Clinical utility of quantitative RT-PCR targeted to α1,4-*N*-acetylglucosaminyltransferase mRNA for detection of pancreatic cancer

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(Received August 29, 2005/Revised October 30, 2005/Accepted November 3, 2005/Online publication January 23, 2006)

α1,4-*N*-Acetylglucosaminyltransferase (α4GnT) is a glycosyltransferase responsible for the biosynthesis of α1,4-GlcNAc-capped *O*-glycans, and is frequently expressed in pancreatic cancer cells but not peripheral blood cells. In the present study, we tested the clinical utility of α4GnT mRNA expressed in the mononuclear cell fraction of peripheral blood as a biomarker of pancreatic cancer. Total RNA isolated from the peripheral blood mononuclear cells from 55 pancreatic cancer patients, 10 chronic pancreatitis patients, and 70 cancer-free volunteers was analyzed quantitatively by reverse transcription-polymerase chain reaction with primers specific for α4GnT, and the expression level of α4GnT mRNA relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured. When the ratio of α4GnT to GAPDH transcripts exceeded a defined cut-off value, patients were considered to have pancreatic cancer. By these standards, 76.4% of the pancreatic cancer patients were detected by this assay. A strong correlation was obtained between positivity in this assay and the expression of α4GnT protein detected immunohistochemically in pancreatic cancer tissues resected subsequently, suggesting that α4GnT mRNA detected in the peripheral blood is derived from circulating pancreatic cancer cells. Although increased levels of α4GnT mRNA was detected in 40.0% of chronic pancreatitis patients and 17.1% of cancer-free volunteers, the expression levels were significantly lower than those seen in pancreatic cancer patients. These results suggest that quantitative analysis of α4GnT mRNA expressed in the mononuclear cell fraction of peripheral blood will contribute to the detection of pancreatic cancer. (*Cancer Sci* 2006; 97: 119–126)

Pancreatic cancer is one of the most intractable malignancies.[1,2] In particular, the 5-year survival rate of this neoplasm is the lowest of all types of cancer, and it is the fifth leading cause of cancer death in Japan.[3] The poor prognosis of pancreatic cancer is largely attributable to the difficulty in diagnosis of the disease at relatively early stages as well as the highly invasive character of the cancer cells, regardless of the tumor size. In fact, the vast majority of pancreatic cancer patients are diagnosed at advanced stages associated with clinical manifestations such as jaundice and back pain, likely due to the limitation of tumor markers available for the diagnosis of pancreatic cancer at potentially curable stages.[4] Several well-established biomarkers, including CEA,[5] CA19-9,[6] DU-PAN-2[7,8] and Span-1,[9] are available for the detection of pancreatic cancer, but it is also true that these biomarkers are not elevated in certain numbers of pancreatic cancer patients. Thus, in order to detect pancreatic cancer more efficiently, it is necessary to identify novel biomarkers that will be useful for its diagnosis.[10,11]

Mucous glycoproteins secreted from the gastroduodenal mucosa are heavily glycosylated and protect the mucosa against various pathogens and physical stresses. Among the oligosaccharides found in human gastrointestinal mucins, α1,4-GlcNAc-capped *O*-glycan is unique because its expression in normal tissues is limited to gastric gland mucous cells, Brunner's gland of the duodenal mucosa and accessory gland of the pancreaticobiliary tract.[12] Interestingly, this unique *O*-glycan is expressed frequently in neoplastic cells such as carcinomas of the stomach, bile duct and pancreas, as well as pancreatic intraepithelial neoplasia (PanIN-I, PanIN-II and PanIN-III),[13] thus it is regarded as a tumor-associated carbohydrate antigen for these tumors.[12] Recently we isolated a cDNA encoding human α4GnT, which is responsible for the biosynthesis of α1,4-GlcNAc-capped *O*-glycans, by expression cloning from a gastric mucosa cDNA library.[14] We subsequently demonstrated that α4GnT is expressed in the Golgi of gastric gland mucous cells and Brunner's glands in normal gastroduodenal mucosa as well as the Golgi of adenocarcinoma cells such as gastric, pancreatic and biliary tract cancers expressing α1,4-GlcNAc-capped *O*-glycans.[15–17]

Our previous study demonstrated that neither α4GnT RNA nor protein is detectable in the normal peripheral blood cells.[17] Thus, we quantitatively measured the expression levels of α4GnT mRNA in the mononuclear cell fraction of peripheral blood obtained from gastric cancer patients using RT-PCR and demonstrated that this assay is useful to detect, as well as monitor, gastric cancer.[17] The present study extends this assay for detection of pancreatic cancer using a technically

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Abbreviations: α4GnT, α1,4-*N*-Acetylglucosaminyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H. pylori, Helicobacter pylori; MTC, multiple tissue cDNA; ROC, receiver operating characteristic; RT-PCR, reverse transcription–polymerase chain reaction; TAMURA, 3′-6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine; TBS, Tris buffered saline.

doi: 10.1111/j.1349-7006.2006.00148.x
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improved modification. Specifically, levels of α4GnT mRNA in the mononuclear cell fraction of peripheral blood from pancreatic cancer patients were determined quantitatively using multiplex PCR employed to detect simultaneously both α4GnT and an internal standard gene in a single reaction tube.

Materials and Methods

Clinical samples

The present study involved 55 pancreatic cancer patients (34 men and 21 women; age range 45–92 years [mean ± SE, 68.5 ± 9.8 years]). For the reduction of jaundice, a drainage tube was placed in the common bile duct of 16 of 32 patients whose tumors were located in the pancreatic head, whereas none of the 23 patients whose tumor was located in the body or tail of the pancreas received such drainage. In addition to the pancreatic cancer patients, samples from 10 chronic pancreatitis patients (10 men; ages ranging from 55 to 75 years [65.8 ± 7.6]) and 70 volunteers (70 men; ages ranging from 31 to 90 years [69.4 ± 1.4]) were analyzed. These volunteers underwent a health screening and were verified to be cancer-free by routine examinations including abdominal ultrasonography. Written informed consent was obtained from all patients and volunteers prior to the study. Peripheral blood samples were taken from patients and volunteers. In pancreatic cancer patients, blood samples were collected before surgical resection of the primary tumor. When patients underwent endoscopic biopsy of the gastric mucosa, blood samples were taken minimally at 2-week intervals after biopsy.

In addition to the patients' samples, the Human Blood Fractions MTC Panel of the first-strand cDNA (Clontech, Palo Alto, CA, USA) was analyzed. This panel is composed of mononuclear cells (B cells, T cells and monocytes) pooled from 50 male or female Caucasians, resting $CD8^+$ cells pooled from 33 male or female Caucasians, resting $CD4^+$ cells pooled from 20 male or female Caucasians, resting $CD14^+$ cells pooled from 36 male or female Caucasians, resting $CD19^+$ cells pooled from 34 male or female Caucasians, $CD19^+$ cells activated with pokeweed mitogen pooled from four male or female Caucasians, mononuclear cells activated with pokeweed mitogen and concanavalin A pooled from four male or female Caucasians, $CD4^+$ cells activated with concanavalin A pooled from 12 male or female Caucasians, and $CD8^+$ cells activated with phytohemagglutinin pooled from eight male or female Caucasians. These samples were analyzed using a real-time quantitative RT-PCR assay. In parallel, tissue specimens of pancreatic cancer obtained from 23 patients who subsequently underwent surgical operation for removal of primary tumors were examined by immunohistochemistry, and the tumor stage was classified according to the tumor node metastasis classification system.[18] In addition, pancreatic tissue specimens of two cases operated for chronic pancreatitis were archived from the pathology files of Shinshu University Hospital, Matsumoto, Japan. The study protocol was approved by the Institutional Review Board of Shinshu University School of Medicine.

RNA extraction and cDNA synthesis

Five milliliters of peripheral blood was collected, treated with ethylene diamine tetraacetic acid to prevent coagulation, and layered on 3 mL of Lymphprep (Nycomed Pharma, Oslo, Norway) in a 15-mL polypropylene tube. The tube was centrifuged at 2000*g* for 30 min at 20°C. The mononuclear cell fraction was transferred to a new tube, resuspended in 5 mL phosphate-buffered saline, and then centrifuged at 3000*g* for 5 min. Total RNA was isolated from the pellet using a RNeasy Mini kit (Qiagen, Valencia, CA, USA), followed by DNaseI treatment. After inactivation of DNaseI, 11 µL of the DNaseI-treated RNA was incubated with 1 µL of 10 mM dNTPs and 1 µL of 0.5 mg/mL oligo$(dT)_{15}$ primer (Promega, Madison, WI, USA) at 65°C for 5 min. After chilling on ice, these mixed samples were then incubated with 4 µL of 5× first strand buffer, 1 µL of 0.1 M dithiothreitol, 1 µL of RNase inhibitor (Promega), and 1 µL of the reverse transcriptase SuperScript 2 (Invitrogen, Carlsbad, CA, USA) at 42°C for 1 h. The reaction was terminated by heating at 70°C for 15 min, and samples were then kept at –20°C until real-time quantitative RT-PCR analysis.

Real-time RT-PCR

Quantitation of α4GnT mRNA expressed in peripheral blood mononuclear cells as well as the Human Blood Fractions MTC Panel was carried out using an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA) as described previously, with minor modifications.[17] On the basis of the published human *α4GnT* sequence,[14] specific primer pairs and probes were designed using the Primer Express program (PE Applied Biosystems). Forward and reverse primers for human *α4GnT* were 5′-GTTTTCCTCTTCCC-TTTGGATATGA-3′ (nucleotides +340 to +364; the first nucleotide of the initiation methionine codon is +1) and 5′-AGCTGATGTGGAGCCAGTTTCT-3′ (nucleotides +427 to +448), respectively. These primers were designed to hybridize to different exons of the *α4GnT* gene to avoid amplifying genomic DNA. The TaqMan probe was synthesized as 5′-TGGTACAATCAAATCAACGCCAGCGC-3′ (nucleotides +397 to +422) by PE Applied Biosystems, and it carried a 5′-6-carboxyfluorescein reporter label and a TAMURA quencher group. To normalize α4GnT mRNA expression levels, a housekeeping gene, *GAPDH*, was quantitatively analyzed simultaneously as a control. To construct a standard curve, 10-fold dilutions of the plasmid cDNA harboring *α4GnT* (pcDNAI-α4GnT) ranging from 3×10^{-2} to 3×10^{-10} µg/mL, corresponding to 5×10^9 to 5×10^1 copies/mL were prepared. Similarly, a 10-fold dilution of the plasmid cDNA harboring a partial cDNA sequence of *GAPDH* (pCR2.1-GAPDH), which was constructed as described previously,[17] was prepared from 2.3×10^{-2} to 2.3×10^{-10} µg/mL, corresponding to 5×10^9 to 5×10^1 copies/mL.

Multiplex PCR was carried out in 50 µL of reaction mixture containing 3 µL of cDNA sample, 25 µL of 1× Universal PCR Master Mix (PE Applied Biosystems), 800 nM of the primer set for α4GnT, 80 nM of the primer for *GAPDH*, 125 nM of the TaqMan probe for *α4GnT*, and 100 nM of the TaqMan probe for *GAPDH* that carries the 5′-VIC reporter label and 3′-TAMURA quencher group (PE Applied Biosystems). Reaction tubes were placed in the ABI PRISM 7700 Sequence Analyzer, preheated at 95°C for 10 min and amplified for 50 cycles of 95°C for 15 s, followed by 60°C for 1 min. The abundance of α4GnT mRNA and GAPDH mRNA was determined by comparison with the standard curves for

doi: 10.1111/j.1349-7006.2006.00148.x
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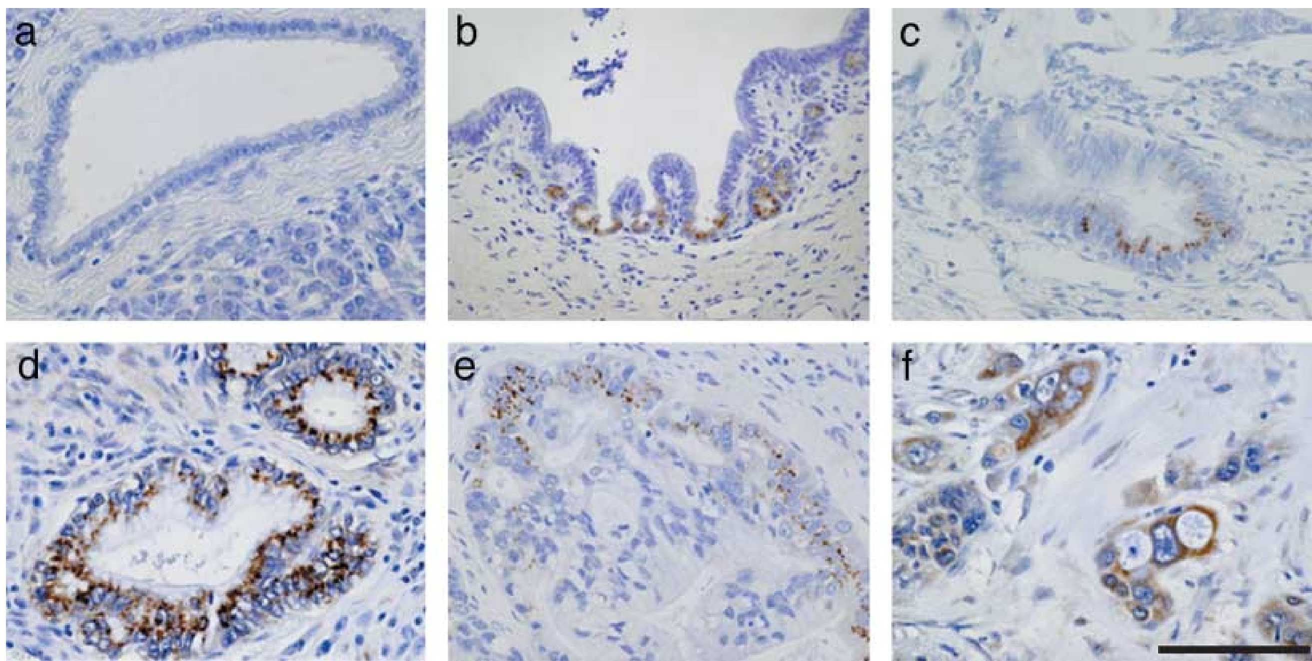

Fig. 1. Expression of α4GnT protein in the normal and neoplastic pancreatic tissues. α4GnT was detected by immunohistochemistry using the antiα4GnT antibody I17K. α4GnT is not expressed in the normal pancreatic duct (a), whereas it is expressed in the Golgi region of pancreatic ducts exhibiting PanIN-IB (b). The α4GnT protein is also expressed in the pancreatic ducts with PanIN-II found in chronic pancreatitis (c). In the pancreatic carcinoma, α4GnT protein is detected in well differentiated (d), moderately differentiated (e), and poorly differentiated (f) adenocarcinomas. Scale bar = 100 μM.

α4GnT and GAPDH, respectively, and the relative expression level of α4GnT mRNA was defined by multiplying the α4GnT : GAPDH mRNA ratio by 1.0×10^7. The assays were carried out in duplicate, and mean values of the two experiments were indicated.

Immunohistochemistry

To detect α4GnT protein in pancreatic cancer cells, 23 cases of the resected pancreatic cancer tissues were subjected to immunohistochemistry with the monospecific antiα4GnT polyclonal antibody, I17K, as described previously.[16] Briefly, 3 μM-thick sections were deparaffinized and treated with 0.3% H_2O_2 in methanol and then blocked with 1% normal goat serum in TBS. The sections were incubated with the antibody for 1.5 h. After washing with TBS, sections were incubated with biotinylated antirabbit IgG and then horseradish peroxidase-labeled streptavidin. The peroxidase reaction was developed with a diaminobenzidine/H_2O_2 solution, and counterstained with hematoxylin. In control experiments carried out by replacing the primary antibody with preimmune serum or omitting the primary antibody from the staining procedure, no specific staining was seen. Tissue specimens containing > 5% positively stained cancer cells were defined as positive, and the others were classified as negative according to previously described criteria.[19]

Enzyme immunoassay of biomarkers in patients' serum

Various biomarkers, including CEA, CA19-9, DU-PAN-2 and Span-1, in pancreatic cancer patients' serum were evaluated by enzyme immunoassay before surgery. CEA (cut-off value, 2.5 ng/mL) was measured using a CEA•Dainapack kit (Dainabot, Tokyo, Japan), and CA19-9 (cut-off value, 37 U/mL) was measured using an AxSYM CA19-9•Dainapack kit (Dainabot). DU-PAN-2 (cutoff value, 150 U/mL) and Span-1 (cutoff value, 30 U/mL) were measured by SRL at Tokyo, Japan.

Statistics

Statistical analyses comparing two independent groups categorized by the clinicopathological variables of pancreatic cancer were carried out using the Mann–Whitney *U*-test. Similarly, comparisons among more than three groups were carried out using the Kruskal–Wallis test. These analyses were performed using StatView 5.0 software (Abacus Concepts, Berkeley, CA, USA). In addition, a cut-off value was determined by constructing a ROC curve using StatMate III (ATMS, Tokyo, Japan). Statistical association between the expression of α4GnT protein in the resected pancreatic cancer tissues and the expression level of α4GnT mRNA determined in the mononuclear cell fraction of peripheral blood was evaluated using Fisher's test (Abacus Concepts). In these analyses, P-values < 0.05 were considered to be statistically significant.

Results

Expression of α4GnT protein in pancreatic cancer cells

In order to determine the expression of α4GnT protein in pancreatic ductal adenocarcinoma cells, immunohistochemistry using the antiα4GnT antibody I17K was undertaken with normal and neoplastic tissues of the pancreas, which were resected surgically at the time of operation. In the normal pancreas, α4GnT was not detected in the main or interlobular pancreatic ducts (Fig. 1a). By contrast, α4GnT protein was

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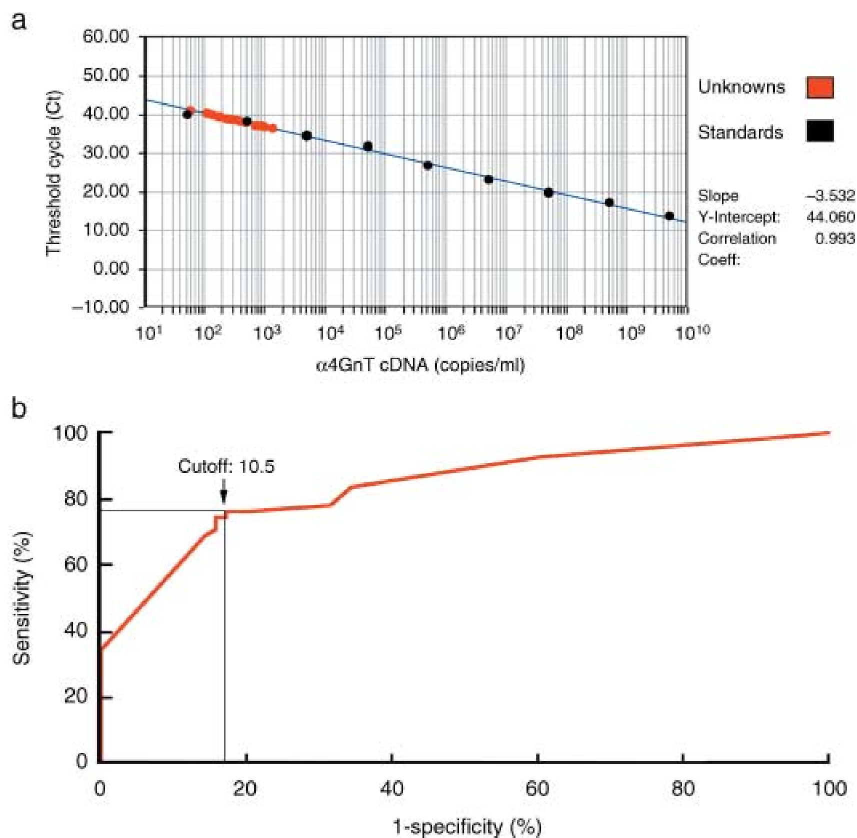

Fig. 2. Quantitative RT-PCR assay targeting α4GnT mRNA. (a) A standard curve for α4GnT was constructed by plotting serially diluted α4GnT cDNA, pcDNAI-α4GnT (black dots), where unknown samples from patients or cancer-free volunteers are indicated as red dots. (b) ROC curve was created by plotting the expression level of α4GnT mRNA in the peripheral blood from 55 pancreatic cancer patients and 70 cancer-free volunteers. Arrow denotes the cutoff value of 10.5, which best discriminates pancreatic cancer patients from cancer-free volunteers with 76.4% sensitivity and 82.9% specificity.

associated with the Golgi region of pancreatic ducts with PanIN-IB (Fig. 1b). α4GnT protein was also expressed in the pancreatic ducts with PanIN-II found in the inflammatory lesions of chronic pancreatitis in both of the two cases examined (Fig. 1c). In pancreatic cancer, α4GnT was detected in the Golgi of adenocarcinoma cells in 73.9% of 23 patients, irrespective of histological tumor type; that is, five of nine patients with well-differentiated adenocarcinoma (Fig. 1d), seven of eight patients with moderately differentiated adenocarcinoma (Fig. 1e) and five of six patients with poorly differentiated adenocarcinoma (Fig. 1f) were positive for α4GnT protein in cancer tissues.

Construction of a standard curve for the quantitative RT-PCR assay

The standard curve for α4GnT mRNA was constructed using 10-fold dilutions of α4GnT cDNA, pcDNAI-α4GnT (Fig. 2a). By defining the cycle number where fluorescence reached a detection threshold as Ct, we obtained a strong linear relationship between Ct and the log of the cDNA concentration. Based on the standard curve, levels of α4GnT mDNA ranging from 5×10^1 to 5×10^9 copies/mL were detected in a reaction tube. Similarly, GAPDH mRNA was detected ranging from 5×10^1 to 5×10^9 copies/mL based on the standard curve for GAPDH constructed using 10-fold dilutions of pCR2.1-GAPDH. Using these standard curves, the expression level of α4GnT mRNA relative to that of GAPDH mRNA was determined.

Determination of a cut-off value distinguishing pancreatic cancer patients from cancer-free volunteers

To most efficiently discriminate pancreatic cancer patients from cancer-free volunteers, a ROC curve was constructed (Fig. 2b). Thus, the α4GnT:GAPDH mRNA ratios multiplied by 1.0×10^7 were defined as the expression level of α4GnT, and the values determined in the mononuclear cell fraction of the peripheral blood from 55 patients with pancreatic cancer versus 70 cancer-free volunteers were plotted. By defining the cut-off value as 10.5, the optimal combination of 76.4% for sensitivity and 82.9% for specificity was obtained. Thus, we regarded a value as positive when expression levels of α4GnT mRNA greater than 10.5 were obtained in this assay.

Determination of the expression levels of α4GnT mRNA in peripheral blood samples from pancreatic cancer patients and cancer-free volunteers

Based on the criterion that the expression level of α4GnT mRNA should exceed the cut-off value of 10.5 for a positive result in this assay, we determined the expression levels of α4GnT mRNA in the mononuclear cell fraction of peripheral blood isolated from 55 pancreatic cancer patients and 70 cancer-free volunteers (Fig. 3).

In pancreatic cancer, 42 (76.4%) of 55 patients examined were positive for this assay, and the expression level of α4GnT mRNA was 37.50 ± 5.44 (mean ± SE). The expression level of α4GnT transcripts was then evaluated by clinicopathological variables including tumor location, stage, venous invasion,

doi: 10.1111/j.1349-7006.2006.00148.x
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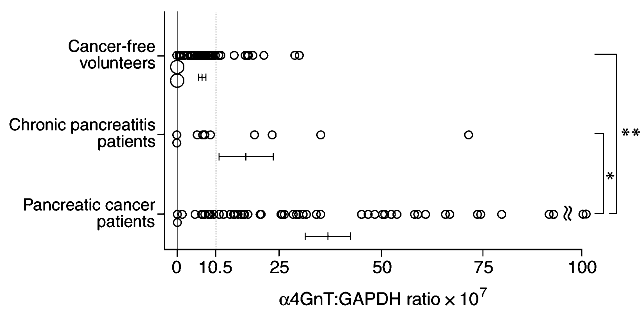

Fig. 3. Scatter plots indicating the expression level of α4GnT mRNA in the mononuclear cell fraction of peripheral blood measured by the quantitative RT-PCR. Small and large circles represent one and 10 individuals, respectively. Horizontal bars indicate mean ± SE. *$P < 0.05$, **$P < 0.001$.

lymphatic invasion and lymph node metastasis determined at subsequent surgical operation. Although the frequency of the positive patients and the expression level of α4GnT seemed to be associated with tumor progression, no significant statistical differences were seen in any clinicopathological variables examined (Table 1).

In addition, the expression level of α4GnT mRNA in the mononuclear cell fraction of blood samples from cancer-free volunteers was determined. Of the 70 cancer-free volunteers examined, 12 (17.1%) volunteers were found to be positive for this assay, but the expression level of α4GnT transcripts was 7.2 ± 0.9, which was significantly lower than that seen in pancreatic cancer patients ($P < 0.001$).

We then tested whether activated lymphocytes express α4GnT mRNA aberrantly by using the Human Blood Fractions MTC Panel, and it was shown that α4GnT mRNA was not detectable in any of the blood fractions examined, including activated lymphocytes.

Detection of α4GnT mRNA in peripheral blood samples from chronic pancreatitis patients

We next measured the expression level of α4GnT in the mononuclear cell fraction of peripheral blood isolated from chronic pancreatitis patients (Fig. 3). Of the 10 patients examined, four (40.0%) were classed as positive by exceeding the defined cutoff of 10.5. However the expression level of α4GnT mRNA was found to be 17.87 ± 6.98, which was again significantly lower than that seen in pancreatic cancer patients ($P < 0.05$). Statistically significant differences between expression levels were not seen between cancer-free volunteers and chronic pancreatitis patients.

Comparison of α4GnT mRNA with well-characterized biomarkers in pancreatic cancer patients

The results of the real-time PCR analysis of α4GnT mRNA expressed in the mononuclear cell fraction of peripheral blood from the pancreatic cancer patients were then compared with the results of enzyme immunoassays for well-characterized biomarkers including CEA, CA19-9, DU-PAN-2 and Span-1. As shown in Table 2, more than 74% of pancreatic patients were positive for either α4GnT or CA19-9, and CEA and DU-PAN-1 were found to less frequently detect pancreatic cancer compared with α4GnT and CA19-9.

Table 1. Frequency of pancreatic cancer patients positive for the α4GnT assay and correlation between expression levels of α4GnT mRNA in peripheral blood mononuclear cells and clinicopathological variables

Variable	Frequency of positive patients†		α4GnT mRNA‡ (mean ± SE)	*P*-value
	n	%		
Tumor location				
Head	25/32	78.1	37.95 ± 7.03	0.9728§
Body and tail	17/23	73.9	36.87 ± 8.78	
Tumor stage				
0	0/1	0	5.290	0.4571¶
II	2/3	66.7	29.44 ± 11.67	
III	7/8	87.5	32.85 ± 8.55	
IV	33/43	76.7	39.68 ± 6.72	
Venous invasion				
Negative	3/5	60.0	29.29 ± 11.20	0.6954§
Positive	17/19	89.5	36.97 ± 10.36	
Lymphatic invasion				
Negative	1/3	33.3	11.81 ± 4.82	0.0606§
Positive	19/21	90.5	38.74 ± 9.44	
Lymph node metastasis				
Negative	5/7	71.4	28.78 ± 9.93	0.8241§
Positive	15/17	88.2	38.09 ± 11.32	

†Expression levels greater than 10.5 were defined as positive. ‡α4GnT:GAPDH mRNA ratios multiplied by 1.0×10^7 are indicated. §Analyzed using the Mann–Whitney *U*-test. ¶Analyzed using the Kruskal–Wallis test.

We then tested whether a combined assay with α4GnT and another biomarker would detect pancreatic cancer patients more efficiently than the enzyme immunoassays targeting single biomarkers (Table 3). Notably, it was found that more than 86% of pancreatic cancer patients were detected when the α4GnT assay was combined with enzyme immunoassay for CEA, CA19-9, DU-PAN-2 or Span-1. In particular, 96.4% of pancreatic cancer patients were positive for either α4GnT mRNA or Span-1 or both, whereas 71.4% of the patients were positive for Span-1 alone.

Detection of α4GnT protein in resected pancreatic cancer tissues

Transcripts of α4GnT are not detectable in peripheral blood cells, including leukocytes, lymphocytes and monocytes.[17] Thus, it is possible that α4GnT mRNA detected in the mononuclear cell fraction of peripheral blood from pancreatic cancer patients is derived from circulating pancreatic cancer cells expressing α4GnT mRNA. To test this hypothesis, the results of real-time RT-PCR of α4GnT mRNA expressed in the peripheral blood were compared with those of α4GnT protein expressed in 23 cases of the subsequently resected pancreatic cancer tissues by immunohistochemistry with the antiα4GnT antibody I17K. In 19 patients positive for α4GnT transcripts in the peripheral blood, 17 were also positive for α4GnT protein in the resected pancreatic cancer tissues. By contrast, α4GnT protein was not detected in pancreatic cancer tissues of three of four patients who were also negative for the α4GnT mRNA assay. These results indicate a significant association between α4GnT mRNA in the peripheral blood

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Table 2. Frequency of pancreatic cancer patients detected using assays for α4GnT mRNA, CEA, CA19-9, DU-PAN-2 and SPan-1

Tumor stage	α4GnT mRNA (> 10.5)		CEA (> 2.5 ng/mL)		CA19-9 (> 37 U/mL)		DU-PAN-2 (> 150 U/mL)		SPan-1 (> 30 U/mL)	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%
0	0/1	0	0/1	0	0/1	0	NE	NE	NE	NE
II	2/3	66.7	0/3	0	0/3	0	0/2	0	0/2	0
III	7/8	87.5	3/8	37.5	6/8	75.0	1/8	12.5	2/7	28.6
IV	33/43	76.7	23/41	56.1	34/42	82.9	16/22	72.7	18/19	94.7
Total	42/55	76.4	26/53	49.1	40/54	74.1	17/32	53.1	20/28	71.4

NE, not evaluated.

Table 3. Frequency of pancreatic cancer patients detected using combined assays†

Biomarker	CEA (> 2.5 ng/mL)		CA19-9 (> 37 U/mL)		DU-PAN-2 (> 150 U/mL)		SPan-1 (> 30 U/mL)	
	n	%	*n*	%	*n*	%	*n*	%
α4GnT mRNA	46/53	86.8	48/54	88.9	30/32	93.8	27/28	96.4
CEA	–	–	43/53	81.1	24/32	75.0	21/28	75.0
CA19-9	–	–	–	–	27/32	84.4	23/28	82.1
DU-PAN-2	–	–	–	–	–	–	21/28	75.0

†Frequency of the patients positive for either or both biomarkers combined is indicated.

and α4GnT protein in pancreatic cancer tissues ($P = 0.0209$), suggesting that α4GnT mRNA detected in patients' peripheral blood is derived from circulating pancreatic cancer cells.

Discussion

α1,4-*N*-Acetylglucosaminyltransferase is a glycosyltransferase that mediates the transfer of GlcNAc with an α1,4-linkage from UDP-GlcNAc to βGal residues, forming α1,4-GlcNAc-capped *O*-glycans.[14] As shown in our previous studies and confirmed here, α4GnT is expressed frequently in pancreatic cancer cells as well as in gastric cancer cells, but not in peripheral blood cells.[15,17] Therefore, we used quantitative RT-PCR to determine the expression level of α4GnT mRNA in tumor cells circulating in the peripheral blood of pancreatic cancer patients. We primarily defined the cut-off value as 10.5 for this assay, based on the ROC curve, and could detect 76.4% of 55 pancreatic cancer patients. The significant correlation between the expression level of α4GnT mRNA in the peripheral blood detected by the RT-PCR assay and α4GnT protein detected in resected pancreatic cancer tissues by immunohistochemistry strongly suggests that α4GnT mRNA detected in the peripheral blood is derived from circulating pancreatic cancer cells. Although 40% of 10 chronic pancreatitis patients and 17.1% of 70 cancer-free volunteers were also positive by this assay, the expression levels of α4GnT mRNA in both groups were significantly lower than those seen in pancreatic cancer patients. These results indicate the clinical utility of real-time RT-PCR targeted to α4GnT mRNA for detection of pancreatic cancer.

The present study also revealed that the location of the pancreatic tumor does not alter the results of the assay (Table 1). It is known that early detection of pancreatic cancer occurring in the tail and body of the pancreas can be particularly difficult because jaundice, which is frequently associated with pancreatic head cancer, is not evident unless the common bile duct is affected by the tumor.[1] Thus, the assay demonstrated here will likely contribute to early detection of pancreatic body and tail cancers that are not associated with jaundice.

In the present study, we have also shown that the expression level of α4GnT mRNA in the peripheral blood from pancreatic cancer patients is elevated in a manner correlated with tumor stage (Table 1), suggesting that the number of cancer cells entering the peripheral blood is increased as the tumor progresses. Most recently, we have shown that α1,4-GlcNAc-capped *O*-glycans secreted from gastric gland mucous cells function as an antibiotic against *H. pylori* infection.[20] The role of these unique *O*-glycans expressed on pancreatic cancer cells remains unknown, and thus further study will be required to address this problem.

There are several biomarkers for pancreatic cancer, including CEA,[5] CA19-9,[6] DU-PAN-2[7,8] and Span-1.[9] Among them, CA19-9 is the most widely used in screening and monitoring of the disease.[21–24] We compared α4GnT with other biomarkers (including CA19-9) and found that the frequency of pancreatic cancer patients detected by α4GnT was much the same as that detected by CA19-9 (Table 2). The same analysis also revealed that DU-PAN-2 and CEA detected pancreatic cancer patients less frequently than α4GnT, CA19-9 and Span-1. It is noteworthy that two of three patients at stage II were positive for α4GnT mRNA, suggesting the possible usefulness of α4GnT mRNA for the early detection of pancreatic cancer. Further study on a larger number of patients with stages 0, I and II will be required to prove this possibility.

The present study demonstrated that the frequency of pancreatic cancer patients detected using enzyme immunoassays for CEA, CA19-9, DU-PAN-2 and Span-1 was increased substantially when combined with the α4GnT assay (Table 3). It is generally accepted that the quantitative RT-PCR assay requires much time and cost compared with enzyme immunoassay.

doi: 10.1111/j.1349-7006.2006.00148.x
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However, the combined assay with α4GnT mRNA can detect pancreatic cancer patients more efficiently when compared with the enzyme immunoassays targeting single biomarkers.

The present study also demonstrated that the expression levels of α4GnT mRNA were elevated in 40% of chronic pancreatitis patients and 17% of cancer-free volunteers, albeit at much lower levels than those of pancreatic cancer patients. Previously, we showed that significant amounts α4GnT mRNA were detected in patients with *H. pylori* infection or chronic gastroduodenal ulcers.[17] It has been reported that unexpected genes such as α-fetoprotein are transcribed in lymphocytes when they are activated,[25] suggesting the possibility that α4GnT mRNA might be induced in the activated lymphocytes circulating in the *H. pylori*-infected patients. However, as shown previously[17] and further confirmed here, we have demonstrated that α4GnT mRNA is not detectable in activated lymphocytes or resting lymphocytes. By contrast, we have also reported that extensive biopsy of the gastric mucosa results in elevation of the α4GnT mRNA level in peripheral blood.[26] These results combined suggest that the gastric gland mucous cells expressing α4GnT mRNA enter the bloodstream through the injured sites of gastric mucosa caused by inflammation or biopsy. Considering the high incidence of *H. pylori* infection in individuals over 40 years of age in Japan,[27,28] α4GnT mRNA detected in the cancer-free volunteers is most likely derived from gastric gland mucous cells that have entered the peripheral blood through injured sites of the gastric mucosa caused by *H. pylori* infection. We previously demonstrated that α4GnT mRNA is not detected in the peripheral blood of healthy volunteers without *H. pylori* infection.[17] Similary, it may also be possible that α4GnT mRNA detected in the chronic pancreatitis patients originated from the α4GnT-positive pancreatic duct epithelia entering the blood circulation, because the disruption of pancreatic ducts could occur in chronic pancreatitis (Fig. 1c).[29] Further studies will be of significance to identify the cells that elevate the α4GnT mRNA level in the peripheral blood of these non-cancerous patients.

Recently we demonstrated that α4GnT is expressed not only in pancreatic carcinoma cells but also in biliary tract carcinoma cells that produce α1,4-GlcNAc-capped *O*-glycans.[15] Thus, the α4GnT assay will also be applicable to the detection of patients with biliary tract cancers. We have shown that α4GnT mRNA was detected in three of five patients with biliary tract cancer.[17] It is of great significance to determine the clinical utility of the α4GnT assay for diagnosis of biliary tract cancer as well.

Collectively, our results obtained in the present study indicate that quantitative analysis of α4GnT mRNA expressed in the peripheral blood allowed us to detect pancreatic cancer cells expressing α1,4-GlcNAc-capped *O*-glycans. In order to clarify the clinical contribution of this assay system, prospective controlled trials are needed in the screening, diagnosis and monitoring of pancreatic cancer patients.

Acknowledgments

This study was supported by Grant-in-Aids for 3rd Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor and Welfare of Japan and Scientific Research on Priority Areas 14030032 and 14082201 from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to JN), and by NIH Grant CA48737 from the National Institutes of Health (to MF). The authors thank Drs Tsutomu Katsuyama and Fukuto Maruta for encouragement during this work. We are also grateful to Dr Elise Lamar for critical reading of the manuscript.

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doi: 10.1111/j.1349-7006.2006.00148.x
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