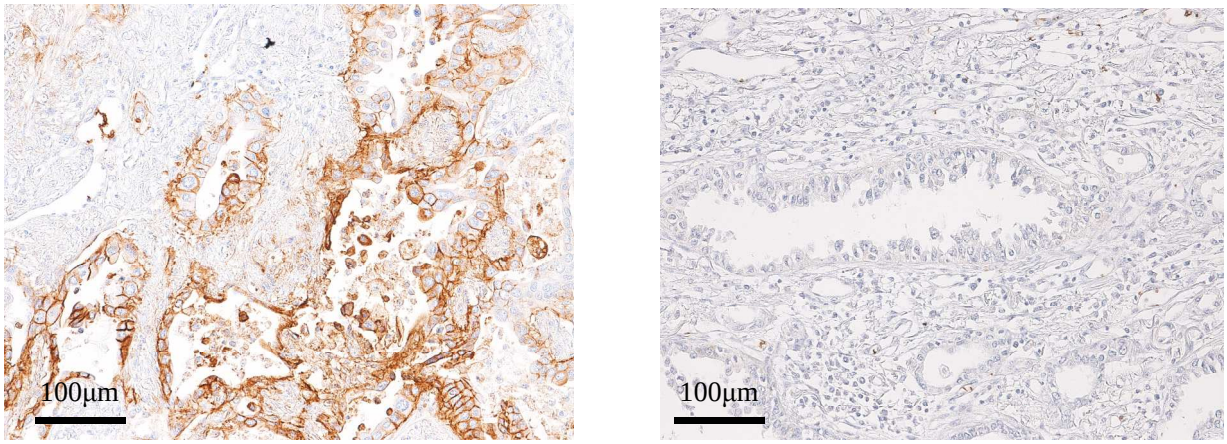
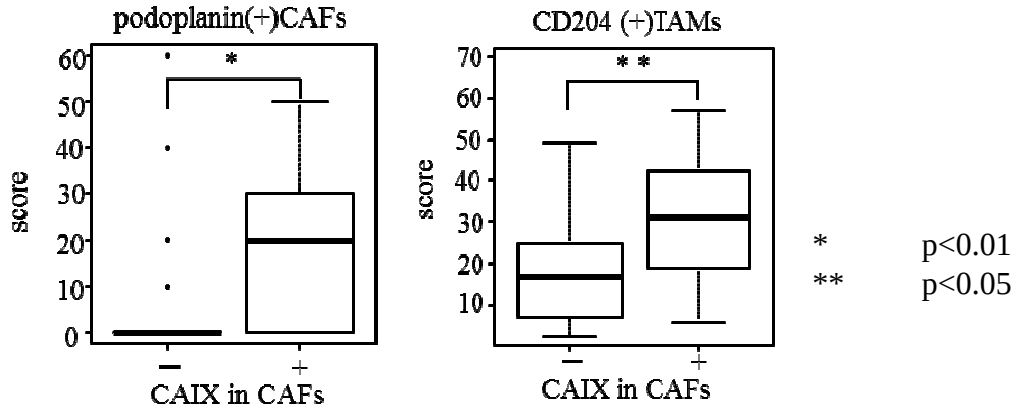
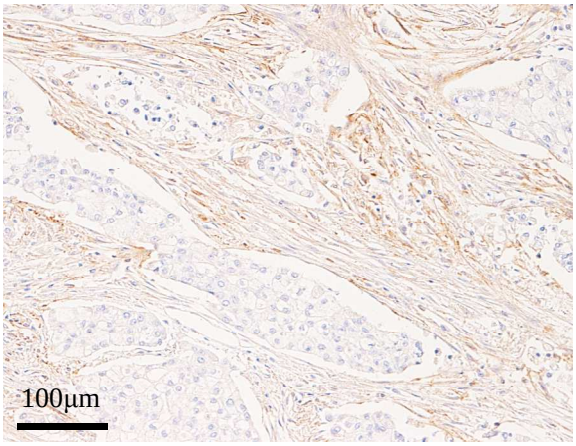

100μm
100μm

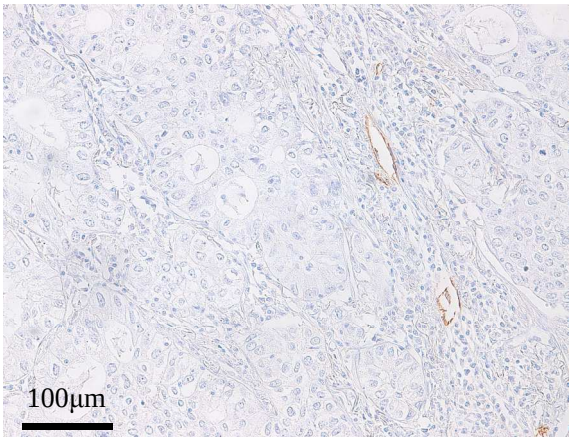
Fig. 4

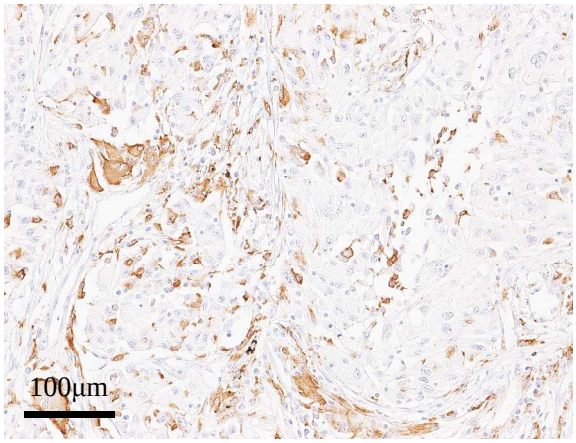
A


podoplanin(+)CAFs
score
60
50
40
30
20
10
0
*
−
+
CAIX in CAFs
CD204 (+)TAMs
score
70
60
50
40
30
20
10
* *
−
+
CAIX in CAFs
* p<0.01
** p<0.05

B


100μm


100μm


100μm

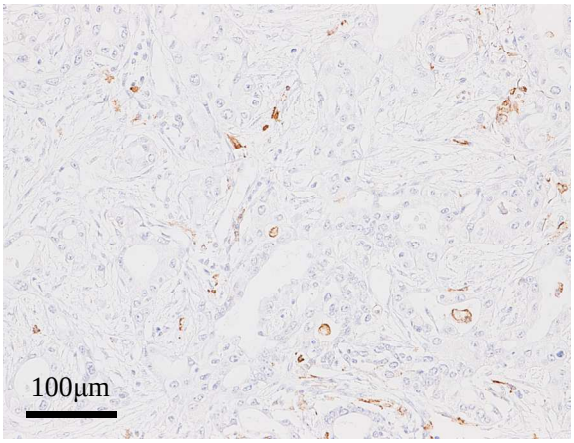

100μm

Table 1 Univariate analysis for recurrence-free survival rate

Characteristics	Patients number	5year survival (%)		*P*
Age				
＜65years	77	48.1		0.68
≧65years	111	48.6		
Sex				
Male	104	45.1		0.09
Female	84	52.3		
Smoking				
Never	78	49.9		0.25
Current/Former	108	47.2		
Tumor size				
≦5cm	153	53.7	<	0.01
>5cm	35	25.7		
Lymph node metastasis				
Absent	125	61.3	<	0.01
Present	63	22.6		
Vascular invasion				
Absent	78	67.9	<	0.01
Present	110	34.3		
Lymphatic permeation				
Absent	100	63.0	<	0.01
Present	88	31.4		
Pleural invasion				
Absent	98	66.3	<	0.01
Present	90	28.5		
CAIX in cancer cells				
Absent	128	53.2	<	0.01
Present	60	34.8		
CAIX in CAFs				
Absent	140	53.2	<	0.01
Present	48	34.1		

Table 2 Multivariate analysis for recurrence-free survival rate

	Unfavorable	Hazard Ratio	95% Cl	*P*
Tumor size	＞5cm	2.20	1.403-3.453	<0.01
Lymph node metastasis	Presence	1.85	1.237-2.773	<0.01
Vascular invasion	Presence	1.66	1.020-2.684	<0.05
Lymphatic permeation	Presence	1.53	1.023-2.281	<0.05
Pleural invasion	Presence	2.00	1.310-3.041	<0.01
CAIX in cancer cell	Presence	1.19	0.816-1.745	0.36
CAIX in CAFs	Presence	1.63	1.069-2.475	<0.05

Supplemental Table 1 Characteristics of CAIX expression in CAFs

Characteristics	CAIX in CAFs		*P*
	(−)	(+)	
	N	N	
total	140	48	
Age			
＜65years	52	25	0.09
≧65years	88	23	
Sex			
Male	76	28	0.74
Female	64	20	
Smoking			
Never	67	13	<0.05
Current/Former	73	35	
Tumor size			
≦5cm	114	39	1
＞5cm	26	9	
Lymph node metastasis			
Absent	95	30	0.60
Present	45	18	
Vascular invasion			
Absent	73	5	<0.01
Present	67	43	
Lymphatic permeation			
Absent	77	23	0.41
Present	63	25	
Pleural invasion			
Absent	80	18	<0.05
Present	60	30	

Supplemental Table 2 Antibodies used in this study

Antibodies		
Antibody	company	Antigen retrieval
CD44	Acris Antibodies GmbH	Microwave
ALDH1	BD Transduction Laboratories	Microwave
EGFR	Ventana Medical Systems, Inc.	Benchmark ULTRA*
Ezrin	Cell Signaling Technology, Inc.	Microwave
E-cadherin	Dako Cytomation	Microwave
PD-L1	Cell Signaling Technology, Inc.	Benchmark ULTRA*
Glut-1	SPRING Bioscience	Microwave
HSF-1	Proteintech	Microwave
Podoplanin	Acris Antibodies	Microwave
CD204	Trans Genic	Microwave

Benchmark ULTRA*: automated staining system