Clinical Significance of Tartrate-resistant Acid Phosphatase Type-5 Expression in Human Gastric Cancer

MIKIO KAWAMURA, KOJI TANAKA, YUJI TOIYAMA, YOSHINAGA OKUGAWA, MASATO OKIGAMI, HIROMI YASUDA, SUSUMU SAIGUSA, MASAKI OHI, YASUHIRO INOUE, KEIICHI UCHIDA, YASUHIKO MOHRI and MASATO KUSUNOKI

Department of Gastrointestinal and Pediatric Surgery, Division of Reparative Medicine, Institute of Life Sciences, Mie University Graduate School of Medicine, Mie, Japan

Abstract. *Aim: The present study investigated the clinical significance of tartrate-resistant acid phosphatase type-5 (ACP5) expression in gastric cancer. Materials and Methods: In 150 specimens of gastric cancer and adjacent normal mucosa, expression of ACP5 protein and mRNA and was determined by immunohistochemical staining and quantitative real-time polymerase chain reaction, respectively. Results: Expression of ACP5 mRNA was significantly higher in cancer tissues than in adjacent normal mucosa. Elevated ACP5 mRNA was associated with lymph node metastasis and peritoneal dissemination. Logistic regression analysis revealed that elevated ACP5 expression was an independent risk factor for peritoneal dissemination and was associated with shorter survival. Immunohistochemical staining of primary carcinomas showed ACP5 to be expressed mainly in the cytoplasm. Conclusion: ACP5 is predictive of peritoneal dissemination in patients with gastric cancer, and might play a crucial role in the establishment of peritoneal dissemination.*

Gastric cancer is the second leading cause of cancer-related death worldwide (1). Patients with distantly metastatic gastric cancer have poor prognoses (2). Peritoneal dissemination is the most common mode of distant metastasis, and of recurrence after curative resection for gastric cancer (2, 3). Although various treatments have been used for peritoneal dissemination, good clinical outcome has not yet been obtained because of rich fibrous components and drug resistance (4-6). Peritoneal dissemination markedly impairs quality of life because of bowel obstruction, ascites retention, and resulting malnutrition (3). Prevention and control of metastasis, including peritoneal dissemination, is therefore expected to maintain quality of life and to improve prognosis.

Before metastasizing, cancer cells acquire biological capabilities enabling them to overcome host barriers. Cell invasion is a complex biological process that correlates with metastatic potential in human malignancies (7). Recently, tartrate-resistant acid phosphatase-5 (*ACP5*), considered to be a classic marker for bone resorption and osteoclast differentiation (8), was reported as a potent proinvasive and oncogenic gene (9, 10). To date, no studies have reported the significance of ACP5 in human gastric cancer. In this study, we evaluated *ACP5* mRNA in gastric cancer tissue and adjacent normal mucosa, and examined its relationship with clinicopathological parameters.

Materials and Methods

Patients and specimens. Specimens of gastric cancer and adjacent normal mucosa were obtained from 150 patients who underwent surgery between January 2000 and December 2009 at our Institution. Patients who received preoperative chemotherapy or radiotherapy were excluded. Samples were frozen in liquid nitrogen immediately after surgical resection, and were stored until RNA extraction at –80˚C. All investigations were performed in accordance with the Helsinki Declaration and approved by our Institutional Review Board (No. 2215). Written informed consent for their tissues to be used in this study was obtained from all participants before surgery. All patients were classified according to the Japanese Classification of Gastric Carcinoma (11).

RNA extraction and cDNA synthesis. Tumor specimens were homogenized with a Mixer Mill MM 300 homogenizer (Qiagen, Chatsworth, CA, USA). Total RNA was isolated using an RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from 5.0 mg total RNA with random hexamer primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Correspondence to: Mikio Kawamura, Department of Gastrointestinal and Pediatric Surgery, Division of Reparative Medicine, Institute of Life Sciences, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507, Japan. Tel: +81 592321111 (ext 5645), Fax: +81 592326968, e-mail: up2d8@clin.medic.mie-u.ac.jp

Key Words: ACP5, gastric cancer, peritoneal dissemination.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCR analysis was performed using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The relative abundance of target transcripts were measured using TaqMan probes for *ACP5* (Assay ID, Hs00356261_m1, TaqMan Gene Expression Assays; Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase: *GAPDH* (Assay ID, Hs02758991_g1; Applied Biosystems) was measured as an internal standard. The cDNA was amplified and quantified using Applied Biosystems StepOne Plus Real-Time PCR System, and analyzed by Software v2.2.2 (Applied Biosystems).

Quantitation of relative expression levels of ACP5. Relative gene expression was determined using the standard curve method. Standard curves and line equations were generated using five-fold serially diluted solutions of cDNA generated by reverse transcription of qPCR Human Reference Total RNA (Clontech, Mountain View, CA, USA). All standard curves were linear in the analyzed range with an acceptable correlation coefficient (R^2). Target gene expression was calculated from the standard curve followed by quantitative normalization of cDNA in each sample using *GAPDH* as an internal control. Assays were performed in duplicate for each sample; mean values were used for analysis.

Immunohistochemistry. Immunohistochemical analysis was performed as previously described (12). Sections were stained with primary mouse monoclonal antibody towards ACP5 (no. 49507, 1:100; Abcam, Cambridge, UK). Proteins were detected using the labeled streptavidin–biotin method (LASB2 kit/HRP; Dako Cytomation, Glostrup, Denmark). Appropriate positive and negative controls were used throughout.

Statistical analysis. Comparisons were performed using the non-parametric Wilcoxon signed-rank test for continuous variables. Analyses of nonparametric receiver operating characteristics (ROC) were performed to calculate cut-off values for *ACP5* expression according to the most accurate value obtained in all patients. Survival was evaluated by the Kaplan–Meier method. Logistic regression analysis was used to evaluate the independent influence of factors on lymph node metastasis and peritoneal dissemination. Differences between two groups were determined by log-rank test. Two-sided *p*-values of less than 0.05 were considered statistically significant.

Results

Patients' characteristics. Data from 150 patients (males: females 118:32; median age=69 years; range=18-90 years) were analyzed to determine associations between *ACP5* and clinicopathological findings and survival. The median follow-up period was 21.5 months (range=0.3-79.9 months) Clinical T stages were T1: n=13 (8.7%), T2: n=39 (26%), T3: n=71 (47.3%) and T4: n=27 (18%). Forty-three patients (28.6%) had lymph node metastasis; 15 (10%) had hepatic metastasis and 25 (16.7%) had peritoneal dissemination. There were 24 patients (16%) with stage I, 22 (14.7%) with stage II, 51 (34%) with stage III, and 53 with stage IV (35.3%) disease.

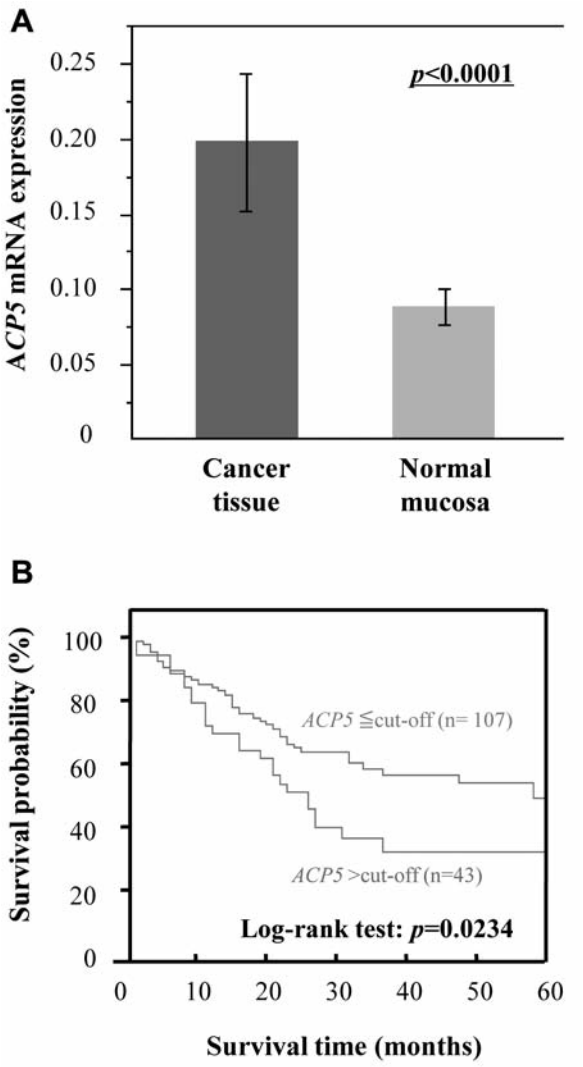

Figure 1. *A: Tartrate-resistant acid phosphatase type-5 (ACP5) expression levels in cancer tissue and normal mucosa. ACP5 mRNA was highly expressed in cancer tissue than adjacent normal mucosa (0.20±0.047 vs. 0.089±0.011; p<0.0001). B: Kaplan–Meier analysis of overall survival according to ACP5 mRNA expression levels. The cut-off value (0.146) was calculated by receiver operating characteristics analysis.*

Association of ACP5 expression with clinicopathological variables. Relative *ACP5* expression in patients with gastric cancer ranged from 0-4.73. *ACP5* expression in gastric cancer tissue was significantly higher than in adjacent normal mucosa (0.20±0.047 *vs.* 0.089±0.011; $p<0.0001$; Figure 1A). Table I shows the relationships between *ACP5* levels in cancer tissue and clinicopathological findings. *ACP5* expression was significantly positively correlated with lymph node metastasis ($p=0.0053$), peritoneal dissemination ($p=0.046$) and advanced stage ($p=0.034$).

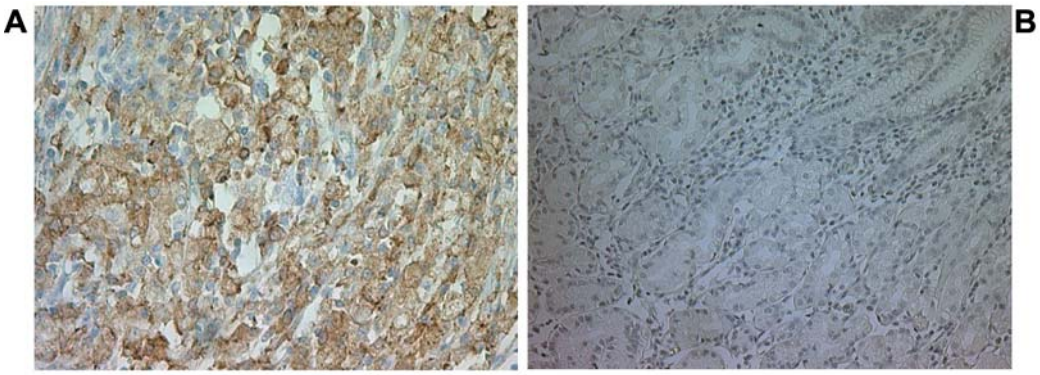

Figure 2. *Typical example of positive immunohistochemical staining of tartrate-resistant acid phosphatase type-5 (ACP5). ACP5 expression is diffusely expressed in the cytoplasm of cancer cells (A), but is practically undetectable in adjacent normal mucosa (B). Original magnification, ×400 (A), ×200 (B).*

To examine the predictive value of *ACP5* expression in prognosis of patients with gastric cancer, we defined the cut-off value according to the best predictive values calculated by ROC analysis (cut-off value=0.146). A higher level of *ACP5* expression was associated with poor prognosis (p=0.0234; Figure 2B).

Association between ACP5 expression and peritoneal dissemination. To examine the predictive value of *ACP5* for peritoneal dissemination and lymph node metastasis, we conducted a χ^2 test (Tables II and III). Significant clinical parameters that predicted peritoneal dissemination were T classification (T3 or 4; p=0.0064), blood vessel involvement (p=0.028) and high *ACP5* expression (p=0.0060). Furthermore, multivariate logistic analysis identified T classification (T3 or 4; p=0.025) and high ACP5 expression (p=0.014) as significant independent clinical parameters associated with peritoneal dissemination (Table II). Although high *ACP5* expression and lymph node metastasis were significantly related in the χ^2 test (p=0.043), *ACP5* expression was not predictive in multivariate analysis (Table III, p=0.1).

Immunohistochemistry for ACP5. We next investigated expression and cellular localization of ACP5 in gastric cancer tissue using immunohistochemistry. ACP5 was predominantly expressed in the cytoplasm of cancer cells (Figure 2A), but was practically undetectable in adjacent normal mucosa (Figure 2B).

Table I. *Relationships between tartrate-resistant acid phosphatase type 5 (ACP5) and clinicopathological factors.*

Variable	n	mRNA expression	*p*-Value
Gender			
Male	118	0.213±0.057	
Female	32	0.144±0.027	0.777
Age (years)			
<69 (median)	70	0.203±0.058	
≥69	80	0.195±0.054	0.821
Histological type			
Intestinal type	76	0.108±0.012	
Diffuse type	74	0.291±0.091	0.244
T Classification			
T1,T2	52	0.112±0.017	
T3,T4	98	0.243±0.069	0.179
Vessel involvement			
Negative	29	0.110±0.029	
Positive	121	0.219±0.056	0.051
Lymphatic vessel involvement			
Negative	12	0.124±0.056	
Positive	138	0.204±0.049	0.132
Lymph node metastasis			
Negative	43	0.094±0.018	
Positive	107	0.240±0.063	0.0053
Peritoneal dissemination			
Negative	125	0.288±0.035	
Positive	25	0.335±0.100	0.046
Hepatic metastasis			
Negative	135	0.203±0.050	
Positive	15	0.147±0.080	0.179
TNM stage			
I, II	46	0.105±0.018	
III, IV	104	0.239±0.065	0.034

Discussion

ACP5 is an enzyme secreted by mature bone-resorbing osteoclasts, and is considered to be a marker for bone resorption and osteoclast differentiation. Therefore, serum ACP5 activity can be used to assess osteolysis owing to bone metastasis in several human malignancies (13, 14). Recent studies have also indicated that ACP5 promotes invasion and distant metastasis of human melanoma and breast cancer cells (9). Overexpression of ACP5 in melanoma cells led to

Table II. *Univariate and multivariate analyses for predictors of peritoneal dissemination by logistic regression analysis.*

	Peritoneal dissemination			Multivariate logistic analysis	
Variable	Yes	No	*p*-Value	Odds ratio (95% CI)	*p*-Value
Histological type					
Diffuse	9	57	0.072		
Intestinal	17	67			
T Classification					
T 3,4	23	75	0.0064	4.35 (1.199-15.81)	0.025
T 1,2	3	49			
Vessel involvement					
Yes	25	96	0.028	5.51 (0.682-44.36)	0.11
No	1	28			
Lymphatic vessel involvement					
Yes	26	112	0.098		
No	0	12			
ACP5 mRNA expression					
≥0.146	13	29	0.0060	3.15 (1.266-7.833)	0.014
<0.146	13	95			

ACP5: Tartrate-resistant acid phosphatase type 5; CI: confidence interval.

Table III. *Univariate-and multivariate analyses for predictors of lymph node metastasis by logistic regression analysis.*

	Lymph node metastasis			Multivariate logistic analysis	
Variables	Yes	No	*p*-Value	Odds ratio (95% CI)	*p*-Value
Histological type					
Diffuse	51	23	0.52		
Intestinal	56	20			
T Classification					
T 3,4	78	20	0.0021	2.445 (1.103-5.418)	0.028
T 1,2	29	23			
Vessel involvement					
Yes	95	26	<0.0001	2.511 (0.876-7.196)	0.087
No	12	17			
Lymphatic vessel involvement					
Yes	104	34	0.0002	4.371 (0.832-22.94)	0.081
No	3	9			
ACP5 mRNA expression					
≥0.146	72	7	0.043	2.252 (0.848-5.978)	0.10
<0.146	35	36			

ACP5: Tartrate-resistant acid phosphatase type 5; CI: confidence interval

decreased focal adhesion kinase autophosphorylation at Tyr397, which promotes cell invasion. Overexpression of ACP5 in melanoma cells also causes morphological changes to spreading shape and increases cell movement (9). Xia *et al*. recently showed that ACP5 was significantly up-regulated in human HCC tissues compared with adjacent normal tissues, and patients with positive ACP5 expression had poorer prognoses than those with negative expression after surgery (15). They further uncovered a close relationship between ACP5 and forkhead box M1 (FOXM1), which is considered to be a master regulator of tumor metastasis by inducing epithelial–mesenchymal transition (16, 17). They showed that the inhibition of ACP5 expression significantly attenuated FOXM1-enhanced cell invasion in vitro and lung metastasis in vivo indicating that ACP5 is essential for FOXM1-mediated HCC metastasis (15). Li *et al*. reported that FOXM1 directly regulates *VEGF* gene expression, which is partially responsible for FOXM1-mediated promotion of

human gastric cancer angiogenesis, growth, and metastasis (18). ACP5 is thus closely related to several metastasis-associated genes. This accumulating evidence indicates that ACP5 might offer gastric cancer cells a microenvironment that promotes invasiveness, followed by distant metastasis.

In the present study, we have shown *ACP5* to be significantly up-regulated in human gastric cancer tissues compared to adjacent non-cancerous tissues. *ACP5* overexpression was significantly correlated to lymph node metastasis, peritoneal dissemination and advanced TNM stage. In multivariate analysis, *ACP5* expression was an independent risk factor for peritoneal dissemination. In addition, patients with elevated *ACP5* expression had shortened survival. As far as we are aware of, no studies have yet reported the clinicopathological significance of *ACP5* in human gastric cancer. Preoperative detection of peritoneal dissemination could aid disease management and selection of optimal therapy. Although further investigation is needed, *ACP5* expression in gastric cancer could be a marker for peritoneal dissemination or poor prognosis.

Acknowledgements

We would like to thank Motoko Ueeda and Chihiro Hibi for providing excellent technical assistance.

References

1 Bray F, Jemal A, Grey N, Ferlay J and Forman D: Global cancer transitions according to the Human Development Index (2008–2030): a population-based study. Lancet Oncology *13*: 790-801, 2012.

2 Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Ruschoff J, Kang YK; ToGA Trial Investigators: Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase III, open-label, randomised controlled trial. Lancet *376*: 687-697, 2010.

3 Coccolini F, Gheza F, Lotti M, Virzi S, Iusco D, Ghermandi C, Melotti R, Baiocchi G, Giulini SM, Ansaloni L and Catena F: Peritoneal carcinomatosis. World J Gastroenterol *19*: 6979-6994, 2013.

4 Fushida S, Kinoshita J, Yagi Y, Funaki H, Kinami S, Ninomiya I, Fujimura T, Nishimura G, Kayahara M and Ohta T: Dual anticancer effects of weekly intraperitoneal docetaxel in treatment of advanced gastric cancer patients with peritoneal carcinomatosis: a feasibility and pharmacokinetic study. Oncol Rep 19: 1305-1310, 2008.

5 Shimada S, Tanaka E, Marutsuka T, Honmyo U, Tokunaga H, Yagi Y, Aoki N and Ogawa M: Extensive intraoperative peritoneal lavage and chemotherapy for gastric cancer patients with peritoneal free cancer cells. Gastric cancer *5*: 168-172, 2002.

6 Tsukada T, Fushida S, Harada S, Terai S, Yagi Y, Kinoshita J, Oyama K, Tajima H, Ninomiya I, Fujimura T and Ohta T: Low-dose paclitaxel modulates tumour fibrosis in gastric cancer. Int J Oncol *42*: 1167-1174, 2013.

7 Liotta LA: Tumor invasion and metastases – role of the extracellular matrix: Rhoads Memorial Award lecture. Cancer Res *46*: 1-7, 1986.

8 Halleen JM: Tartrate-resistant acid phosphatase 5B is a specific and sensitive marker of bone resorption. Anticancer Res *23*: 1027-1029, 2003.

9 Scott KL, Nogueira C, Heffernan TP, van Doorn R, Dhakal S, Hanna JA, Min C, Jaskelioff M, Xiao Y, Wu CJ, Cameron LA, Perry SR, Zeid R, Feinberg T, Kim M, Vande Woude G, Granter SR, Bosenberg M, Chu GC, DePinho RA, Rimm DL and Chin L: Proinvasion metastasis drivers in early-stage melanoma are oncogenes. Cancer cell *20*: 92-103, 2011.

10 Endo-Munoz L, Cumming A, Rickwood D, Wilson D, Cueva C, Ng C, Strutton G, Cassady AI, Evdokiou A, Sommerville S, Dickinson I, Guminski A and Saunders NA: Loss of osteoclasts contributes to development of osteosarcoma pulmonary metastases. Cancer Res *70*: 7063-7072, 2010.

11 Sano T and Aiko T: New Japanese classifications and treatment guidelines for gastric cancer: revision concepts and major revised points. Gastric cancer *14*: 97-100, 2011.

12 Saigusa S, Toiyama Y, Tanaka K, Yokoe T, Okugawa Y, Kawamoto A, Yasuda H, Inoue Y, Miki C and Kusunoki M: Stromal CXCR4 and CXCL12 expression is associated with distant recurrence and poor prognosis in rectal cancer after chemoradiotherapy. Ann Surg Oncol *17*: 2051-2058, 2010.

13 Yao NS, Wu YY, Janckila AJ, Ku CH, Hsieh AT, Ho CL, Lee SH and Chao TY: Serum tartrate-resistant acid phosphatase 5b (TRACP5b) activity as a biomarker for bone metastasis in non-small cell lung cancer patients. Clin Chim Acta *412*: 181-185, 2011.

14 Wu YY, Janckila AJ, Ku CH, Yu CP, Yu JC, Lee SH, Liu HY, Yam LT and Chao TY: Serum tartrate-resistant acid phosphatase 5b activity as a prognostic marker of survival in breast cancer with bone metastasis. BMC cancer *10*: 158, 2010.

15 Xia L, Huang W, Tian D, Chen Z, Zhang L, Li Y, Hu H, Liu J, Chen Z, Tang G, Dou J, Sha S, Xu B, Liu C, Ma J, Zhang S, Li M, Fan D, Nie Y and Wu K: *ACP5*, a direct transcriptional target of FOXM1, promotes tumor metastasis and indicates poor prognosis in hepatocellular carcinoma. Oncogene 2013.

16 Raychaudhuri P and Park HJ: FoxM1: a master regulator of tumor metastasis. Cancer Res *71*: 4329-4333, 2011.

17 Bao B, Wang Z, Ali S, Kong D, Banerjee S, Ahmad A, Li Y, Azmi AS, Miele L and Sarkar FH: Over-expression of FoxM1 leads to epithelial–mesenchymal transition and cancer stem cell phenotype in pancreatic cancer cells. J Cell Biochem *112*: 2296-2306, 2011.

18 Li Q, Zhang N, Jia Z, Le X, Dai B, Wei D, Huang S, Tan D and Xie K: Critical role and regulation of transcription factor FOXM1 in human gastric cancer angiogenesis and progression. Cancer Res *69*: 3501-3509, 2009.

Received March 13, 2014
Revised May 13, 2014
Accepted May 14, 2014