Expression of METH-1 and METH-2 in Pancreatic Cancer

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ABSTRACT

Purpose: METH-1/hADAMTS-1 and METH-2/hADAMTS-8 are recently identified genes that inhibit angiogenesis, and the murine homologue, ADAMTS-1, shows metalloproteinase function. Because the significance of METH-1 and METH-2 has not been determined in solid tumors, we examined the mRNA expressions of these molecules in pancreatic cancer and hepatocellular carcinoma (HCC).

Experimental Design: METH-1 and METH-2 mRNA expressions were identified in six pancreatic cancer cell lines and were quantified by TaqMan reverse transcription-PCR in 18 paired samples of pancreatic cancer and surrounding noncancerous pancreas, and in 14 samples of pancreatic cancer. METH-1 mRNA expression was also examined in 16 noncancerous pancreas, and in 14 samples of pancreatic cancerous pancreas, but METH-2 was not. METH-1 expression was not shown to be involved in progression of pancreatic cancer through local invasion and lymph node metastasis.

Results: Four of six pancreatic cancer cell lines expressed METH-1, and 1/6 expressed METH-2. METH-1 was substantially expressed in both pancreatic cancer and noncancerous pancreas, but METH-2 was not. METH-1 expression in pancreatic cancer tissue was significantly lower than that in noncancerous pancreas (P = 0.002), and a similar result was obtained between HCC and cirrhotic liver (P = 0.003). METH-1 expression did not show a significant correlation with vascularity in pancreatic cancer or in HCC. However, pancreatic cancer with higher expression of METH-1 showed significantly severe lymph node metastasis or retroperitoneal invasion (P = 0.033 and P = 0.018, respectively) and worse prognosis (P = 0.038).

Conclusions: METH-1, which was initially reported to have a potent antiangiogenic effect, does not seem to be a predominant determinant of tumor vascularity in pancreatic cancer. Rather, METH-1 seems to be involved in progression of pancreatic cancer through local invasion and lymph node metastasis.

INTRODUCTION

METH-1/hADAMTS1 and METH-2/hADAMTS8 belong to the recently described metalloproteinase/ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) family of proteins. ADAMTS family represents a sequence similarity to a reprotysin subfamily, such as ADAM genes. Unlike other ADAM family members, the ADAMTS family does not have a transmembrane region and is secreted from cells in a catalytically active form. In addition to ADAM family sequence, the ADAMTS family contains TSP type 1 motif at its COOH-terminal half region. At this moment, eight ADAMTS genes, including aggrecanase and procollagen I N-proteinase, have been identified. METH-1/hADAMTS-1 and METH-2/hADAMTS-8 are proteins of Mr 110,000 and 98,000 respectively, and their structures include the signal peptide for secretion into the endoplasmic reticulum, a prodomain, a catalytic metalloprotease domain, a cysteine rich/disintegrin domain, and a variable number of TSP-like domains; three for METH-1 and two for METH-2 (1).

The murine homologue, called ADAMTS1 gene, which has 83.4% homology with METH-1, was first identified as a transcript expressed highly in a cachexigenic colon 26 cell line and induced by the inflammatory cytokine interleukin 1 (2). It has also been suggested to be an active metalloprotease by means of the proteinase trapping mechanism of α2macroglobulin (3) and of the induction of cellular degradation and dissolution of the follicle wall in the ovary (4), although the metalloprotease function of METH-1 has not been reported thus far. Furthermore, both METH-1 and METH-2 block angiogenesis more strongly than other angioinhibitory molecules such as angiotatin, endostatin, and TSP1 in the CAM and cornea pocket assay in a dose-dependent manner (1). Another study demonstrated that expression of METH-1 in tubular MVEC was much stronger than in simply proliferating MVEC, which may indicate an angioinhibitory effect of METH-1 (5).

In humans, METH-1 is widely expressed in various organs, whereas METH-2 is seen only in the lung (1). As far as we know, no reports have been published on the expression of METH-1 and METH-2 in solid cancer specimens. In the present study, we investigated the expression of METH-1 and METH-2 in pancreatic cancer tissue and in surrounding noncancerous pancreatic tissue specimens. We also inquired expression of METH-1 in a highly vascularized tumor, HCC, and in cirrhotic
METH1/hADAMTS1 and METH2/hADAMTS8 in Pancreas Cancer

To evaluate the clinical significance of METH-1 in pancreatic cancer, we analyzed the relationship between METH-1 and clinicopathological factors.

MATERIALS AND METHODS

Cell Line. Pancreatic cancer cell lines AsPC-1, BxPC-3, Capan-2, CFPAC-1, HPAC, and Panc-1 were purchased from the American Type Culture Collection. Cells were cultured as monolayers in the appropriate medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humid atmosphere of 5%CO2/95% air.

Patients and Tumor Samples. Thirty-two patients with pancreatic ductal adenocarcinoma, who had undergone pancreaticoduodenectomy at the Department of Surgery and Surgical Basic Science, Kyoto University, Japan, between January 1996 and June 2000 were included in this study. Patients with other pancreatic malignancies such as intraductal papillary mucinous adenocarcinoma, acinar cell carcinoma, and endocrine tumor were excluded. The average age at surgery was 65.2 years (range, 46–76 years). We chose only those patients who had survived ≥60 days after surgery to exclude perioperative mortality-related bias. Follow-up data were updated in December 31, 2000 [median follow-up was 10.4 months (range, 3.0–43.9 months)]. At that time, 20 patients had died of pancreatic cancer, 3 had died because of other factors, and 9 were alive. In this study, 18 paired samples of pancreatic cancer and surrounding noncancerous pancreas and 14 samples of pancreatic cancer were examined.

Sixteen patients with HCC who had undergone liver resection at the Department of Surgery and Surgical Basic Science, Kyoto University, between January 1993 and June 2000 were included in this study. All of the liver samples from patients were cirrhotic. Sixteen paired samples of HCC and cirrhotic liver were examined.

Histopathological diagnosis was confirmed by the Department of Pathology, Kyoto University Hospital. Pancreatic cancer was staged according to the pTNM (Unio Internationale Contra Cancrum) system (6). Tumor specimens were collected with a mixture consisting of cDNA derived from 100 ng of RNA, 0.2 μM each of upstream and downstream primers for the sequences of the METH-1 gene and METH-2 gene, 0.2 μM of deoxynucleotide triphosphate at a final concentration respectively, and 2.5 units of Taq DNA polymerase with reaction buffer (TaqKaRa, Kyoto, Japan) in a final volume of 50 μl. The reaction of PCR was performed for 35 cycles in a thermal cycler (Gene Amp PCR system 2400; PE Applied Biosystems, Foster City, CA) as follows: 15 s at 94°C for denaturation, 30 s at 58°C for annealing, and 30 s at 72°C for extension. Oligonucleotide primers to amplify METH-1 and METH-2 transcripts were designed on the human gene sequence METH-1, upstream primer: 5′-GGATGTCTATCAGCGCATC-3′ and downstream primer: 5′-TCACCGGCTCAGCTGCATCTC-3′; and METH-2 upstream primer: 5′-GGATGTCTATCAGCGCATC-3′ and downstream primer: 5′-TCACCGGCTCAGCTGCATCTC-3′, giving origin to a 312-bp band in the case of METH-1 and a 257-bp band in the case of METH-2.

TaqMan RT-PCR. To monitor gene expression, we used quantitative real-time RT-PCR analysis. This novel approach makes use of the 5′ exonuclease activity of the DNA polymerase (AmpliTaq Gold) (7–9). Briefly, within the amplification process defined by a gene-specific PCR primer pair, an oligonucleotide probe labeled with two fluorescent dyes is created and designated as TaqMan probe. As long as the probe is intact, the emission of the reporter dye (FAM) at the 5′ end is quenched by the second fluorescence dye (TAMRA) at the 3′ end. During the extension phase of PCR, the polymerase cleaves the TaqMan probe resulting in a release of the reporter dye. The increasing amount of reporter dye emission is detected by an automated sequence detector combined with analysis software (ABI Prism 7700 Sequence Detection System; PE Applied Biosystems). The algorithm normalizes the reporter signal to a passive reference. Next, the algorithm multiplies the SD of the background reporter signal in the first few cycles (in most PCR systems cycles 3–15, respectively) by a default factor of 10 to determine the threshold. The cycle at which this baseline level is exceeded is defined as the threshold cycle. The threshold cycle has a linear relationship with the logarithm of the initial template copy number.

RNA Extraction and RT-PCR. To perform RT-PCR, total cellular RNA was prepared using TRIZOL Reagent (Life Technologies, Inc., Rockville, MD) and cDNA was prepared by random priming from 1 μg of total RNA using a First-Strand cDNA Synthesis kit (Pharmacia Biotech, North Peapack, NJ) according to the manufacturer’s instructions. PCR was carried out with a mixture consisting of cDNA derived from 100 ng of RNA, 0.2 μM each of upstream and downstream primers for the non-tumor ratio; ADAMTS, a disintegrin and metalloprotease with thrombospondin motifs; METL, metalloprotease and thrombospondin domains.
cycles of 95°C for 15 s and 60°C for 1 min. Assessment of β-actin RNA for quality and normalization was done with the TaqMan β-actin Control Reagent kit (PE Applied Biosystems), which uses standard TaqMan probe chemistry. Direct sequence of the PCR product was performed with the automatic DNA sequencer AVI PEIAM 310 Genetic Analyzer (PE Applied Biosystems) using ABI dye-terminator chemistry according to the manufacturer’s protocol. The sequencing reactions were repeated at least twice.

**Immunohistochemical Staining for CD34.** Tissue samples were fixed in 4% paraformaldehyde or 10% formaldehyde in PBS, embedded in paraffin, and cut into consecutive, 4 μm-thick sections. Immunohistochemical staining was performed by the immunoperoxidase technique (10). In brief, paraffin sections were deparaffinized with xylene and graded ethanol and finally rehydrated in PBS. Trypsin digestion was done to unmask the antigen for CD34. Endogenous peroxidase was blocked by incubating the sections in 1% hydrogen peroxide in methanol for 15 min at room temperature. The sections were then incubated with appropriate dilutions of the primary antibody at 4°C overnight. As the antibody, a mouse monoclonal IgG antibody (QB-END/10; Novocastra Lab., Newcastle, United Kingdom) for CD34 at a 1:25 dilution was used. Then, the sections were incubated with the appropriate peroxidase-labeled secondary antibody for 1 h at room temperature, and the sections were incubated with diaminobenzidine substrate for 5 min. The sections were counterstained with Mayer’s hematoxylin solution.

**Evaluation of Immunostaining and Vessel Counting.** Vessels within the tumor were counted under the light microscopy after staining for CD34, based on the criteria of Weidner et al. (11). Three areas with the highest number of discrete microvessels were identified by scanning tumor sections at a low power (×100). After the areas of highest neovascularization were identified, photographs of each area were taken at 20 objective and 200 magnification (×20 objective and ×10 ocular, corresponding to a 0.75-mm² area) to accurately count microvessels, and the average counts of the three areas were calculated. Two investigators (T. M. and Y. M.), without the knowledge of clinicopathological features of the patients, counted microvessels simultaneously.

**Statistical Analyses.** Clinicopathological characteristics were compared in 32 patients with high and low METH-1 using the χ² test (or Fisher’s exact probability test). Patients with low and high METH-1 were classified by the estimated value under and over the median value. The microvessel count and METH-1 expression correlations were evaluated by Student’s t test. Kaplan-Meier method was used to calculate survival curves, and log-rank and generalized Wilcoxon test were performed to compare differences in survival rates of the patient who were subjected to curative surgery (15 patients versus 14 patients). Three patients subjected to noncurative surgery were excluded. All of the statistical analyses were done using JMP statistical software (version 3.02) for Macintosh. A P of < 0.05 was considered statistically significant.

**RESULTS**

**METH-1 and METH-2 Expression in Pancreatic Cancer Cell Lines.** METH-1 expression and METH-2 expression in pancreatic cancer cells were analyzed by semiquantitative RT-PCR (Fig. 1). METH-1 was detected as a single band corresponding to the product size of 321 bp, which was the expected size of the METH-1 product. METH-2 subunit was detected as a single 257-bp band, which was compatible with the expected size of the METH-2 product. METH-1 was expressed in BxPC-3, CFPAC-1, and Panc-1, whereas METH-2 was minimally expressed in HPAC and was not detected in the other five pancreatic cell lines.

**Validity of TaqMan RT-PCR.** Because abundant RNase in pancreatic tissue decreased the quality of mRNA when the sample was harvested after pancreatectomy, we used TaqMan RT-PCR instead of Northern blot analysis to quantify METH-1 mRNA. Fig. 2 shows the METH-1:β-actin ratio estimated by TaqMan RT-PCR and by semiquantitative RT-PCR.
with the mRNA samples from pancreatic cancer cell lines. There was a good correlation between them ($R^2 = 0.981$). We additionally analyzed the TaqMan RT-PCR product (76 bp) of METH-1 by direct sequencing, and the sequence totally coincided with METH-1 mRNA (National Center for Biotechnology Information nucleotide number AF060152) cds from 2105 to 2181.

**METH-1 Expression in Pancreatic Cancer and HCC.**

We then quantified METH-1 mRNA expression in 18 paired samples of pancreatic cancer and surrounding noncancerous pancreas and in 14 samples of pancreatic cancer by TaqMan RT-PCR. Because METH-1 was reported to have an antiangiogenic property, we also examined METH-1 expression in 16 pairs of cirrhotic livers and HCC, a tumor with potentially higher vascularity than pancreatic cancer. Fig. 3A shows METH-1 expression in pancreatic cancer and HCC, and Fig. 3B shows T:N ratio of METH-1 expression. T:N ratio represents a value dividing METH-1 mRNA in tumor by the one in paired noncancerous pancreas or paired cirrhotic liver. METH-1 mRNA expression in pancreatic cancer was $0.72 \pm 0.18$ when the $\beta$-actin expression was set to be 1, whereas that in noncancerous pancreas was $1.76 \pm 0.28$; METH-1 expression was significantly low in cancer tissues ($P = 0.002$; Fig. 2A). METH-1/$\beta$-actin ratio in HCC was $0.51 \pm 0.16$ and that in cirrhotic liver was $1.52 \pm 0.26$. HCC also expressed distinctly less METH-1 than cirrhotic liver did ($P = 0.003$). On the other hand, there was no difference in T:N ratio between pancreatic cancer and HCC ($0.268 \pm 0.070$ versus $0.341 \pm 0.076$).

**METH-2 Expression in Pancreatic Cancer.** Because METH-2 was seen in only one pancreatic cancer cell line, we expected the expression of METH-2 in pancreatic cancer tissues to be also minimal. Fig. 4 shows the METH-2 expression in six pancreatic cancers and noncancerous pancreas assessed by TaqMan RT-PCR. METH-2/$\beta$-actin ratio in pancreatic cancer was $0.02 \pm 0.02$, whereas that in noncancerous pancreas was $0.10 \pm 0.11$; values were quite lower compared with METH-1/$\beta$-actin ratios.

**Relationship between METH-1 Expression and Vascularity of the Tumor.** Because METH-1 has been suggested to be an angioinhibitory factor, we estimated the relationship between METH-1 expression and vascularity of the tumor (Fig. 5). Twenty pancreatic cancer specimens were stained with CD34, and the MVD, was determined. The range of MVD was between 12.8 and 72.7 vessels/mm², and the average was 39.4 vessels/mm². There was no correlation between METH-1 expression and MVD in pancreatic cancer. When similar analysis was done in HCC, there was no significant correlation between METH-1 expression and MVD, either. However, MVD of HCC was substantially higher compared with pancreatic cancer (data not shown).

**METH-1 Expression and Clinicopathological Feature.** The METH-1/$\beta$-actin ratio ranged from 0.07 to 5.05, and the median value was 0.32 in 32 pancreatic cancer samples. When
a median value of 0.32 was chosen as the cutoff point for discrimination of the 32 patients, 17 patients were categorized as showing high METH-1 expression and 15 as showing low METH-1 expression. Among the clinicopathological variables examined, age, gender, histological type, tumor size, pT, pM, and tumor stage, were equally distributed in these two subgroups. However, retroperitoneal invasion and lymph node metastasis were significantly more frequent in the high-METH-1 subgroup than in the low-METH-1 subgroup (47% versus 7% and 94% versus 60%, respectively; Table 1). When survival curves of the high-METH-1 subgroup and low-METH-1 subgroup subjected to curative surgery were compared, the survival curve for the 14 patients with tumors that highly expressed METH-1 was significantly worse than that of the 15 patients with tumors showing low expression of METH-1 (Fig. 6; log-rank test, \( P = 0.024 \); Wilcoxon’s test, \( P = 0.038 \)).

**DISCUSSION**

METH-1 and METH-2, which are members of the ADAMTS family, were first identified in 1999 by Vazquez et al. (1). METH-1 is a human orthologue of ADAMTS-1 by 83.4% amino acid identity. ADAMTS-1 is a member of the ADAM protein family that is involved in proteolytic modification of cell surface proteins and intercellular matrices (12–16). The unique structure of ADAMTS-1, characterized by the presence of thrombospondin type 1 motifs, is shared by other newly identified proteins in mammals and in *Caenorhabitis elegans* (17), which constitute the ADAMTS subfamily that perform well-conserved biological functions. On the other hand, METH-2 is a human orthologue of ADAMTS-8, and its biological function has not been elucidated as yet. METH-1 and METH-2 proteins also have antiangiogenic properties and inhibit the proliferation of endothelial cells but not of smooth muscle cells or fibroblasts (1).

In this study, we used TaqMan RT-PCR to quantify METH-1 and METH-2 mRNA expression. This technique has been used recently to evaluate mRNA expression both in the clinical field and in the research field, (18–21) and has the advantage that in a reaction where one is dealing with degraded template attributable to tissue processing or abundant RNase in the tissue, the amplicon size is kept to a minimum (typically 50–150 bp; Refs. 9, 22). Furthermore, to compensate for possible variations in the level of degradation among samples, parallel amplification of the housekeeping gene \( \beta \)-actin (assumed to be expressed at a constant level) was performed to

**Table 1** Univariate analysis of the associations between METH-1 expression and tumor characteristics

<table>
<thead>
<tr>
<th>Tumor characteristics</th>
<th>Low ((n = 15))</th>
<th>High ((n = 17))</th>
<th>( P^a )</th>
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<tr>
<td>Tumor size</td>
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<td>&lt;2 cm</td>
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<td>2–5 cm</td>
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<td>16 (94%)(^b)</td>
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<td>9</td>
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<tr>
<td>severe</td>
<td>1 (7%)(^b)</td>
<td>8 (47%)(^b)</td>
<td>0.018</td>
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\(^a\) \( P \) was calculated by \( \chi^2 \) test or Fisher’s exact test.

\(^b\) Figures in parentheses are the percentage of node involvement or severe retroperitoneal invasion in each subgroup.

**Fig. 6** Survival curve of the patients with high-METH-1 and low-METH-1 pancreatic cancer. The survival curve of patients with high-METH-1 tumors \((n = 15)\) was significantly worse than that of patients with low-METH-1 tumors \((n = 14)\); mean survival time: 11.9 months versus 24.0 months). \( P = 0.027 \) by log-rank test; \( P = 0.038 \) by Wilcoxon’s test.
generate a normalized METH-1 and METH-2. This index gives a quantitative figure for the degree of METH-1 and METH-2 expression in tissues. Because METH-1 and METH-2 genes were identified recently and because of the difficulties of Northern blot analysis of pancreatic cancer specimens, we applied this real-time RT-PCR to quantify METH-1 and METH-2 expression.

In this study, four of six pancreatic cancer cell lines expressed METH-1, whereas one expressed METH-2. METH-1 mRNA was substantially expressed in both pancreatic cancer and noncancerous pancreas, but METH-2 mRNA was not. When quantified with TaqMan RT-PCR, METH-1 mRNA expression in pancreatic cancer tissue was significantly lower than that in noncancerous pancreatic lesions, and similar results were obtained between HCC and cirrhotic liver. Reduction rate of METH-1 mRNA expression in pancreatic cancer was nearly equal to that in HCC. The expression of METH-1 showed no significant correlation with the MVD either in pancreatic cancer tissue or HCC. As for other angioinhibitory factors, it has been shown that TSP-2 expression itself was not related to vascularity in colon cancer, but when with VEGF expression, it inversely related with vascularity (23). TSP-1 expression demonstrated no correlation with MVD in glioma (24). Thus, together with these results, METH-1, which was initially reported to potently inhibit angiogenesis, does not seem to be a predominant determinant of the tumor vascularity in pancreatic cancer and HCC, but low levels of METH-1 might be involved in tumor angiogenesis.

We next analyzed the relationship between METH-1 mRNA expression and clinicopathological factors. Pancreatic cancer with lymph node metastases or severe retroperitoneal invasion showed significantly higher expression of METH-1. There were no significant correlations between METH-1 expression and tumor size or histological grade. Patients with high METH-1 expression showed significantly worse prognosis after curative surgery than those with low METH-1. The result might indicate a possible association between METH-1 and invasiveness of pancreatic cancer.

Recently, ADAMTS-1 was reported to cleave aggrecan, which is one of the major proteoglycans (25) found in the ECMs of articular cartilage. The core protein of aggrecan is substantially modified with chondroitin sulfate and keratan sulfate glycosaminoglycans, which serve to hydrate the cartilage tissue, providing properties of compression and elasticity (26, 27). Other ADAMTS family members, ADAMTS-4 and ADAMTS-5, have also been shown to cleave aggrecan at the Glu373-Ala374 bond within the interglobular domain (28–30). Moreover, ADAMTS-4 cleaves brevican (31), the newest member of the lecitan family of chondroitin sulfate proteoglycans, a family that locates at Glu395-Ser396, which bears striking homology to the aggrecanase cleavage site in aggrecan. Brevican expression is high at times and in places where glial cells are highly motile (32) and is dramatically increased in primary tumors (gliomas) of the central nervous system (33). On the other hand, Kuno and Matsushima (34) suggested that the ECM binding of ADAMTS-1 is mediated by the TSP motifs that possess high affinity for sulfated glycosaminoglycans in the ECM such as heparan sulfate. Syndecan-1 expression, one of the members of the cell surface transmembrane heparan sulfate proteoglycan family, was reported to be up-regulated in pancreatic cancers (35). Another heparan sulfate proteoglycan, glypican-1, is also overexpressed in pancreatic cancers, and transfection of the glypican-1 antisense construct attenuated the mitogenic response to epidermal growth factor 2 and heparin binding-epidermal growth factor in pancreatic cancer cell lines (36, 37). As reported in glioma, METH-1 may contribute to tumor invasion and lymph node metastasis by cleaving an unknown substrate in surrounding ECM such as heparan sulfate, and this may explain why pancreatic cancers with high METH-1 expression show severe retroperitoneal invasion and lymph node metastases.

In conclusion, this is the first study that demonstrates mRNA expression of METH-1/ADAMTS-1 and METH-2/ADAMTS-8 in pancreatic cancer tissue. Although METH-1 expression did not correlate with tumor vascularity, our clinicopathological results suggest that METH-1 is involved in the progression of pancreatic cancer through local invasion and lymph node metastases.

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