Clinicopathological characterization of duodenal adenocarcinoma with high CD44 variant 9 expression

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Short title: CD44v9 expression in duodenal adenocarcinoma

Summary

Background: CD44 has been identified as a cancer stem cell (CSC) biomarker in various malignancies.

Aims:To elucidate the clinicopathological features of CD44v9-positive cells in DA.

Results: Twenty-nine DA patients were selected from medical archives at our hospital. CD44v9 expression was analyzed together with that of MUC2, MUC5AC, and MUC6 by immunohistochemistry. High levels of CD44v9 expression weakly correlated with inflammatory cell infiltration ($r = 0.431$, $p = 0.020$) and MUC6 expression ($r = 0.425$, $p = 0.022$). Furthermore, double immunofluorescence staining of CD44v9 and Ki67 or cleaved caspase 3 (CC3) was performed. High- and low-density areas of CD44v9-positive cells were designated as CD44v9-positive and -negative areas, respectively. Within CD44v9-positive areas, the level of Ki67-positivity among CD44v9-positive cells was significantly lower than that of CD44v9-negative cells ($p = 0.002$). Meanwhile, the level of CC3-positivity among CD44v9-positive cells was significantly lower than that of CD44v9-negative cells ($p < 0.001$).

Conclusions: CD44v9 expression may be affected by inflammatory cell infiltration and mucin phenotype in DA. CD44v9-positive cells also have a low mitotic activity and resist apoptosis. These characteristics suggest that CD44v9-positive cells possess CSC-like properties in DA.

Key words: duodenal adenocarcinoma, CD44v9, Ki67, cleaved caspase 3

INTRODUCTION

Although the incidence of non-ampullary duodenal adenocarcinoma (DA) is uncommon, such tumors regularly undergo distant metastasis and have a poor prognosis [1-3]. While factors including tumor stage and tumor metastasis have prognostic value in many carcinomas, more robust prognostic factors are required.

Recent reports suggest that the presence of cancer stem cells (CSCs) is associated with clinical prognosis [4, 5]. CSCs have self renewal and can differentiation characteristics similar to normal stem cells. CSCs represent a small population of cells within a tumor, having low mitotic activity and exhibiting resistance to apoptosis. Furthermore CSCs promote tumor development [6]. CSCs are resistant to chemotherapy, promoting tumor recurrence and metastasis [7]. Efforts to detect and characterize CSC markers have identified CD44 [8], CD133 [9], CD166 [8], EpCAM [8], Lgr5 [10], BMI1 [11], Olfm4 [12], ALDH [13], Sox9 [14], Musashi-1 [15], NESTIN [16], and CD54 [17] as potential candidates in gastrointestinal carcinomas. However, few studies have reported CSC markers in DA.

CD44 is a single chain, single-pass, transmembrane glycoprotein which functions in cellular adhesion to the extracellular matrix. CD44 has been implicated in wide variety of physiological processes including leukocyte homing and activation, wound healing and cell migration, and tumor cell invasion and metastasis [18, 19]. CD44 is expressed on the surface of cancer cells where it assists hematogenous spread and metastasis through interaction with P- and L-selectins [20]. CD44 is also involved in epithelial-to-mesenchymal transition [21]. Recently, CD44 has been identified as a CSC marker in various solid carcinomas [22-26].

Alternative splicing yields several variant (CD44v) isoforms, and the expression

level of variant 9 (CD44v9) is correlated with the tumor stage, recurrence, and metastasis in gastric carcinoma [27-29]. However, whether CD44v9 is involved in CSC function is unknown. Binding of CD44v9 to the cysteine transporter subunit xCT can stabilize xCT expression and promote glutathione synthesis [30]. CD44v9 expression in cancer can therefore control accumulation of reactive oxygen species (ROS). Consistently, CD44v9 can promote tumor growth and chemoresistance [30, 31]. Therefore, CD44v9 has a functional pathophysiological role in cancer and serves as a useful pathological marker. As the expression level and role of CD44v9 in DA is poorly understood, we investigated the clinicopathological features of CD44v9-positive DA cells.

MATERIALS AND METHODS

Patients and materials

We identified 33 DA patients treated at Shinshu University Hospital, Matsumoto, Japan from 1997 to 2013. Of these, 4 cases were excluded as it was difficult to distinguish adenocarcinoma from dysplasia in these samples. This study was approved by the Ethics Committee of Shinshu University, Japan.

Histopathology, immunohistochemistry, and immunofluorescence

All specimens were fixed in 20% formaldehyde and embedded in paraffin. Serial 4-μm-thick sections were cut from these blocks and stained with hematoxylin and eosin, or immunostained. Antibodies used were anti-CD44v9 (1:5000; CosmoBio, Tokyo, Japan), anti-MUC2 (1:100; Novocastra, Newcastle upon Tyne, United Kingdom), anti-MUC5AC

(1:100; Novocastra), anti-MUC6 (1:100; Novocastra), anti-Ki67 (1:50, Dako, Glostrup, Denmark), and anti-cleaved caspase 3 (CC3; 1:50; Cell Signaling Technology, Tokyo, Japan).

The CD44v9-positive score was calculated as percentage of the total area of the carcinoma staining positive for CD44v9 under high-power magnification: negative, 0; <10%, 1+; 10–50%, 2+; and >50%, 3+. MUC2, MUC5AC, and MUC6 also were scored in a similar manner.

Double immunofluorescence staining was performed using CD44v9 and Ki67, or CD44v9 and CC3. Sections were incubated with each primary antibody for 1 h at room temperature followed by washing with phosphate-buffered saline. Sections were then incubated with anti-rat antibody conjugated with Alexa Fluor 488 and anti-mouse antibody conjugated with Alexa Fluor 568 (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature in the dark. Microscopic analysis was performed using an Axioplan II imaging microscope (Zeiss, Germany) equipped with an HBO 100 mercury lamp and filter sets for DAPI, FITC, and Texas Red. Images were captured and processed using the Isis/mFISH imaging system (Metasystems, Germany). The degree of CD44v9-positive staining for each patient was based on the number of CD44v9-positive cells per high power field (400× magnification) from three sites within the tissue section, and the mean value of CD44v9-positivity for each case was used in statistical calculations. For each patient, expression levels of Ki67 and CC3 were also measured within the CD44v9-positive areas. The number of cells staining double-positive for CD44v9 and Ki67 or CD44v9 and CC3 was also determined.

The grade of inflammation was scored using the updated Sydney system (Houston classification) of gastritis, with scores ranging from 0–3.

Statistical Analysis

Statistical analysis was performed using JMP version 10 (SAS Institute Japan, Tokyo, Japan). The Spearman's rank correlation coefficient was used to assess correlation. The Wilcoxon rank sum test was applied to assess the statistical significance of associations between CD44v9 expression and that of Ki67 or CC3. Univariate survival analysis was carried out according to the Kaplan–Meier method, and differences in survival curves were assessed using the log-rank test. Multivariate analyses were calculated according to the Cox regression model. A p value < 0.05 was considered significant.

RSULTS

Clinicopathological data, localization, and frequency of CD44v9

Clinicopathological data is depicted in Table 1. CD44v9-positive cells were detected in 25 of 29 (86.2%) of DA cases. The amount of CD44v9-positive cells varied from diffusely positive to scattered positive (Fig. 1A–H). The distribution of CD44v9-positive cells varied from the marginal region to the central region (Fig. 1F, 1G). Tumor cells expressing CD44v9 were also identified at the invasive front (Fig. 1H).

CD44v9 expression and clinicopathological data

Correlations between CD44v9 expression and clinicopathological characteristics of patients with DA are summarized in Table 2. There was moderate correlation between CD44v9 expression and mononuclear cell infiltration (r = 0.431, p = 0.02) (Fig. 1D, 1H), and CD44v9 expression and MUC6 expression (r = 0.425, p = 0.022). However,

CD44v9 expression was not correlated with age, grade, tumor stage, mucin phenotypes , neutrophil infiltration except MUC6 expression, or mononuclear cell infiltration.

Double immunofluorescence staining of CD44v9 and Ki67 or CC3

Within CD44v9-positive areas, 27.2% (7.3–34.4%) of CD44v9-positive cells were Ki67-positive and 55.3% (27.2–88.7%) of CD44v9-negative cells were Ki67-positive (Fig. 2A). Of the Ki67-positive cells in the CD44v9-positive area, significantly fewer were CD44v9-positive than were CD44v9-negative ($p = 0.002$; Fig. 3A). Of the CC3-positive cells within the CD44v9-positive area, very few were CD44v9-positive (0–0.2%), while 5.8 (1.2–27.0%) were CD44v9-negative (Fig. 2B). Of the CC3-positive cells in the CD44v9-positive area, significantly fewer were CD44v9-positive than were CD44v9-negative ($p < 0.001$; Fig. 3B).

Correlation of tumor markers with clinicopathological data

We next assessed staining of several markers using a univariate log-rank test and a multivariate Cox proportional hazards model. For these analyses, CD44v9, MUC2, MUC5AC, and MUC6 staining, and neutrophil and mononuclear cell infiltration were scored as negative (0 or 1) or positive (2 or 3). The histological grade was classified low-grade (G1 or G2) or high-grade (G3). Tumor stage was also defined as low-stage (I or II) or high-stage (III or IV). Patient survival was analyzed using a log-rank test and Kaplan–Meier survival curves. Overall survival (OS) was significantly influenced by histological grade (Fig. 4A) and tumor stage (Fig. 4B). The mean OS of G3 patients was significantly lower than that of G1 and G2 patients ($p < 0.001$), and the mean OS of stage III and IV patients was much lower than that of stage I and II patients ($p < 0.001$).

However, there was no significant association between OS and CD44v9 status (p = 0.964; Fig. 4C). Furthermore, there was no significant association between OS and the score for MUC2, MUC5AC, MUC6, neutrophils, or mononuclear cells (p = 0.563, p = 0.075, p = 0.213, p = 0.797, and p = 0.698, respectively). Multivariate survival analysis was performed under inclusion of all factors that were significantly prognostic in univariate analyses (Table 3). Subsequently, only tumor stage was found to be an independent prognostic factor in all survival analyses (hazard ratio: 21.20, 95% CI: 2.78–431.83, p = 0.003).

DISCUSSION

We have evaluated the clinicopathological characteristics of CD44v9 expression in DA and identified that CD44v9 expression may be influenced by inflammatory cell infiltration and mucin phenotype in DA. Furthermore, CD44v9-positive cells exhibit a low mitotic activity and resist apoptosis. This suggests that CD44v9-positive DA cells may possess CSC-like features such as those of other CD44v9-positive carcinoma cells described previously [28, 32, 33]. Therefore, CD44v9 expression might represent a useful target for CSC therapy.

The conditions of oxidative stress present in the tumor environment may influence CD44v9 expression. Under such conditions, an excess of ROS is associated with cancer cell death [34]. ROS accumulation during the chronic inflammatory conditions of DA may therefore remove CD44v9-negative cells and facilitate survival of CD44v9-positive cells. This is supported by our finding that CD44v9 expression was positively-correlated with inflammatory cell infiltration. Moreover, Ishimoto et al. have reported

that CD44v9-positive cells demonstrated an enhanced ability to suppress the ROS production in human gastrointestinal cancer cells [30]. Together, these findings further support the notion that CD44v9-positive cells have CSC-like features.

CSCs are also resistant to apoptosis. We have identified a low level of CC3 expression by CD44v9-positive cells within CD44v9-positive areas, which suggests a low rate of apoptosis for these cells [35]. This finding further supports the notion that CD44v9-positive cells have CSC-like features.

Further evidence of the CSC-like properties of CD44v9-postive DA cells is provided by their low mitotic rate. While CSC mitotic rates have yet to be identified in most solid cancers, Rebecca et al. have reported that breast cancer CSC populations possess relatively few cells in the mitotic stage [36]. In hematopoietic disease, CSCs also survive in a dormant state after chemotherapy [37, 38]. We now describe CD44v9 as a functional marker that can be used to identify potentially dormant CSC in DA. The mitotic ability of CSCs likely depends on various factors, including cancer type, gene mutation, and the microenvironment.

Others have previously reported that CD44v9 expression is associated with prognosis in cancer of the stomach and tongue [29, 39]. However, CD44v9 expression was not associated with tumor stage in our current study. We also found that histological grade did not correlate with CD44v9 expression, similar to results from Yasui et al. [28]. Therefore, CD44v9 expression is not a valuable prognostic marker for DA.

Studies examining the utility of tumor stage and histological grade as prognostic indicators have yielded inconsistent results. Nodal metastasis has been reported to be an independent predictor of decreased survival in DA patients [3]. However, our analysis identified tumor stage as the only independent predictor of survival. Faisal et al. have

also reported that high tumor stage conferred poor prognosis in DA [40]. Furthermore, Ushiku et al. reported that MUC6 expression conferred a poor prognosis in DA [3]. We identified a positive correlation between CD44v9 expression and MUC6 expression, suggesting that higher CD44v9 expression might be associated with poor prognosis.

Our study is limited by its small sample size, which included only patients who underwent surgery at our hospital. Our results should therefore be verified using larger cohorts.

CD44v9-positive cells may possess CSC-like properties in DA. CD44v9 expression might also affect other prognostic indicators, and may itself be a marker of prognosis. Further studies are warranted to elucidate the precise role of CD44v9 expression in DA and its utility as a prognostic indicator.

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Conflict of interest

The authors declare that they have no conflict of interest.

Figure Legends

Fig. 1

Hematoxylin and eosin staining and immunohistochemical staining for CD44v9 in DA. A–D, Invasive adenocarcinomas were seen in each patient sample examined. E, More than 50% of tumor cells exhibited CD44v9 expression. F, Less than 10% of tumor cells exhibiting CD44v9 expression were found in the central area of DA. G, Less than 10% of tumor cells exhibiting CD44v9 expression were found in the marginal area of DA. H, Tumor cells exhibiting CD44v9 expression were identified at the invasive front. Original magnification for all micrographs 100×

Fig. 2

Immunofluorescence double-staining in DA. A, Immunofluorescence double-staining for CD44v9 (green) and Ki67 (red) within the CD44v9-positive area. Double-positive cells are indicated (arrows). Nuclear staining: blue. B, Immunofluorescence double-staining for CD44v9 (green) and CC3 (red) within the CD44v9-positive area. Double-positive cells are indicated (arrow). Nuclear staining: blue. Original magnification for all micrographs 200×

Fig. 3

Ki67 and CC3 staining within the CD44v9-positive area. A, The ratio of Ki67-positive cells that were CD44v9 positive compared with those that were CD44v9-negative. B, As for A, with CC3-positive cells.

Scores are expressed as minimum, 25^{th} and 75^{th} (percentiles), and maximum.

*, $p = 0.002$ (Wilcoxon rank sum test).

**, $p < 0.001$ (Wilcoxon rank sum test).

Fig. 4

Overall survival of patients with DA. A, Kaplan–Meier survival curves of patients with low-grade or high-grade tumors as assessed by examination of the CD44v9-positive area. B, Kaplan–Meier survival curves of patients with low-stage or high-stage tumors as assessed by examination of the CD44v9-positive area. C, Kaplan–Meier survival curves of patients with CD44v9-positive or CD44v9-negative tumors.

Table 1 Clinicopathological characteristics of 29 DA patients

Variable	Value	Number of patients
Median age (years)		
	≤64	15
	>64	14
Sex		
	Male	18
	Female	11
Histological Grade		
	G1	21
	G2	1
	G3	7
Tumor Stage		
	I	15
	IIA	3
	IIIA	6
	IIIB	2
	IV	3
CD44v9		
	0	4
	1	15
	2	4
	3	6
MUC2		
	0	14
	1	9
	2	1
	3	5
MUC5AC		
	0	10
	1	6
	2	0
	3	13
MUC6		
	0	8

	1	4
	2	5
	3	12
Neutrophil infiltration		
	0	23
	1	4
	2	2
	3	0
Mononuclear cell infiltration		
	0	0
	1	12
	2	10
	3	7

Table 2 Clinicopathological correlations of CD44v9 in DA

Variable	*r*	*p*
Age	−1.323	0.494
Grade	0.131	0.499
Stage	0.138	0.114
MUC2	0.023	0.906
MUC5AC	0.300	0.284
MUC6	0.425	0.022
Neutrophil infiltration	−0.028	0.886
Mononuclear cell infiltration	0.431	0.020

r = Spearman's rank correlation coefficient;
p = *p*-value

Table 3 Multivariate analysis for prognostic factors in patients with DA

Variable	HR	95% CI	*p*
Grade	3.02	0.61–22.60	0.182
Stage	21.20	2.78–431.83	0.003

HR: hazard ratio
CI: confidence interval
p = *p*-value

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