The Relationship of CD133, Histone Deacetylase 1 and Thrombospondin-1 in Gastric Cancer

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Abstract. *Background/Aim. Gastric cancer is one of the most common types of cancer. Cancer stem cells (CSCs) have been reported to play important roles in multiple cancer types. This study investigated the correlation between cluster of differentiation 133 (CD133), histone deacetylase 1 (HDAC1) and thrombospondin-1 (THBS1) expression in advanced gastric cancer. Materials and Methods: The study included 65 patients with gastric cancer with recurrence after surgery. Expression of CD133, HDAC1 and THBS1 was examined by immunohistochemistry. Prognostic factors were investigated by multivariate analysis using Cox's proportional hazard model. Results: Clinicopathological variables, including survival, of patients positive for CD133 expression (n=6, 23%), were compared with those without CD133 expression (n=20, 77%). Positive HDAC1 expression and THBS1 expression were observed in 34 (52%) and 17 (26%) patients, respectively. Using univariate analysis, positive expression of CD133 and negative expression of THBS1 predicted significantly worse prognosis. Multivariate analysis revealed CD133-positive and THBS1-negative expression were independent prognostic indicators. Conclusion: CD133 expression and THBS1 expression were prognostic factors, and a negative relationship between HDAC and THBS1 was observed in advanced gastric cancer. These biomarkers may help determine postoperative treatment in patients with gastric cancer.*

Gastric cancer is one of the most common types of cancer in the world. The majority of patients with gastric cancer are diagnosed at an advanced tumour stage. Despite the significant progress made in complete local tumour resection, the 5-year survival rate of patients with advanced gastric cancer remains less than 30% (1, 2).

Recently, cancer stem cells (CSCs) have been reported to play important roles in certain cancer types. cluster of differentiation 133 (CD133) was originally reported as a surface marker of haematopoietic stem cells and progenitor cells, and is known to be an important marker in solid cancer such as colonic cancer and glioma (3-7). Importantly, a CD133-positive subpopulation of colonic cancer cells was recently demonstrated to be highly enriched in tumour-initiating CSCs that have the ability to self-renew and recapitulate the bulk tumour population (3-5).

Another emerging feature of CSCs is the involvement of histone deacetylases (HDACs), which are enzymes implicated in the epigenetic modifications of transcription and regulate the expression of genes during cancer development and progression (8, 9). HDACs are known to play important roles in stem cell self-renewal, commitment and differentiation assessment. In fact, one strategy to target cancer cells involves the pharmacological inhibition of HDACs. Several HDAC inhibitors, of natural and synthetic origin, have been described to induce cell-cycle arrest in, and differentiation and apoptosis of human tumour cells (10, 11).

Thrombospondin-1 (THBS1) is a high-molecular-weight (450-kDa) multifunctional glycoprotein which was first described as a product of thrombin-stimulated platelets (12, 13). The function of THBS1 remains controversial. While some reports demonstrate that THBS1 has anti-angiogenic effects, opposing reports suggest it is pro-angiogenic. The anti-angiogenic effects of THBS1 have been reported in colorectal, lung, bladder, and breast cancer (14-16).

The aim of this study was to clarify the correlation between CD133, HDAC1 and THBS1 expression in advanced gastric cancer.

Materials and Methods

Patients. The subjects of this study were 65 patients with gastric cancer who experienced recurrence after they underwent surgical treatment between 2000 and 2010 at The University of Tokushima. Surgical specimens were examined pathologically using haematoxylin and

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Key Words: Gastric cancer, histone deacetylase, HDAC, CD133, thrombospondin-1, THBS1.

Table I. *Characteristics of study patients with advanced gastric cancer.*

Parameter	
Median age (range), years	64 (20-87)
Gender: male/female	52/13
Primary tumor (T): 2-4a/4b	49/16
Regional lymph node status (N): 0-2/3	50/15
Hepatic metastasis: 0/1	50/15
Perineural invasion: 0/1	24/41
Distant metastasis (M): 0/1	58/7
Differentiation: differentiated/undifferentiated	40/25

eosin-stained tissue preparations. This study was authorized in advance by the Institutional Review Board of The University of Tokushima Graduate School of Medical Science,(Approved number 1517) and all the patients provided written informed consent.

Immunohistochemistry. Formalin-fixed, paraffin-embedded samples were used in the study. Sections were serially cut at 5 μm, then dewaxed, deparaffinized in xylene, and rehydrated through a series of graded alcohols. For better antigen retrieval, the samples were boiled for 20 min in a microwave oven in a citrate buffer (pH 6.0). Endogenous peroxidases were blocked by 0.3 % hydrogen peroxidase treatment for 30 min. The samples were incubated in 5% goat serum for 60 min to prevent nonspecific antigen binding. The slides were incubated with primary antibodies overnight at 4˚C. We used the following primary antibodies and dilutions: 1:100 dilution of a mouse monoclonal antibody for CD133 (Abcam, Cambridge, Cambridgeshire, UK), 1:100 dilution of a goat polyclonal antibody for HDAC1 (Santa Cruz Biotechnology, Dallas, TX, USA), 1:100 dilution of a mouse monoclonal antibody for THBS1 (Sigma-Aldrich, St. Louis, MO, USA). The secondary peroxidase-labelled polymer conjugated to goat anti-mouse immunoglobulins was applied for 60 min. The sections were developed in 3,3-diaminobenzidine and counterstained with Mayer's haematoxylin. The slides were dehydrated through graded alcohols and coverslips were applied. For unbiased immunohistochemical staining, two investigators decided independently on the presence of positive cells on each slide. CD133 positivity was recorded if any cells of the tumour were stained in the cytoplasm (17,18). The frequency of positively stained cells in the tumour was as low as 0.5-2.0%, consistent with previous reports (Figure1A) (3,17,18). HDAC1-positive expression was determined by counting the number of tumour cells with nuclear staining; those in which 10% or more of the cells were positive were regarded as being HDAC1-positive expression according to previous reports (Figure 1B) (19, 20). THBS1 immunostaining was divided into two categories based on the percentage of stained cells and their staining intensities. The cases were classified as negative when <30% of the entire population of cells stained weakly or moderately, or positive when ≥30% of the population stained moderately to strongly) (Figure 1C) (12). For all items, three slides were examined per case.

Statistics. For comparison of continuous variables, the Mann-Whitney U-test was used, and the chi-squared test was applied for categorical data. Patient survival was calculated by the product limit method of Kaplan and Meier, and differences in survival between the groups were compared using the log-rank test. Prognostic factors were examined using univariate and multivariate analyses (Cox proportional hazards regression model). The continuous variables were generally classified into two groups, according to the median value of each variable. All statistical analysis was performed using statistical software (JMP 8.0.1; SAS, Cary, NC, USA). Statistical significance was defined as a *p*-value less than 0.05.

Table II. *Comparison of background variables between CD133-positive and -negative groups.*

Variable*	Patients (n=26)		*p*-Value (n=6)
	Negative	Positive (n=20)	
T4b	20%	17%	0.68
N3	25%	33%	0.9
H1	32%	0%	0.33
P•CY1	42%	67%	0.5
M1	60%	33%	0.5
ly2•3	80%	67%	0.9
v2•3	15%	17%	0.59
Undifferentiated	60%	67%	0.85

*Variables were defined by the 14th edition of the Japanese Classification of Gastric Carcinoma. T4b Tumor invades adjacent structures (SI); N3: metastasis in 7 or more regional lymph nodes; H1: hepatic metastasis; P1: peritoneal metastasis; CY1: peritoneal cytology positive for carcinoma cells; M1: distant metastasis; ly2: moderate lymphatic invasion; ly3: marked lymphatic invasion; v2: moderate venous invasion; v3: marked venous invasion; Undifferentiated: Poorly differentiated adenocarcinoma (por), Signet-ring cell carcinoma (sig) and Mucinous adenocarcinoma (muc).

Table III. *Comparison of background variables between HDAC1-positive and -negative groups.*

Variable	Patients (n=26)		*p*-Value
	Negative (n=31)	Positive (n=34)	
T4b	23%	26%	0.94
N3	23%	24%	0.84
H1	13%	32%	0.06
P•CY1	71%	56%	0.21
M1	10%	12%	0.79
ly2•3	44%	68%	0.07
v2•3	22%	15%	0.62
Undifferentiated	32%	44%	0.33

Results

Table I shows a comparison of background variables. Table II shows a comparison of background variables between the CD133-positive and -negative groups. A total of 26 patients were underwent immunohistochemistry of CD133. The clinicopathological variables, including survival of patients

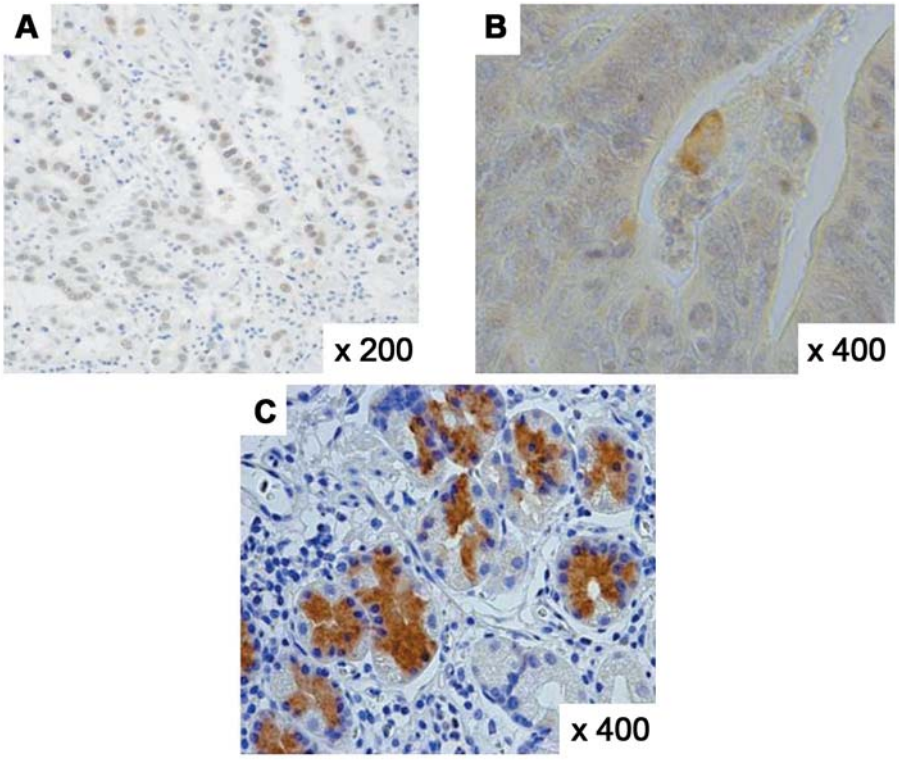

Figure 1. *Immunohistochemistry in gastric cancer. A: Positive expression for cluster of differentiation 133 (CD133) was detected in cytoplasm of cancer cells. B: Positive expression histone deacetylase 1 was determined by counting the number of tumour cells with nuclear staining. C: Positive expression for and thrombospondin-1 was determined by division into two categories based on the percentage of stained cells and their staining intensities.*

Table IV. *Comparison of background variables between THBS1-positive and -negative groups.*

Variable	Patients (n=65)		*p*-Value
	Negative (n=48)	Positive (n=17)	
T4b	23%	29%	0.84
N3	27%	12%	0.19
H1	25%	18%	0.54
P•CY1	76%	58%	0.18
M1	16%	0%	0.07
ly2•3	64%	41%	0.09
v2•3	24%	16%	0.54
Undifferentiated	71%	58%	0.54

Table V. *Univariate analysis of prognostic factors.*

Variable		3-Year survival rate (%)	*p*-Value
T4b	–/+	14.1/14.3	0.89
N3	–/+	14.7/10.0	0.48
H1	–/+	14.3/13.3	0.76
P•CY1	–/+	8.5/17.6	0.1
M1	–/+	18.4/11.1	0.12
ly2•3	–/+	24.2/7.9	0.43
v2•3	–/+	15.4/8.3	0.79
Undifferentiated	–/+	8.0/18.3	0.67
HDAC1	–/+	19.9/8.8	0.13
CD133	–/+	15.6/0	<0.05
THBS1	–/+	6.3/38.5	<0.05

who had positive CD133 expression (n=6, 23%) were compared with those without CD133 expression (n=20, 77%). There was no difference in variables except for the frequency of histological intrahepatic metastasis.

Table III gives a comparison of background variables between HDAC1-positive and -negative groups. The clinicopathological variables, including survival of patients who had positive HDAC1 expression (n=34, 52%) were compared with those without HDAC1 expression (n=31, 48%). Hepatic metastasis (p=0.06) and lymphatic invasion (p=0.07) in the HDAC1-positive group tended to be higher than that in the HDAC1-negative group.

Table IV compares the THBS1-positive and -negative groups. The clinicopathological variables, including survival

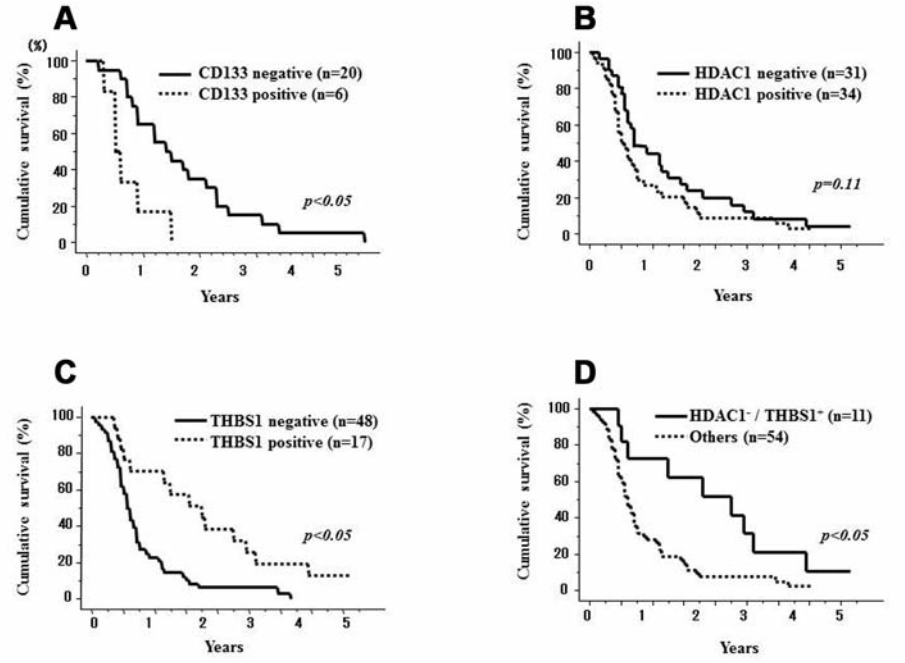

Figure 2. *Cumulative survival rate of patients by expression of CD133 (A), histone deacetylase 1 (HDAC1) (B), thrombospondin-1 (THBS1) (C) and combined expression of HDAC1 and THBS1 (D). The survival rate of the CD133-positive group was significantly worse than that of the CD133-negative group, as was that of the HDAC1-positive group. In contrast, the survival rate of the THBS1-positive group was significantly better than that of the THBS1-negative group. Survival was significantly better for those both HDAC1-negative and THBS1-positive.*

Table VI. *Multivariate analysis of prognostic factors.*

Variable	Relative risk	*p*-Value
CD133-positive *vs*. CD133-negative	3.47	<0.05
THBS1-negative *vs*. THBS1-positive	2.7	<0.05

of patients who had positive THBS1 expression (n=17, 26%) were compared with those without THBS1 expression (n=48, 74%). Distal metastasis in the THBS1-negative group tended to be higher than that in the THBS1-positive group (p=0.07).

Table V provides the univariate analysis of prognostic factors. Among clinicopathological variables, positive CD133 expression and negative THBS1 expression were significantly associated with a worse prognosis.

Figure 2 shows the comparison of survival curves. The 1-year survival rate in the CD133-positive group (16.7%) was significantly worse than that in the CD133-negative group (55%) (Figure 2A). The 5-year survival rate was not significantly different according to HDAC1 expression (Figure 2B). The 5-year survival rate of the THBS1-positive group (33.3%) was significantly better than that of the THBS1-negative group (4.2%) (Figure 2C). The 5-year survival rate of those who were both HDAC1-negative and THBS1-positive (18.1%) was significantly better than those with other expression (5.6%) (Figure 2D).

Table VI shows the result of the multivariate analysis of prognostic factors. Among the prognostic factors significant by the univariate analysis, positive CD133 expression and negative THBS1 expression were independent factors conferring a poor prognosis (relative risk of 3.47 and 2.70, respectively).

Discussion

As far as we are aware, this is the first report to clearly demonstrate the clinical role of the correlation between CD133, HDAC1 and THBS1 expression in advanced gastric cancer. This study shows two things. Firstly, that CD133 and THBS1 expression were independent prognostic factors. Secondly, that the group of patients with HDAC1-negative expression and THBS1-positive expression had significantly better prognosis than others.

CD133 expression has been reported to be a CSC marker of solid tumours and has been detected in several types of cancer (21-24). In gastric cancer, CD133-positive expression predicts a worse patient prognosis, relative to CD133-negative expression, suggesting CD133 expression might be a useful prognostic factor (21). In the current study, we

validated CD133 as a useful biomarker for gastric cancer prognosis. Previous studies have shown that CSCs are characteristically resistant to chemotherapy and radiotherapy. Therefore, patients with gastric cancer with high expression of CD133 might have less sensitivity to chemotherapy compared to patients with low CD133 expression.

HDACs can reverse epigenetic traits that characterize genes involved in the regulation of self-renewal or differentiation and improve embryonic developmental potential (25, 26). Moreover, the effect of HDACs on chromatin organization is associated with the regulation and maintenance of stem cell pluripotency in coordination with numerous signalling pathways (27, 28). One important pathway reported to be regulated by HDACs is the hypoxia-inducible factor 1 alpha (HIF1α) pathway. A previous report showed that HDAC inhibitors repress the function of HIF1α through inducing hyperacetylation of histones (19, 29). In addition, another study reported that HIF1α plays a crucial role in the expansion of CSCs in intrahepatic cholangiocarcinoma (3, 30, 31). Taken together, these data suggest HDAC1 might regulate cancer cell stemness through HIF1α activation.

In previous reports, THBS1 has been shown to have an antiangiogenic effect in various types of cancer. In contradiction, other reports suggest that THBS1 has a proangiogenic effect dependent on tumour type and environment (12-16). In the current study, THBS1 expression was a good prognostic factor, and dual HDAC-negative expression and THBS1-positive expression were indicative of a better prognosis.

In conclusion, CD133 expression and THBS1 expression represent good biomarkers. And, a relationship between HDAC1 and THBS1 was important in advanced gastric cancer. These biomarkers have the potential role in determining postoperative treatment of patients with gastric cancer.

Conflicts of interest

The Authors declare that they have no conflicts of interest in regard to this study.

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