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Characterisation and protein expression profiling of annexins in colorectal cancer

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The annexins are family of calcium-regulated phospholipid-binding proteins with diverse roles in cell biology. Individual annexins have been implicated in tumour development and progression, and in this investigation a range of annexins have been studied in colorectal cancer. Annexins A1, A2, A4 and A11 were identified by comparative proteomic analysis to be overexpressed in colorectal cancer. Annexins A1, A2, A4 and A11 were further studied by immunohistochemistry with a colorectal cancer tissue microarray containing primary and metastatic colorectal cancer and also normal colon. There was significant increase in expression in annexins A1 ($P=0.01$), A2 ($P<0.001$), A4 ($P<0.001$) and A11 ($P<0.001$) in primary tumours compared with normal colon. There was increasing expression of annexins A2 ($P=0.001$), A4 ($P=0.03$) and A11 ($P=0.006$) with increasing tumour stage. An annexin expression profile was identified by *k*-means cluster analysis, and the annexin profile was associated with tumour stage ($P=0.01$) and also patient survival. Patients in annexin cluster group 1 (low annexin expression) had a better survival (log rank = 5.33, $P=0.02$) than patients in cluster group 2 (high annexins A4 and A11 expression). In conclusion, this study has shown that individual annexins are present in colorectal cancer, specific annexins are overexpressed in colorectal cancer and the annexin expression profile is associated with survival.

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The annexins are a multigene family of calcium-regulated phospholipid-binding proteins (Gerke and Moss, 2003; Gerke *et al*, 2005). The annexins are classified into five groups (A–E), and within each of these groups, individual annexins are identified numerically. Annexins in group A are human annexins, with group B referring to animal annexins without human orthologs, group C to fungi and moulds, group D to plants and group E to protists (Liemann and Huber, 1997; Rand, 2000; Hayes and Moss, 2004; Rescher and Gerke, 2004; Lim and Pervaiz, 2007). The characteristic annexin structural motif is a 70-amino-acid repeat, called the annexin repeat. Four annexin repeats packed into an α-helical disk are contained within the C-terminal polypeptide core (Gerke and Moss, 2003). While all annexins share this core region, the N-terminal varies widely between annexins, and it is this diversity of N-terminal amino-acid sequence that gives the individual annexins their functional differences and biological activities (Gerke and Moss, 2003; Gerke *et al*, 2005). There are 12 human annexin subfamilies (A1–A11 and A13) that have been found to have various intra- and extracellular roles in a range of cellular processes such as cell signalling, ion transport, cell division and apoptosis (Gerke and Moss, 2003; Gerke *et al*, 2005).

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All annexins share an ability to bind to negatively charged phospholipid membranes in a calcium-dependent manner. This property is found within the annexin core motif where the calcium- and membrane-binding sites are located. Annexins bind to the cytosolic surface of the plasma membrane and to organelle membranes such as the Golgi apparatus. This binding can be reversed by the removal of calcium, freeing the annexin from the phospholipid membrane. However, the functional significance of their reversible membrane-binding ability remains unknown in many annexins, although in some it is thought to be important for vesicle aggregation and membrane organisation (Liemann and Huber, 1997; Rand, 2000; Rescher and Gerke, 2004; Lim and Pervaiz, 2007). Although all annexins share this binding property, there is variation in calcium sensitivity and phospholipid specificity between individual annexins. For example, within one cell there can be differences in the distribution of annexins, with annexin A1 having an endosomal localisation, A2 to be found in cytosol and A4 being associated with the plasma membrane (Liemann and Huber, 1997).

Some annexins are capable of calcium-independent binding and several have roles in vesicle aggregation. Annexins A1, A2 and A11 function in cooperation with other calcium-binding proteins to form complexes while annexins A1, A2 and A5 interact with cytoskeletal proteins. Many annexins are involved in exocytic and endocytic pathways and some have roles in ion channel regulation (Gerke and Moss, 2003). Extracellularly, annexin A1 has a role in controlling the inflammatory response while annexin A2 is present on the external surface of endothelial cells, where it may act as a receptor for ligands, including plasminogen and tissue plasminogen

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His595Tyr Polymorphism in the Methionine Synthase Reductase (*MTRR*) Gene Is Associated With Pancreatic Cancer Risk

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Background & Aims: **This study attempts to elucidate a part of the genetic predisposition to the sporadic invasive ductal adenocarcinoma of the pancreas focusing on the genes implicated in the gene-environment interactions in carcinogenesis.** ***Methods:*** **First, 227 single nucleotide polymorphisms (SNPs) of 46 genes were genotyped on 198 cases and 182 controls. The SNPs, which showed a significant association, were further genotyped on additional samples to perform a joint analysis (total 317 cases vs 1232 controls). The gene selected by joint analysis was resequenced for a high-density SNP typing and a haplotype analysis on 702 cases and 785 controls. Function of the risk and wild-type haplotypes was assessed using cells transfected with complementary DNA (cDNA).** ***Results:*** **The joint analysis with multiple testing adjustment identified 2 SNPs on the methionine synthase reductase (*MTRR*) gene: rs162049 (intronic SNP), Fisher exact test, $P = .0018$; OR, 1.33; 95% CI: 1.11–1.60 and rs10380 (His595Tyr), Fisher exact test, $P = .0063$; OR, 1.45; 95% CI: 1.11–1.88. The SNPs remained significant in the recessive model after the permutation test for multiple testing (rs162049, $P = .024$; rs10380, $P = .023$) in the high-density analysis. Stable transfectants of the risk haplotype *MTRR* cDNA showed significantly elevated homocysteine levels in a culture medium, a lower level of the LINE-1 methylation, and a lower expression of the MTRR protein than did the transfectants with the wild-type haplotype cDNA.** ***Conclusions:*** **Our study suggested a common missense SNP of the *MTRR* gene as a novel pancreatic cancer susceptibility factor with a functional significance in folate-related metabolism and the genome-wide methylation status.**

Pancreatic cancer is the fifth leading cause of cancer mortality in males and the seventh in females in Japan. The 5-year survival rate is below 5%, making pancreatic cancer one of the most lethal cancers, with mortality approximately equal to its incidence rate. However, the prognosis of the curatively resectable cases is clearly better than the inoperable cases,[1] suggesting that an early diagnosis should be a valid approach to reduce pancreatic cancer mortality. New powerful biomarkers are actively being investigated, such as those based on proteomics technologies,[2] and identification of a high risk group of pancreatic cancer would have clinical importance in addition to advancing our basic understanding of pancreatic carcinogenesis.

Obvious familial aggregation with or without cancer-prone hereditary syndromes is considered to be present in less than 10% of pancreatic cancer cases.[3] Other factors, including diabetes mellitus,[4] chronic pancreatitis,[5] obesity,[6] and unidentified environmental factors[7] have been suggested as possible risk factors, but no consistent results have been observed. Although the pathogenesis of sporadic pancreatic cancer is still largely unknown, environmental and lifestyle-related risk factors have suspected roles, as they do for many other types of cancers. The only established risk factor for sporadic pancreatic cancer is cigarette smoking, with reported odds ratios ranging from 1.5 to 3.0.[8] On the other hand, a high consumption of fruit and vegetables, the major dietary folate sources, has been associated with a lower risk of pancreatic cancer,[9] suggesting a potential role of dietary factors that influence methyl-group availability in the development of pancreatic cancer.[10]

Even though most of specific carcinogenic or anticarcinogenic compounds are yet to be identified, molecular epidemiologic studies during the past decade have suggested that genetic polymorphisms of some phase I or phase II metabolizing enzymes are associated with the risk of several cancers.[11] Associations have been independently tested between pancreatic cancer risk and certain candidate gene polymorphisms,[12–15] and positive associ-

Abbreviations used in this paper: LD, linkage disequilibrium; MTRR, methionine synthase reductase; SNP, single nucleotide polymorphism.

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ations have been reported for the UDP-glucuronosyltransferase (*UGT1A7*)[16]; 5, 10-methylenetetrahydrofolate reductase (*MTHFR*)[17]; *NAT1*[18]; or x-ray repair cross-complementing group 1 (*XRCC1*).[19] However, a systemic survey has been lacking on the impact of candidate gene polymorphisms on the risk of pancreatic cancer.

Here, we present the results of a case-control study of pancreatic ductal adenocarcinoma to explore the association with 227 single nucleotide polymorphisms (SNPs) of 46 selected genes based on their possible involvement in gene-environment/lifestyle interactions. We report for the first time that a polymorphic variant of methionine synthase reductase (*MTRR*) is associated with pancreatic cancer susceptibility. Moreover, we suggested a functional significance of the SNP in the cells transfected with the *MTRR* complementary DNA (cDNA).

Patients and Methods

Patients

Between May 2001 and December 2007, 532 patients with pancreatic ductal adenocarcinoma were newly ascertained at the National Cancer Center Hospital, Tokyo, Japan. All the patients had histopathologically confirmed primary invasive ductal adenocarcinoma. In addition, 170 pancreatic cancer samples that were recruited in the BioBank Japan Project (http://www.src.riken.go.jp/eng/src/project/person.html) were used for a high-density genotyping (third typing). Control subjects consisted of 623 healthy volunteers at Keio University, Tokyo, Japan, 104 outpatients of the National Cancer Center Hospital, and 505 participants of a cancer screening program at the Iwata Hospital, Shizuoka, Japan. To participate in the genetic study, the control subjects were confirmed to have no past or present history of malignancy at the time of the unlinkable anonymization. Information on the family history of cancer and on smoking history was collected by a clinical research coordinator using a questionnaire, but the family history of pancreatic cancer was not available for our control group and for the case samples donated from BioBank Japan. Categories for smoking status were defined as the number of packs of cigarettes smoked per day multiplied by the number of years smoked (pack-years). The characteristics of the subjects are summarized in Table 1. The meaning of first to third genotyping is described in the Study Design and Statistics below. All the case and control subjects provided informed consent, and the study was approved by the Ethical Committee of the National Cancer Center.

SNPs Selection and Genotyping

The 46 genes and their 227 SNPs analyzed in the first screening of this study are listed in Supplementary Table 1 (see Supplementary Table 1 online at www.gastrojournal.org.) We previously published the selection and basic genetic epidemiologic characterization of 42 of the 46 genes,[20] which were selected from those encoding xenobiotic metabolic enzymes, DNA repair enzymes, and other stress-related proteins.

Genomic DNA was extracted from the peripheral blood leukocytes using the standard proteinase K/phenol-chloroform method. Genotyping was performed using either a mass spectroscopy-based method, MassARRAY (Sequenom, Inc, San Diego, CA), or a fluorogenic probe and 5′-nuclease-based method, TaqMan (Applied Biosystems, Foster City, CA.).

Study Design and Statistical Analyses

We performed the genotyping in a stepwise manner; the first genotyping was done on 227 SNPs of the 46 genes using 198 case and 182 control subjects. The SNPs that showed a significant difference ($P < .05$) in the allele distributions between the cases and controls were further genotyped in the additional 119 case and 1050 control subjects. For those SNPs subjected to the second typing, the data of the first and second additional typings were merged to identify SNPs by joint analysis[21,22] on the total of 317 cases vs 1232 controls. Power of the second stage, if it were analyzed independently, would be merely 28%. Therefore, we decided in our statistical analysis plan not to do any analysis on the second stage samples alone but to choose a joint analysis method. The following third typing was a high-density SNP typing to find the most likely candidate for a functionally responsible SNP of the finally selected gene using the SNPs identified by resequencing in this study. The third typing was done on 702 cases (317 cases used in the first and second typing plus additional 385 cases) and the 785 sex-matched controls selected from the 1232 controls used in the joint analysis.

Hardy-Weinberg equilibrium testing was used as a quality control for genotyping. Fisher exact test, crude odds ratios (OR), and 95% confidence intervals (CI) were used to compare allele, dominant, and recessive models between groups. In this study, the dominant model was defined as Aa+aa vs AA, where "A" and "a" are major and minor alleles, respectively, and the recessive model as aa vs AA+Aa. In the third typing, conditional logistic regression analyses were applied to evaluate differences in dominant and recessive inheritance distributions after adjustment for age and smoking status.

To identify disease-associated SNPs in a 2-stage association study by taking into consideration the proper balance of false-positive and false-negative errors, the following criteria were used: candidate SNPs were selected in the first typing by Fisher exact test at the significance level of 0.05 on the allele model. Next, disease-associated SNPs were identified in the joint analysis at the nominal significance level of 0.05 by a permutation test for multiple testing on the number of the SNPs analyzed in the joint analysis. In the third high-density SNP typing, the significance level of 0.05 was used after

Table 1. Characteristics of the Study Population

	First typing			Second typing			Third typing		
	Cases	Controls	[a]*P* value	Cases	Controls	[a]*P* value	Cases	Controls	[a]*P* value
Total number	198	182		119	1050		702	785	
Age, y									
<45	12	19		5	453		30	143	
46–55	49	86		21	130		120	174	
56–65	64	77		51	117		259	154	
>66	73	0	.49	42	350	<.0001	293	314	<.0001
Sex									
Male	115	157		69	635		432	470	
Female	83	25	<.0001	50	415	.62	270	315	.49
Smoking status									
Nonsmokers	76	116		44	621		259	385	
Smokers	122	65	<.0001	74	420	<.0001	439	400	<.0001
Unknown	0	1		1	9		4	0	
Pack-years[b]									
0	76	116		44	621		259	385	
>0 to <20	31	33		25	197		97	172	
≥20	91	32	<.0001	49	223	<.0001	265	228	<.0001
Smokers but pack-years unknown	0	0		0	0		77 (72[c])	0	
Unknown	0	1		1	9		4	0	
Diabetes									
Nondiabetic	189	182		108	1023		471	759	
Diabetic	9	0	.004	11	26	.001	61	25	.001
Unknown	0	0		0	1		170 (170[c])	1	
Location of the primary tumor									
Head	91	—		54	—		254	—	
Body or tail	100	—		63	—		255	—	
Whole pancreas	2	—	NA[d]	1	—	NA	13	—	NA
Unknown	5	—		1	—		180 (170[c])	—	
Clinical stage									
Stage I	1	—		1	—		9	—	
Stage II	4	—		2	—		20	—	
Stage III	21	—		5	—		72	—	
Stage IV	166	—	NA	111	—	NA	421	—	NA
Unknown	6	—		0	—		180 (170[c])	—	
Pancreatic cancer in first-degree relatives									
Yes	12	—		8	—		31	—	
No	159	—	NA	104	—	NA	460	—	NA
Information missing	27	—		7	—		211 (170[c])	—	

[a]*P* value by Fisher exact test.
[b]Pack-years defined as number of packs of cigarettes smoked per day times number of years of smoking.
[c]Number of the pancreatic cancer samples obtained from the BioBank Japan Project.
[d]Statistical calculation is not applicable because of no information for control group.

the permutation test for a multiple testing on the number of the SNPs analyzed in the third genotyping.[23] In a multiple testing problem that includes correlated hypotheses, a permutation test could achieve a higher power than the Bonferroni correction while controlling a family wise error rate. The permutation test is particularly relevant for the third stage typing because the high-density SNPs were in strong linkage disequilibrium (LD) with each other.

Haplotype structure and frequencies were inferred using the expectation-maximization algorithm.[24] Pair-wise LD measures D' and r^2 were estimated in the high-density SNP typing of *MTRR*, and, also, the LD map[25] was constructed from the genotype data of the control subjects. The haplotype-based association analysis was performed by Wald statistics and likelihood ratio tests for testing equality of haplotype frequencies between cases and controls.[26]

In the functional analysis of *MTRR* SNPs, Tukey multiple comparison test was applied to compare the vector-transduced cells. All statistical analyses were carried out using the Statistical Analysis System (SAS) software version 9.1 (SAS Institute Inc, Cary, NC).

Construction of MTRR *Expression Vector*

To construct the *MTRR* cDNA expression vectors to transduce the entire *MTRR* coding exons representing either the risk or wild-type haplotype for pancreatic can-

cer, cDNA was amplified from an individual who is homozygous for each haplotype using the published primers.[27] The polymerase chain reaction (PCR) products were cloned into the pcDNA3.1 D/V5-His vector (Invitrogen, Carlsbad, CA) downstream of the human cytomegalovirus promoter to express the MTRR protein fused with a V5-epitope tag on its C-terminus. Site-directed mutagenesis was performed according to the manufacturer's protocol (Promega, Madison, WI). The resulting pcDNA3.1 D/V5-His/MTRR vectors were designated pMTRR-L and pMTRR-H, and they harbor His595 (wild type) or Tyr595 (risk type) variant of the *MTRR* cDNA, respectively. A pcDNA3.1 D/V5-His/LacZ (named *pLacZ*) served as a negative control.

Transfection of MTRR *Expression Vectors into Cells*

The human 293 embryonic kidney cells were selected because of their high efficiency of transfection and low level of endogenous *MTRR* expression (Figure 1). Lipofectamine 2000 (Invitrogen) was used for a transfection, and, 48 hours later, Geneticin (G418) selection was started, and all the G418-resistant colonies in the plates were pooled and used for further analysis. Twenty-four hours after replating 5×10^5 G418 resistant 293 cells on 60-mm plates, homocysteine levels in the culture medium were measured, and Western blotting, reverse transcription (RT)-PCR and DNA methylation analyses were performed as described below.

Western Blot and RT-PCR Analyses on Transfected Cells

Western blot analyses of the G418-resistant 293 cells transfected by either pMTRR-H, pMTRR-L, or pLacZ were performed essentially as described.[28] The protein samples were size fractionated by 8%–16% SDS-polyacrylamide gel, and a rabbit anti-MTRR serum raised against synthetic MTRR C-terminal fragments (NH2-C+KTLATLKEEKRYLQ-COOH) was used for the immunoblotting. The specificity of the antiserum was validated by an enzyme-linked immunosorbent assay (ELISA) against the peptides used for the immunization. Commercially available antibodies were used for the detection of the V5 (Invitrogen) and β-actin (Sigma Chemical Co, St. Louis, MO) proteins.

Primers for RT-PCR analyses were as follows: for *MTRR*, the same primers as described in "Construction of *MTRR* expression vector"; for V5, 5′-GGGTCAAGACAATTCTG-CAG-3′ and 5′-TGGTGATGGTGATGATGA-3′; and, for β-actin, 5′-GACTACCTCATGAAGATCCT-3′ and 5′-GCG-GATGTCCACGTCACACT-3′. The cDNA was subjected to real-time RT-PCR (ABI Prism 7900 Sequence Detection System; Applied Biosystems) using β-actin as an internal control to normalize template concentration.

Homocysteine Levels in Culture Medium

Total homocysteine levels in the culture medium were measured using an IMX homocysteine assay (Abbott Diagnostics, Abbott Park, IL).[29] The limit of detection was 0.5 μmol/L of the culture medium. When 5 μmol/L to 25 μmol/L of homocysteine was added to the culture medium, the recovery rate was 98.74% ± 4.96% (mean ± SD). The between-assay coefficients of variation were lower than 7%.

Global Methylation Level Analysis by Combined Bisulfite Restriction Analysis

Sodium bisulfite treatment of genomic DNA from cells was performed using CpGenome DNA Modification Kit according to the manufacturer's instructions (Millipore, Billerica, MA). The bisulfite-treated DNA samples were PCR amplified for 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds using the primers for the LINE-1 repetitive elements[30]: 5′-CCGTA-AGGGGTTAGGGAGTTTTT-3′ and 5′-RTAAAACCCTC-CRAACCAAATATAAA-3′. The amplified bisulfite-treated DNA was digested in 10 μL reaction volume with 2 U of Hinf-1 and fractionated by the WAVE dHPLC system (Transgenomic, Inc, Omaha, NE). Image Quant software of the system was used to quantify the methylated and unmethylated sequences. Genomic DNA from Daudi and K562 cells were used as hyper- and hypomethylation controls, respectively, for LINE-1 methylation.[30]

Radioimmunoassay for MTRR

The rabbit antiserum raised against MTRR in this study binds to 30% of labeled MTRR at final dilutions of ×12,000. The synthetic MTRR C-terminal fragments were iodinated by the chloramine-T method, and the labeled peptide was purified by gel filtration on a column of Bio Gel P-4 and eluted with 0.1 N acetic acid. The specific activity was approximately 1 MBq/μg, and the lower limit of detection for MTRR was 100 pg/tube. The inter- and intraassay coefficients of variation were 5.0% and 5.7%, respectively.

Results

Pancreatic Cancer Association Studies

The allele frequencies of the 227 SNPs on the 46 candidate genes were analyzed first in the 198 patients with pancreatic ductal adenocarcinoma and in the 182 controls (first typing). Among the 227 SNPs, 8 SNPs in the *CYP19A1*, *NAT2*, *COMT*, and *MTRR* genes showed statistical significant differences ($P < .05$) in the allele distributions between cases and controls. The allele frequencies of the controls in this study were similar to those reported from the general population in 5 areas of Japan.[20]

The 8 SNPs selected by the first screening were genotyped on additional cases with newly diagnosed pancreatic ductal adenocarcinoma (n = 119) and control sub-

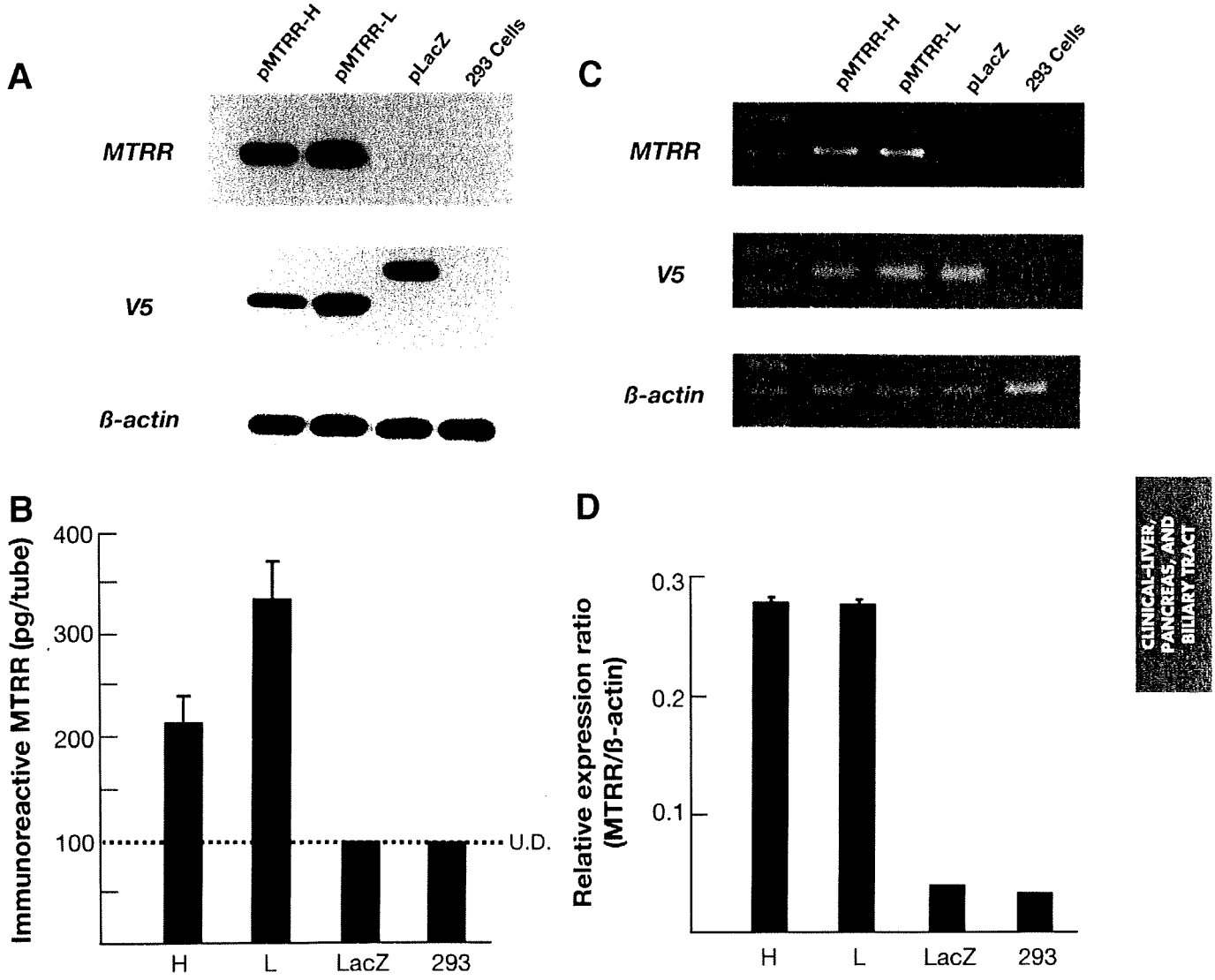

Figure 1. (*A*) MTRR protein expression in the 3 G418-resistant 293 cells transfected with the *MTRR* variants (L for His595 and H for Tyr595) or LacZ control vector analyzed by Western blotting using antibodies against MTRR or V5. Cell lysate from the mock- or *MTRR*-transfected 293 cells was fractionated by SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes and then probed with antibodies to MTRR, V5, and β-actin. β-actin was used as control for loading. (*B*) MTRR immunoreactivity of the cDNA-transfected cells. All samples were identical to those in *A*. The concentration of MTRR protein was measured by radioimmunoassay as described in the Patients and Methods section. The assays were performed in triplicate, and means ± SD were plotted. *U.D.*, undetectable range. (*C*) RT-PCR analysis of total cellular RNA obtained from the 3 G418-resistant 293 cells transfected with the *MTRR* variant cDNAs or LacZ control vector. The RT-PCR products amplified by specific primers for *MTRR* (*top*) and V5 (*middle*) were analyzed on agarose gels. β-actin was used as control for the sample loading. (*D*) Expression of *MTRR* by real-time RT-PCR. All samples were identical to those in *C*. The relative expression ratio is defined as the ratio of *MTRR* gene expression level to that of the internal reference gene β-actin. The assays were performed in triplicate, and means ± SD were plotted.

jects (n = 1050) to perform a joint analysis. Two SNPs, one in the coding (rs10380; His595Tyr) and the other in the intronic (rs162049; intronic SNP G/A) regions of the *MTRR* gene, were significantly associated with a risk of pancreatic cancer even after multiple testing adjustment (Table 2). The SNPs within the *MTRR* gene were in strong LD (ie, a nonrandom association between the alleles at 2 or more genetic loci in a natural breeding population) (Figure 2). After adjustment for age and smoking status, the *P* values for the recessive models of rs162049 and rs10380 SNPs were .002 and .012, respectively, in the joint analysis of a total of 317 cases and 1232 controls. The multivariate analysis showed that rs162049 remained statistically significant under multiple testing adjustment, indicating that the *MTRR* gene is associated with pancreatic cancer risk.

The possible interaction between the *MTRR* SNPs and smoking status was preliminarily examined in the strat-

Table 2. Genotype and Allele Frequencies of the 8 SNPs on the 4 Genes Selected by the First Screening

		Cases		Controls					
	Genotype	n	(%)	n	(%)	OR	95% CI	Fisher exact test,[a] *P* value	Permutation test,[b] *P* value
CYP19	Arg264Cys								
rs700519	G	454	71.8	1840	75.0				
	A	178	28.2	612	25.0	1.18	0.96–1.44	.10	.51
	GG	164	51.9	695	56.7				
	GA	126	39.9	450	36.7				
	AA	26	8.2	81	6.6				
	GG	164	51.9	695	56.7				
	GA+AA	152	48.1	531	43.3	1.21	0.94–1.57	.13	.61
CYP19	C/T								
rs10046	C	370	58.9	1276	54.5				
	T	258	41.1	1066	45.5	0.83	0.70–1.00	.052	.29
	CC	115	36.6	359	30.7				
	CT	140	44.6	558	47.7				
	TT	59	18.8	254	21.7				
	CC	115	36.6	359	30.7				
	CT+TT	199	63.4	812	69.3	0.77	0.59–1.00	.048	.28
CYP19	T/C								
rs1870049	T	484	77.3	1931	78.4				
	C	142	22.7	533	21.7	1.06	0.86–1.32	.59	.99
	TT	185	59.1	767	62.3				
	TC	114	36.4	397	32.2				
	CC	14	4.5	68	5.5				
	TT	185	59.1	767	62.3				
	TC+CC	128	40.9	465	37.7	1.14	0.88–1.48	.33	.93
NAT2	Val94Tyr								
rs1041983	C	461	72.9	1720	70.8				
	T	171	27.1	708	29.2	0.90	0.74–1.10	.32	.93
	CC	164	51.9	613	50.5				
	CT	133	42.1	494	40.7				
	TT	19	6.0	107	8.8				
	CC	164	51.9	613	50.5				
	CT+TT	152	48.1	601	49.5	0.95	0.73–1.22	.66	.99
COMT	His62His								
rs4633	C	464	73.4	1680	68.6				
	T	168	26.6	770	31.4	0.79	0.65–0.96	.020	.13
	CC	166	52.5	588	48.0				
	CT	132	41.8	504	41.1				
	TT	18	5.7	133	10.9				
	CC	166	52.5	588	48.0				
	CT+TT	150	47.5	637	52.0	0.83	0.65–1.08	.17	.71
MTRR	Ile22Met								
rs1801394	A	421	68.8	1764	71.8				
	G	191	31.2	692	28.2	1.16	0.95–1.41	.15	.66
	AA	145	47.4	638	52.0				
	AG	131	42.8	488	39.7				
	GG	30	9.8	102	8.3				
	AA	145	47.4	638	52.0				
	AG+GG	161	52.6	590	48.1	1.20	0.93–1.56	.16	.69
MTRR	G/A								
rs162049	G	310	50.7	1422	57.8				
	A	302	49.4	1040	42.2	1.33	1.11–1.60	.0017	.011
	GG	75	24.5	407	33.1				
	GA	160	52.3	608	49.4				
	AA	71	23.2	216	17.6				
	GG	75	24.5	407	33.1				
	GA+AA	231	75.5	824	66.9	1.52	1.14–2.06	.0038	.027
MTRR	His595Tyr								
rs10380	C	513	84.7	2181	88.9				
	T	93	15.4	273	11.1	1.45	1.11–1.88	.0051	.036
	CC	220	72.6	973	79.3				
	CT	73	24.1	235	19.2				

Table 2. Continued

Genotype	Cases n	Cases (%)	Controls n	Controls (%)	OR	95% CI	Fisher exact test,[a] P value	Permutation test,[b] P value
TT	10	3.3	19	1.6				
CC	220	72.6	973	79.3				
CT+TT	83	27.4	254	20.7	1.44	1.07–1.94	.013	.086

[a,b]*P* values [a]without or [b]with adjustment for multiple comparison.
[b]The *P* value of the permutation test is the number of statistics from randomly permuted data that happen to show the test statistics equal or higher than that of the original data, divided by the total number of the permutations performed (ie, a probability to observe the association by chance).

ified analyses. It was suggested that the association in women between minor allele and pancreatic cancer risk was greater among nonsmokers ($P = .016$) than among smokers ($P = .65$) as shown by the data for the rs162049 SNP (Table 3).

An increased risk of pancreatic cancer was found to be associated with the diabetes status, with an OR of 2.57, 95% CI: 1.34–4.87, $P = .0041$. However, there was no statistically significant association between diabetes status and allele frequency for *MTRR* polymorphisms (rs162049: OR, 1.51; 95% CI: 0.77–2.96; $P = .305$; rs10380: OR, 2.58; 95% CI: 0.58–11.3; $P = .405$) in the case group.

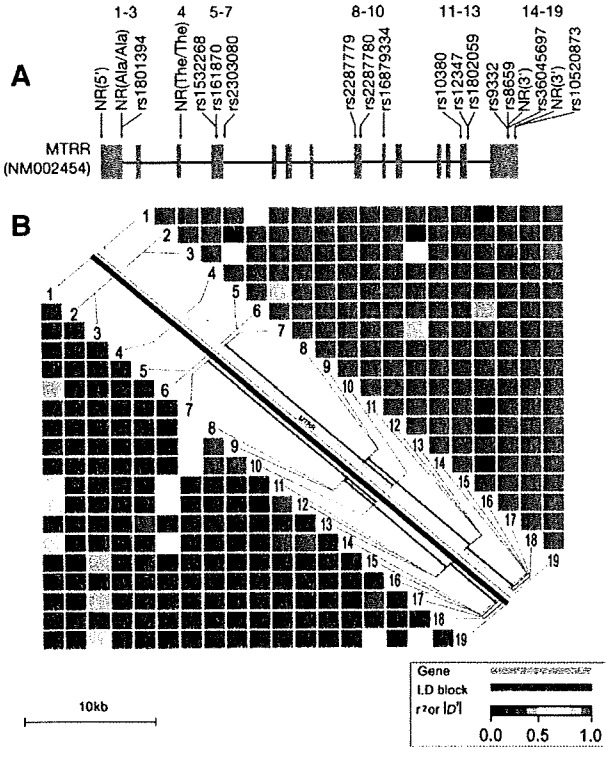

Figure 2. (*A*) Positions of the SNPs in *MTRR* gene identified by the resequencing of the coding region in this study. *Shaded boxes* indicate exons in the *MTRR* gene. *NR*, not registered, means that the SNPs were not found in the NCBI SNP database, dbSNP (http://www.ncbi.nlm.nih.gov/SNP/index.html). (*B*) Linkage disequilibrium (LD) between pairs of SNPs, as measured by *D'* (*upper panel*) and r^2 (*lower panel*) statistics in the 785 controls. High *D'* or r^2 value means that the SNP pair has a high chance to be transmitted together over the generation (ie, they are in LD). *A* and *B* were drawn to the same scale.

Because it is possible that germ-line polymorphisms influence not only development of cancer but also its progression and other tumor phenotypes, we also examined a possible association of the *MTRR* SNPs with TNM stage or location of the primary tumor (Table 1). However, a statistically significant association was not detectable in our case series for TNM stage (rs162049: $P = .19$, rs10380: $P = .54$) or for tumor location (rs162049: $P = .47$, rs10380: $P = .14$).

High-Density SNP Typing of the MTRR *Gene*

To gain further insights into the functionally responsible SNP, the coding region of the *MTRR* gene was resequenced on 76 cases and 20 controls for a comprehensive understanding of the polymorphic structure of the gene. The 19 SNPs identified (Table 4 and Figure 2) and the rs162049 (intronic SNP) selected by the joint analysis (first and second typings) were further analyzed in 702 patients, which consisted of the 317 cases used in the joint analysis and 385 additional patients, and the 785 controls matched with cases for sex. Of the 20 SNPs, the rs10380 (His595Tyr) and rs162049 SNPs showed a statistically significant difference between cases and controls (rs10380: recessive model OR, 3.03; 95% CI: 1.42–6.49; $P = .023$; rs162049: recessive model OR, 1.40; 95% CI: 1.08–1.82; $P = .024$) by the permutation test for the multiple testing (Table 4).

Haplotype Analysis of the MTRR *Gene*

Of the 19 SNPs identified by resequencing, 6 SNPs cause amino acid substitutions. Seven haplotypes (ie, a combination of alleles at multiple linked loci that are transmitted together to offspring) were inferred from the 6 missense SNPs, with the ACTCCT haplotype being associated with the highest risk of pancreatic cancer (OR, 1.28; 95% CI: 1.02–1.58; $P = .033$) (Table 5). Although the association was based on the inferred haplotype frequencies, the OR and its *P* value were not more substantially

Table 3. Smoking Status and Genotype and Allele Frequencies of *MTRR*

		Cases		Controls				
	Genotype	n	(%)	n	(%)	OR	95% CI	Fisher exact test, *P*-value
MTRR (rs162049)								
Pack-years[a] = 0, male	G	27	54.0	436	57.9			
	A	23	46.0	316	42.1	1.17	0.66–2.08	.65
	GG	7	28.0	125	33.2			
	GA+AA	18	72.0	251	66.8	1.28	0.52–3.14	.66
Pack-years = 0, female	G	89	47.9	417	57.9			
	A	97	52.2	303	42.1	1.49	1.08–2.07	.016
	GG	19	20.4	113	31.4			
	GA+AA	74	79.6	247	68.6	1.78	1.02–3.09	.040
>0 Pack-years to <20, male	G	32	50.0	199	56.5			
	A	32	50.0	153	43.7	1.30	0.76–2.21	.34
	GG	9	28.1	59	33.5			
	GA+AA	23	71.9	117	66.5	1.28	0.56–2.96	.68
>0 Pack-years <20, female	G	21	52.5	69	63.8			
	A	19	47.5	39	36.1	1.61	0.77–3.34	.25
	GG	7	35.0	20	37.0			
	GA+AA	13	65.0	34	63.0	1.09	0.37–3.19	1.00
≥20 Pack-years, male	G	125	51.6	274	58.1			
	A	117	48.4	198	41.9	1.29	0.94–1.76	.11
	GG	29	24.0	86	36.4			
	GA+AA	92	76.0	150	63.6	1.81	1.10–2.98	.017
≥20 Pack-years, female	G	16	57.1	18	47.3			
	A	12	42.9	20	52.6	0.67	0.25–1.80	.46
	GG	4	28.6	3	15.8			
	GA+AA	10	71.4	16	84.2	0.46	0.086–2.54	.42
MTRR (rs10380)	*His595Tyr*							
Pack-years = 0, male	C	41	82.0	662	88.0			
	T	9	18.0	90	12.0	1.61	0.75–3.43	.26
	CC	16	64.0	295	78.5			
	CT+TT	9	36.0	81	21.4	2.04	0.87–4.80	.13
Pack-years = 0, female	C	152	84.4	642	89.1			
	T	28	15.6	78	10.9	1.51	0.95–2.42	.092
	CC	67	74.4	287	79.3			
	CT+TT	23	25.6	73	20.7	1.34	0.78–2.31	.31
>0 Pack-years to <20, male	C	55	88.7	309	87.7			
	T	7	11.3	43	12.3	0.90	0.38–2.12	1.00
	CC	6	19.3	42	23.9			
	CT+TT	25	80.7	134	76.1	0.76	0.29–1.97	.65
>0 Pack-years to <20, female	C	34	85.0	101	93.4			
	T	6	15.0	7	6.6	2.54	0.80–8.10	.11
	CC	6	30.0	7	12.9			
	CT+TT	14	70.0	47	87.1	2.87	0.83–9.97	.16
≥20 Pack-years, male	C	206	84.4	417	89.9			
	T	38	15.6	47	10.1	1.64	1.03–2.59	.038
	CC	35	71.3	43	18.5			
	CT+TT	87	28.7	189	81.5	1.76	1.05–2.95	.031
≥20 Pack-years, female	C	23	82.1	33	86.8			
	T	5	17.9	5	13.2	1.43	0.37–5.52	.73
	CC	4	28.6	5	26.3			
	CT+TT	10	71.4	14	73.7	1.12	0.23–5.25	1.00

[a]Pack-years defined as number of packs of cigarettes smoked per day times number of years of smoking.

enhanced than the His595Tyr SNP alone, suggesting that the T allele of His595Tyr is the first candidate for the functional assay to explain the observed association with pancreatic cancer risk.

Functional Analysis of MTRR *SNPs*

To assess the functional difference among the nonsynonymous polymorphisms, expression plasmids of *MTRR* variants, designated pMTRR-H, pMTRR-L, and pLacZ (control vector), were each transfected into 293 cells. To avoid any bias associated with clonal variations, all G418-resistant cells that survived in the plates were pooled and analyzed. As shown in Figure 1, Western blot analysis using the MTRR antibody and V5-tagged antibody showed a reduction of the MTRR protein in the pMTRR-H cells as compared with the pMTRR-L cells.

Table 4. High-Density Genotyping of 20 SNPs in the *MTRR* Gene

				Dominant model[a]				Recessive model[a]			
rs[b]	Type	Codon[c]	Alleles	OR	95% CI	Fisher exact test, *P* value	Permutation test,[d] *P* value	OR	95% CI	Fisher exact test, *P* value	Permutation test,[d] *P* value
NR[e]	5′		t/c	1.03	0.82–1.29	.82	1.00	1.49	0.88–2.55	.14	.84
NR	*Ala/Ala*	9	t/c	1.15	0.58–2.31	.69	1.00	NA[f]	NA	NA	NA
1801394	*Ile/Met*	22	a/g	1.20	0.97–1.48	.094	.36	1.45	1.01–2.08	.042	.079
NR	*The/The*	126	t/c	0.94	0.06–15.5	.97	1.00	NA	NA	NA	NA
1532268	*Ser/Leu*	175	c/t	1.06	0.81–1.38	.74	1.00	1.14	0.42–3.12	.79	1.00
161870	*Leu/Leu*	179	t/c	1.13	0.89–1.43	.33	.96	2.10	1.09–4.03	.026	.14
2303080	*Ser/Thr*	257	t/a	0.75	0.57–1.00	.044	.41	0.86	0.18–4.06	.85	1.00
2287779	*Leu/Leu*	385	g/a	0.86	0.68–1.09	.22	.93	0.43	0.19–0.97	.042	.40
2287780	*Arg/Cys*	415	c/t	0.85	0.67–1.07	.17	.78	0.39	0.17–0.90	.027	.25
16879334	*Pro/Arg*	450	c/g	0.86	0.68–1.09	.21	.90	0.43	0.19–0.96	.040	.39
10380	*His/Tyr*	595	c/t	1.21	0.94–1.56	.14	.66	3.03	1.42–6.49	.0043	.023
12347	*Val/Val*	625	g/a	1.20	0.93–1.54	.16	.67	2.29	1.03–5.09	.041	.35
1802059	*Ala/Ala*	637	g/a	1.05	0.80–1.39	.71	1.00	0.98	0.35–2.73	.96	1.00
9332	3′-1		g/a	1.20	0.93–1.55	.16	.67	2.33	1.05–5.17	.037	.34
8659	3′-2		t/a	1.18	0.96–1.46	.12	.38	1.39	0.99–1.95	.055	.11
NR	3′-3		g/t	1.08	0.38–3.02	.89	1.00	NA	NA	NA	NA
36045697	3′-4		c/*	1.17	0.95–1.45	.14	.45	1.35	0.96–1.89	.086	.16
NR	3′-5		t/c	0.95	0.36–2.49	.92	1.00	NA	NA	NA	NA
10520873	3′-6		t/c	1.19	0.96–1.47	.12	.34	1.54	0.95–2.49	.081	.28
162049[g]	Intron		g/a	1.39	1.10–1.75	.0066	.013	1.40	1.08–1.82	.012	.024

[a]Age- and smoking-adjusted OR, its 95% CI, and *P* value calculated by exact logistic regression for recessive and dominant models. The dominant model was defined as Aa+aa vs AA and the recessive model as aa vs AA+Aa. A and a are the major and minor alleles, respectively.
[b]SNP reference number of the NCBI SNP database, dbSNP (http://www.ncbi.nlm.nih.gov/SNP/index.html).
[c]Amino acid position based on RefSeq NM_002454 (*MTRR*).
[d]*P* value by multiple testing using a permutation test.
[e]Not registered in dbSNP.
[f]Statistical calculation is not applicable because of zero subjects in a cell of a contingency table.
[g]Intronic SNP selected by the joint analysis.
*Null allele.

MTRR radioimmunoassay confirmed that the pMTRR-H cells contained lower amounts of the MTRR protein than did the pMTRR-L cells. The RT-PCR data (Figure 1C and *D*) showed no difference in the level of the *MTRR* gene expression between the pMTRR-L and pMTRR-H cells. There were no cloning artifacts in the vector sequences, and the entire transfection experiments were repeated twice, showing the same difference in the MTRR protein level in the transfected cells.

Homocysteine Levels in Culture Mediums of MTRR-*Transfected Cells*

The mean of total homocysteine levels at 24 hours in the culture medium collected from pMTRR-H, pMTRR-L, and pLacZ control cells were 4.02 ± 0.21 μmol/L/μg protein (mean ± SD; n = 4), 3.65 ± 0.21 μmol/L/μg protein, and 4.35 ± 0.16 μmol/L/μg protein, respectively (Figure 3*A*). The results indicated that the homocysteine level was significantly higher in the

Table 5. Case-Control Association of the *MTRR* Haplotypes Constructed by 6 Nonsynonymous SNPs

	Cases		Controls				
Haplotypes	n	%	n	%	Fisher exact test, *P* value	OR	95% CI
ACTCCC	528	37.8	672	42.8	.0050	0.82	0.70–0.94
GCTCCC	343	24.5	328	20.9	.020	1.23	1.03–1.46
ACTCCT	191	13.7	173	11.0	.033	1.28	1.02–1.58
ACATGC	120	8.6	161	10.3	.13	0.82	0.64–1.05
GTTCCC	115	8.2	116	7.4	.41	1.12	0.85–1.46
ACTTGC	66	4.8	84	5.4	.45	0.88	0.63–1.22
ATTCCC	26	1.9	30	1.9	1.00	0.97	0.57–1.65

NOTE. Odds ratio of each haplotype against the rest of the haplotypes was calculated on the 6 nonsynonymous SNPs summarized in Table 4.

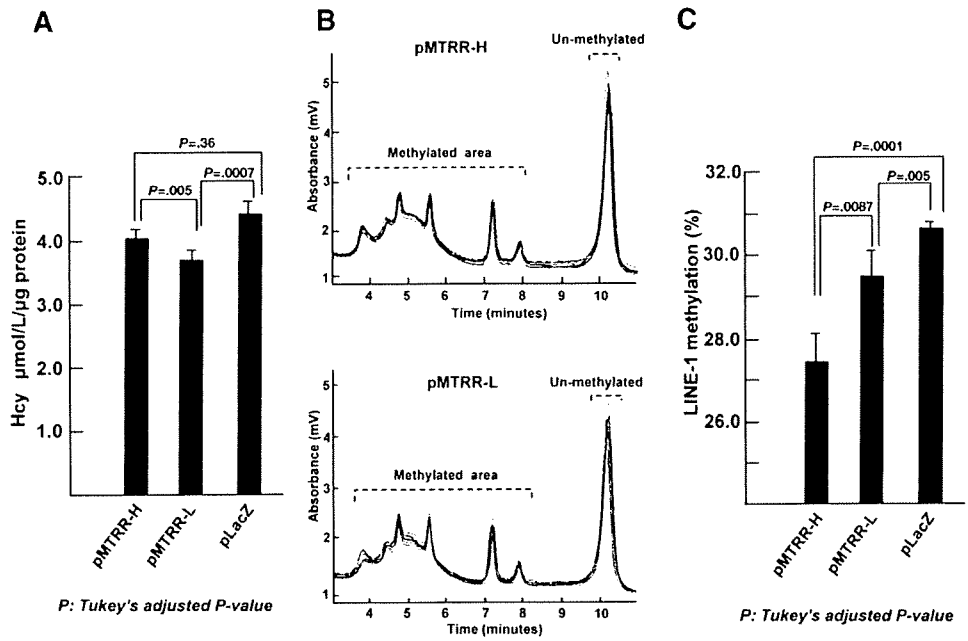

Figure 3. (*A*) Total homocysteine (*Hcy*) levels in the culture medium of the *MTRR* cDNA-transfected 293 cells. The results shown in the Figure are the means ± SD obtained with 4 samples with Tukey adjusted *P* values. *pMTRR-H*, Tyr595 variant (risk type) vector of the *MTRR* cDNA; *pMTRR-L*, His595 variant (wild type) vector of the *MTRR* cDNA; *pLacZ*, control vector of the LacZ. (*B*) LINE-1 methylation status in the cDNA-transfected 293 cells analyzed by COBRA[30] and WAVE dHPLC. The data on the 8 samples were overlaid for pMTRR-H and pMTRR-L, demonstrating small variance of the analysis. (*C*) LINE-1 methylation level in *B* quantified by Image Quant software of the WAVE system. LINE-1 methylation level was calculated as a ratio (in percentage) of the area under the curve for the methylated LINE-1 to those of the sum of methylated and unmethylated LINE-1. The results are shown as means ± SD obtained with 8 samples with Tukey adjusted *P* values.

pMTRR-H cells (Tukey adjusted, $P = .005$) compared with the pMTRR-L cells.

Global Methylation Analysis of LINE-1 in MTRR-Transfected Cells

To assess the global level of DNA methylation, LINE-1 methylation was analyzed by the combined bisulfite restriction analysis (COBRA) method on the *MTRR*-transfected cells. The mean levels of methylated cytosine in the LINE-1 sequences of the pMTRR-H, pMTRR-L, and pLacZ cells were 27.58% ± 0.55% (mean ± SD; n = 8), 28.80% ± 0.83%, and 30.11% ± 0.65%, respectively (Figure 3*C*). There was a statistically significant difference between the pMTRR-L and pMTRR-H cells (Tukey adjusted, $P = .0087$).

Discussion

We examined 227 SNPs on the 46 genes that are considered to interact with environmental and lifestyle-related factors, for an association with pancreatic ductal adenocarcinoma risk in the Japanese population. Only the *MTRR* gene showed a statistically significant association after multiple testing adjustment, and in vitro functional assays on cDNA representing the haplotypes inferred from the high-density SNP typing suggested that missense SNP (rs10380, His595Tyr) is a likely candidate for the functionally responsible polymorphism.

MTRR, which is an enzyme involved in the folate metabolism, maintains the MTR enzyme in an active state for the remethylation of homocysteine to methionine[31] and serves as a molecular chaperone for MTR and an aquacobalamine reductase.[32] Folate is an important dietary methyl-group donor involved in DNA methylation as well as the DNA synthesis pathways.[31] A folate deficiency may cause uracil misincorporation in human DNA and chromosomal breaks, leading to genomic instability and increased mutation rates.[31] Patients with the cblE complementation group of disorders of the folate/cobalamin metabolism are defective in the reductive activation of MTRR and may develop megaloblastic anemia, homocysteinuria, hyperhomocysteinemia, and hypomethioninemia.[33] No previous studies have reported an association between polymorphisms in the *MTRR* gene and pancreatic cancer. Whereas most studies on *MTRR* polymorphisms have focused on the Ile22Met variant (rs1801394), which has been described in association with circulating homocysteine concentration in a healthy male population,[34] and with folate or vitamin levels in colorectal cancer,[35] the Ile22Met SNP showed no statistically significant association with pancreatic cancer risk in our study. Moreover, the SNP was neither in a signif-

icant LD with the His595Tyr SNP nor did it tag the risk haplotype identified in this study (Table 5).

One report each has shown a significant association between the *UGT1A7*,[16] *MTHFR*,[17] or *NAT1*[18] polymorphisms and risk of pancreatic cancer, but we could not replicate the associations in our study population for *UGT1A7* (rs11692021: OR, 1.09; 95% CI: 0.89–1.34; P = .43), *MTHFR* (rs1801133: OR, 0.96; 95% CI: 0.80–1.15; P = .64), or *NAT1* (rs11777998: OR, 0.96; 95% CI: 0.80–1.15; P = .68; rs15561: OR, 0.90; 95% CI: 0.67–1.21; P = .53). This inconsistency may be due to a lack of statistical power of the case-control study design, racial variation, smoking or dietary habits, or difference in marker SNPs.

In several epidemiologic studies, a low intake of folate has been associated with an increased risk of pancreas,[10,36] colorectal,[37] and breast[38] cancers. It is plausible that a variation in the availability of folate-derived methyl groups affects the individual risk of pancreatic cancer through altered cellular capacity for mutation or epigenetic methylation.[36] In addition, animal studies have shown that diets deficient in methyl group donors cause abnormal pancreatic acinar cell differentiation.[39]

Although the mechanisms for a higher risk associated with the *MTRR* SNP remains unknown, the SNP might interact with environmental/ lifestyle-related factors, particularly those related to folate metabolism. One limitation of the present study is that we had no quantitative information on the dietary habits of the subjects enrolled. Possible interaction with sex, smoking status, and other lifestyle-related factors needs to be evaluated in further studies.

To elucidate the functional significance of the *MTRR* variants, we stably transfected the plasmid vector expressing the *MTRR* cDNA to the human embryonic kidney 293 cells, which express an undetectable level of endogenous *MTRR*. We found that the exogenous MTRR protein was produced at lower levels in the pMTRR-H transfectants harboring the risk haplotype cDNA, as compared with the pMTRR-L transfectants harboring the wild-type haplotype (Figure 1). There seems to be lower translation and/or stability of the MTRR-V5 fusion protein derived from the risk haplotype cDNA than the wild-type counterpart, but a precise mechanism is not known.

The functional activity of MTRR can be assessed by the level of the MTR substrate homocysteine which shows a strong inverse association with the dietary and/or blood folate levels in an epidemiologic study.[40] In the present study, the homocysteine levels in the culture medium of the pMTRR-H transfectants were higher than those of the pMTRR-L and pLacZ transfectants (Figure 3), a situation similar to a relative folate deficiency, which is implicated in the increased risk of cancers, including those of the pancreas. Because the important role of the folate is in the methyl group availability to the cellular methylation processes, possible effect of the *MTRR* polymorphisms was investigated on DNA methylation. In addition to the number of the regional hypermethylation-mediated inactivation of tumor suppressor and mutator genes, global hypomethylation has been recognized as a cause of oncogenesis, including a hypomethylation-mediated reactivation of oncogenes,[41] which has been suggested as a frequent epigenetic event in pancreatic cancer.[42] It is intriguing that our in vitro assay showed that the levels of LINE-1 methylation in the pMTRR-H transfectants were significantly lower than those of the pMTRR-L cells. It is plausible that the *MTRR* polymorphism affects cancer susceptibility through the individual variation in the DNA methylation level by interacting with the environmental/lifestyle-related factors.

The above results on the protein expression, homocysteine level in the culture medium, and methylation status of the repetitive element of the *MTRR* cDNA-transfected cells suggest that the haplotype represented by the His595Tyr SNP has functional significance on the *MTRR* gene. Our study provides the first piece of evidence to suggest that the *MTRR* gene polymorphism influences susceptibility for pancreatic cancer. The His595Tyr and the rs162049 intronic SNP should be included in future replication studies, which entail an assessment of the gene-environmental interactions by collecting quantitative information such as dietary folate intake, plasma homocysteine levels,[43] and evaluation of *MTRR* expression and activity in appropriate clinical samples.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.04.016.

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Conflicts of interest: There are no conflicts of interest to disclose.

Supplementary Table 1. The 46 Candidate Genes and Their SNPs Analyzed in the First Typing of This Study

	SNP (rs No.)	Amino acid change
Metabolizing enzymes		
Cytochrome P450		
CYP1A1	1048943	Ile/Val
	4646421	
	936229	
	936228	
CYP1A2	762551	
	2472304	
CYP1B1	10012	Arg/Gly
	1056827	Ala/Ser
	1056836	Leu/Val
	10916	
	162556	
CYP2A6	1137115	Val/Val
	28399433	
CYP2C9	4918758	
	10509679	
CYP2C19	1322179	
	1505	
	4917623	
CYP2E1	3813867	
	2031920	
	2070673	
	2249695	
CYP17A1	6162	His/His
	6163	Ser/Ser
	743572	
	619824	
	4919685	
	743575	
CYP19A1	700518	Val/Val
	700519	Arg/Cys
	10046	
	4646	
	1902586	
	2470176	
	1870049	
	2236722	Trp/Arg
Glutathione S-transferase		
GSTM2	655315	
	569998	
	428434	
GSTM3	1537234	
	7483	Ile/Val
	1571858	
	1332018	
	1537236	
GSTT2	1622002	Met/Ile
	2719	
	2267047	
	140186	
GSTP1	1695	Ile/Val
	4891	Ser/Ser
	1871042	
	762803	
	612020	
N-acetyltransferase		
NAT1	15561	
	7845127	
NAT2	1041983	Tyr/Tyr
	1801280	Ile/Thr
	1799929	Leu/Leu
	1799930	Arg/Gln
	1208	Lys/Arg
	1799931	Gly/Glu
	1495744	
Catechol-O-methyltransferase		
COMT	4633	His/His
	4680	Val/Met
	6267	Ala/Ser
	2075507	
	2020917	
	2239393	
	4646312	
	740603	
	5993882	
	933271	
Epoxide hydrolase		
EPHX1	1051741	Asn/Asn
	1051740	Tyr/His
	6965	
	2292566	Lys/Lys
	2234922	His/Arg
	2078341	
	2292568	Pro/Pro
EPHX2	751141	Arg/Gln
	1042032	
	891401	
	747276	
	2291635	
	1126452	Pro/Pro
NADPH dehydrogenase quinone		
NQO1	1800566	Pro/Ser
	2965757	
	689452	
	1437135	
	689456	
Endocrine		
Estrogen receptor		
ESR1	1913474	
	932479	
	2011885	
	974276	
	1062577	
	2273206	
	2228480	Thr/Thr
	2234693	
	9340799	
ESR2	1256054	Leu/Leu
	1256049	Val/Val
	1256027	
	1256030	
	944459	
	2274705	
Estrogen-related receptor γ		
ESRRG	1498283	
	1339343	
	945453	Ser/Ser
	6604632	
	1857407	
	2820879	

Supplementary Table 1. Continued

	SNP (rs No.)	Amino acid change
Progesterone receptor		
PGR	484389	
	481883	
	511484	
	578938	
	566351	
Glucocorticoid receptor		
GCCR	6194	His/His
	258751	Asp/Asp
	6196	Asn/Asn
	174050	
	33388	
	33389	
	2918419	
Hydroxysteroid 17-β dehydrogenase		
HSD17B2	2042429	
	1017243	
	1424151	
	996752	
	4445895	
HSD17B3	1927886	
	2066480	Val/Ile
	375944	
	280654	
	867807	
	912462	
	2066479	Gly/Ser
Nutrition		
Alcohol dehydrogenase		
ADH1A	931635	
	1229967	
	1229970	
	975833	
	1618572	
	2276332	
ADH1B	17033	
	1159918	
	1042026	
ADHIC	1789924	
	1693430	
	2009181	
	2298755	
	3216150	
	4147542	
Aldehyde dehydrogenase		
ALDH2	671	Glu/Lys
	2238151	
	2238152	
	441	
	4646778	
DNA synthase and methylation		
5, 10-Methylenetetrahydrofolate reductase		
MTHFR	2066470	Pro/Pro
	1801133	Ala/Val
	1801131	Glu/Ala
	2066471	
	2274976	Arg/Gln
Methionine synthase		
MTR	3795708	
	1770449	
	1050993	
Methionine synthase reductase		
MTRR	1801394	Ile/Met
	326121	
	162049	
	10380	His/Tyr
	327592	
Thymidylate synthetase		
TYMS	16948322	
	2298581	
Behavior		
Dopamine receptor		
DRD2	1076560	
	1124491	
	6277	Pro/Pro
	6275	His/His
	1076563	
	1116313	
	1079596	
	1801028	Ser/Cys
	7117915	
	10891556	
DRD3	6280	Ser/Gly
	1800828	
	2087017	
	167770	
DRD4	936460	
	1800955	
	747303	
	752306	
	936461	
Serotonin transporter		
SLC6A4	1042173	
	25528	
	2020939	
	2020936	
	717742	
	1872924	
	140701	
Aryl hydrocarbon receptor		
AHR	2066853	Arg/Lys
	713150	
	2074113	
	2237297	
	2237299	
	2282886	
Oxidative stress		
Nitric oxide synthase		
NOS2A	1060822	Gly/Gly
	2072324	
	2297513	
	2297518	Ser/Leu
	2297520	
	1137933	Asp/Asp
NOS3	1800783	
	1800779	
	1549758	Asp/Asp
	1799983	Glu/Asp
	1800780	
	1007311	

Supplementary Table 1. Continued

	SNP (rs No.)	Amino acid change
DNA repair		
Nudix-type motif		
NUDT1	4866	Val/Met
	1799832	Asp/Asp
	1062492	
	10281945	
8-Oxoguanine DNA glycosylase		
OGG1	2075747	
	1052133	Cys/Ser
	2072668	
	1801129	
	2304277	
Human homolog of the *Saccharomyces cerevisiae* mutagenesis protein Rev		
REV1L	3087399	Asn/Ser
	3087386	Phe/Ser
Immune		
Interleukin		
IL1A	17561	Ala/Ser
	1800587	
	1800794	
	2071374	
	2071375	
IL1B	1071676	
	1143637	
	1143629	
	1143627	
	1143634	Phe/Phe
	16944	

Minimally Invasive Intraductal Papillary-mucinous Carcinoma of the Pancreas: Clinicopathologic Study of 104 Intraductal Papillary-mucinous Neoplasms

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Abstract: Invasive intraductal papillary-mucinous carcinoma (I-IPMC) is a heterogeneous entity with various postoperative outcomes. The aim of this study is to characterize early-stage I-IPMC with nonaggressive characteristics. One hundred and four patients with intraductal papillary-mucinous neoplasm (IPMN) were clinicopathologically investigated. The lesions were classified into 53 noninvasive IPMNs (adenoma, borderline, and noninvasive IPMC) and 51 I-IPMCs on the basis of the WHO classification. I-IPMCs were divided further into 26 minimally invasive IPMCs (MI-IPMCs) and 25 invasive carcinomas originating in IPMC (IC-IPMCs) by new diagnostic criteria proposed in this study. We examined invasiveness of I-IPMC on 4 patterns, and defined simple and practical diagnostic criteria of minimal invasion for each invasive pattern. The disease-specific survival rates after 3, 5, and 10 years were 100%, 100%, and 100% for both noninvasive IPMN and MI-IPMC, and 51%, 38%, and 0% for IC-IPMC. The overall and disease-specific survival rates for MI-IPMC were both significantly better than those for IC-IPMC ($P < 0.001$), but there was no significant difference between noninvasive IPMN and MI-IPMC. Multivariate analysis showed that the factors indicative of poor prognosis were a diagnosis of I-IPMC classified as IC-IPMC and a high level of serum carbohydrate antigen 19-9. The prognosis of IC-IPMC was not significantly different from that of pancreatic ductal carcinoma in each of the corresponding tumor-node-metastasis stages. These findings suggest that a category of MI-IPMC provides more accurate and useful information of the stage and the aggressiveness of I-IPMC.

Key Words: intraductal papillary-mucinous neoplasms of the pancreas, minimal invasion, prognosis, clinicopathologic analysis, invasive pattern

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Intraductal papillary-mucinous neoplasm (IPMN) of the pancreas is a well-characterized clinical and pathologic entity. IPMNs are characterized by intraductal proliferation of neoplastic mucinous cells, which usually form papillae and lead to cystic dilation of the pancreatic ducts, forming clinically and macroscopically detectable masses.[15] Similarly to the well-defined adenoma-carcinoma sequence in colorectal cancer,[5] IPMNs progress from intraductal papillary-mucinous adenoma (IPMA) to borderline IPMN, then to intraductal papillary-mucinous carcinoma (IPMC), and eventually to invasive adenocarcinoma.[2,9,10] According to the WHO classification,[13,15] IPMC is classified as either "noninvasive" or "invasive." It is reported that noninvasive IPMN shows a favorable postoperative outcome in comparison with invasive IPMC (I-IPMC), with 5-year survival rates ranging from 77% to 100%.[4,6,16,20,22,24] With regard to the prognosis of I-IPMC, there is a substantial variation in the 5-year survival rates from 24% to 60% in previous reports.[4,6,16,20,22–24] This may be due to heterogeneity of I-IPMCs, including an invasive component of various sizes and biologic behavior. Our hypothesis is that the prognosis of I-IPMC can be substantially determined by the degree and type of invasion, and thus I-IPMC can be classified as either aggressive or nonaggressive by categorization according to the extent and pattern of invasion. Such a classification would be clinically relevant for deciding whether surgery is indicated, for selecting the most appropriate surgical procedure, and for prediction of postoperative outcome.

The concept of minimally invasive cancer was originally introduced for uterine cervical cancer showing very early invasion and a favorable prognosis.[26] Minimally invasive IPMC (MI-IPMC) has been categorized within the classification of pancreatic carcinoma used by the Japan Pancreatic Society (JPS) since 1993.[12] In the JPS classification, I-IPMC is classified into 2 categories: MI-IPMC and invasive carcinoma originating in IPMC (IC-IPMC), the latter being more advanced. A few reports have indicated that MI-IPMC has a better surgical outcome than IC-IPMC.[19,25,27] However, the definition of "minimal invasion" has not been clear. In the original JPS text, it is described only as "slight invasion beyond the pancreatic duct wall."[12]

In this retrospective study, we evaluated the invasiveness of I-IPMC by the examination of 4 invasive

patterns, and tried to define simple and practical diagnostic criteria of minimal invasion for each invasive pattern. The clinical relevance of this subdivision was then evaluated in terms of postoperative survival.

MATERIALS AND METHODS

Study Population

This study was approved by the Ethics Committee of the National Cancer Center, Japan. Between January 1984 and December 2005, 111 patients underwent pancreatic resection for IPMNs at the National Cancer Center Hospital, Japan. There were no operation-related deaths, and all patients underwent macroscopically curative resection without any residual tumor. Seven cases also had ductal carcinoma of the pancreas, which was not directly associated with IPMNs. Excluding these patients, 104 cases of IPMN were included in this study. The patients comprised of 56 males and 48 females with a median age of 66 (41 to 84) years. The operative procedures included 12 pancreatoduodenectomies (PDs), 59 pylorus-preserving PDs (PPPDs), 24 distal pancreatectomies, 3 total pancreatectomies, 5 partial pancreatectomies, and 1 PPPD with distal pancreatectomy. These procedures accounted for 18.9% of all pancreatectomies (n = 551) performed at our institution for pancreatic tumors during the same period.

Every patient was followed up in the outpatient clinic every 1 to 3 months during the first postoperative year, and every 6 to 12 months thereafter. No patient dropped out during follow-up. Clinical or radiologic data and follow-up information for every patient were obtained from the medical records. The median follow-up period after surgery was 37.2 (4.2 to 210) months for all patients, 52.9 (4.2 to 171) months for noninvasive IPMN, 43.4 (13.2 to 210) months for MI-IPMC, and 20.4 (7.1 to 87.7) months for IC-IPMC.

Pathologic Examination

All of the IPMNs were pathologically reexamined and the diagnosis of IPMN was confirmed. Surgically resected specimens were fixed in 10% formalin and cut into serial 5-mm-thick slices, horizontally in the pancreas head, and sagittally in the pancreas body and tail. All the sections were stained with hematoxylin and eosin for pathologic examination. If necessary, additional staining for elastic fibers (elastica stain) was performed. After histopathologic examination of all the sections, the lesion was classified as IPMA, borderline IPMN, noninvasive IPMC, or I-IPMC according to the WHO classification.[13,15] The lesion was graded by the highest degree of atypia. I-IPMCs were divided further into MI-IPMC or IC-IPMC according to our proposed criteria (Table 1) described later. We evaluated the invasiveness of I-IPMC, and the 4 invasive patterns were examined: "infiltrative growth," "mucous rupture," "expansive growth," and "intra-abdominal rupture." The criterion of minimal invasion was proposed for each invasive pattern. I-IPMC showing some features of minimal invasion without any features categorized in IC-IPMC was classified as MI-IPMC. I-IPMC showing at least one invasive feature beyond minimal invasion is classified as IC-IPMC. For example, if an I-IPMC shows mucous rupture and infiltrative growth of tubular adenocarcinoma with 6-mm length of invasion, this tumor is diagnosed as IC-IPMC.

An infiltrative growth pattern, which is commonly found in conventional invasive ductal carcinoma of the pancreas, is considerably aggressive (Figs. 1A–D). Among the 6 patients with IC-IPMC, in whom the depth of infiltration of carcinoma cells ranged from 6 to 20 mm, 3 patients (including a patient with 6-mm-length infiltration of carcinoma cells) had recurrence in the liver or peritoneal cavity, and died of the disease. This suggests that infiltrative growth is strongly associated with a high rate of recurrence and mortality, even if the size of invasion is limited. On the other hand, none of the 17 patients with a maximum infiltration of 5 mm or less had recurrence except 2 patients, 1 of them had 2-mm-length infiltration of tubular adenocarcinoma and the other had 2-mm-length infiltration of pure mucinous carcinoma. Therefore, we adopted a threshold of 5 mm as a diagnostic criterion for minimal invasion in infiltrative growth (Table 1). Lymphatic, venous, and neural invasion are treated as a part of infiltration of cancer cells. Invasion of 5 mm or less is sometimes difficult to detect. In such cases, elastica staining was helpful for differentiating infiltrating carcinoma from intraductal spreading of carcinoma (Figs. 1C, D).

IPMN is characterized by its prominent mucus secretion into the lumen, in some cases, into the space between epithelial cells and basement membrane due to inverted cellular polarity, which subsequently causes disruption of the pancreatic duct wall and spilling of mucus into the interstitial space.[1,7] This is referred to mucous rupture (Fig. 2) and is diagnosed as minimal invasion if mucous lakes are not associated with mucinous carcinoma showing infiltrative growth (Table 1). Mucous rupture was observed only in the vicinity of the pancreatic ductal system, although the location was not confined to the pancreas. We considered mucus lakes near noninvasive IPMC as mucous rupture regardless of the presence of viable cancer cells within it, because viable cancer cells may be present floating in the mucus lake. When viable cancer cells floating in mucus lake are apparently present and are scant (there is very small number of cancer cells or their clusters floating in only the limited mucus lakes. The representative feature was shown in Fig. 2D), this situation is called as "mucous rupture with cellular component." This subcategory includes a kind of pure mucinous carcinoma (alternatively colloid carcinoma)[1] showing a very low cellularity, and nonmucinous cancer cells which are simply detached from the duct wall and are floating in mucus lake. Mucous rupture without floating cancer cells represented the suspected lesion of mucous rupture with cellular component. When there are many cancer cells (more than "scant" level) floating in mucus lake, it is judged as infiltrative growth of mucinous carcinoma (Figs. 2E, F).

TABLE 1. Growth Patterns of I-IPMCs and Diagnostic Criteria for Minimal Invasion

Growth Pattern	Pathologic Features	Minimal Invasion	Beyond Minimal Invasion (IC-IPMC)
Infiltrative growth	Carcinoma cells from the pancreatic duct occupied by IPMC invade the surrounding stroma. Disappearance of the basement membrane or desmoplastic change around the ducts implies interstitial invasion. Infiltrative distance is defined as the length from the deepest point of invasion to the stroma surface of the nearest non-invasive IPMC	1. An infiltrative distance of 5 mm or less is regarded as minimal invasion 2. Venous, lymphatic, or neural invasion within the area (≤5 mm of the infiltrative distance) is also counted in this category 3. This category includes the invasion of various histologic types of cancer, such as tubular adenocarcinoma, mucinous carcinoma, etc	1. An infiltrative distance more than 5 mm is regarded as IC-IPMC 2. Venous, lymphatic, or neural invasion within the area (> 5 mm of the infiltrative distance) is also counted in this category 3. This category includes the invasion of various histologic types of cancer, such as tubular adenocarcinoma, mucinous carcinoma, etc
Mucous rupture	"Mucous rupture" and "expansive growth" are unique features of IPMC that grows intraductally and secretes large amounts of mucus. The mucus spills out, forming a mucus lake around the pancreatic duct, due to rupture of the dilatated pancreatic duct occupied by IPMN through high pressure caused by the hypersecreted mucin. This is referred to mucous rupture. Mucous lakes of various sizes are seen, sometimes containing scanty floating cancer cells	1. If mucous lakes are not associated with mucinous carcinoma showing infiltrative growth, this feature is diagnosed as minimal invasion, regardless of the size and location of the mucus lakes 2. If a mucus lake contains scanty floating cancer cells (there is very small number of cancer cells or their clusters floating in only the limited mucus lakes. The representative feature was shown in Fig. 2D), it is additionally described as "mucous rupture with cellular component." This subcategory includes a kind of pure mucinous carcinoma associated with IPMC 3. If many cancer cells (more than "scant" level) are floating in mucus lakes (Fig. 2E), it is treated as "infiltrative growth" of mucinous carcinoma and the infiltrative distance of 5 mm or less is regarded as minimal invasion	1. If many cancer cells are floating in mucus lakes, it is treated as "infiltrative growth" of mucinous carcinoma and the infiltrative distance of more than 5 mm is classified as IC-IPMC
Expansive growth	A pancreatic duct is markedly dilated, becoming ductectatic or cystic in shape. The basement membrane and subepithelial elastic fibers may be elongated and disrupted. Cystic IPMC may grow expansively into peripancreatic connective tissues, and eventually involves the bowel or major vessels [portal vein (PV), SPV, SMV, or splenic artery (SPA)]	1. Loss of the basement membrane of the pancreatic duct with IPMC is regarded as minimal invasion 2. If I-IPMC grows expansively, even if it ruptures into the bowel, or even if it erodes a major vessel wall unless cancer cells enter the lumen of the major vessel, it is still regarded as minimal invasion 3. If I-IPMC has this type of growth as predominance, it is corresponded to a kind of pure mucinous carcinoma associated with IPMC	1. If I-IPMC forms a fistula with a major vessel, it is regarded as IC-IPMC
Intra-abdominal rupture	IPMC ruptures into the abdominal cavity, and mucus-secreting cancer cells are scattered in it	Peritoneal dissemination may occur. Therefore, this finding should be distinguished from ordinary IPMN and classified separately as ruptured IPMN. MI- or IC-IPMC should be noted additionally	
Judgement		An I-IPMC showing some features of minimal invasion without any features categorized in IC-IPMC is classified as MI-IPMC	An I-IPMC showing at least one invasive feature beyond minimal invasion is classified as IC-IPMC

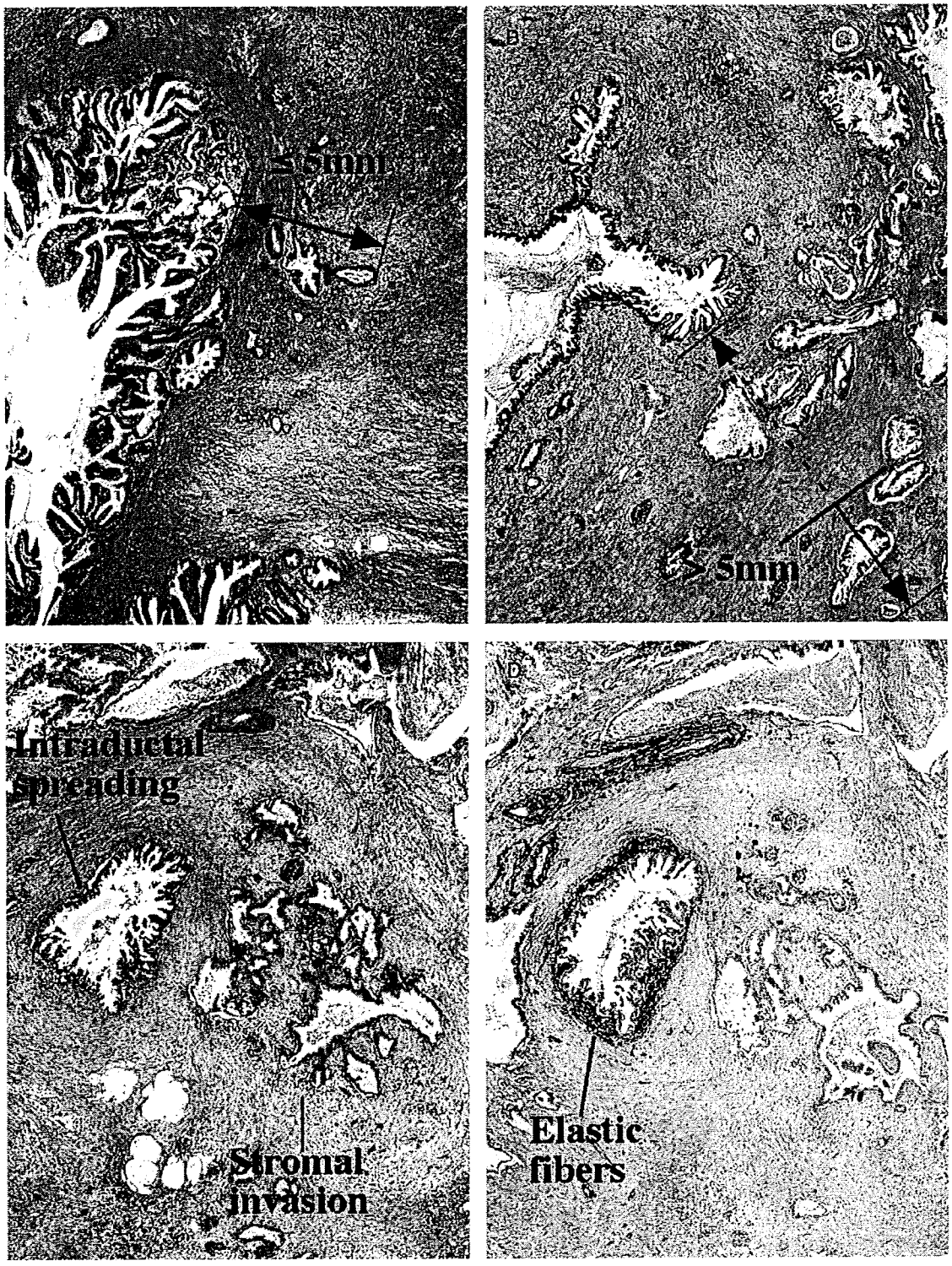

FIGURE 1. Histopathologic features of "infiltrative growth" in I-IPMC. A and B, Histologic features resemble those of conventional invasive ductal carcinoma of the pancreas. The arrows indicate the depth of infiltration of invasive carcinoma. If the depth is less than 5 mm, it is regarded as minimal invasion (A), and if the depth is more than 5 mm it is regarded as IC-IPMC (B). C and D, Elastica stain (D) helps to discriminate infiltrative growth from intraductal spread of carcinoma. The former lacks a positively stained sheath of elastic fibers (black) around the pancreatic duct. Hematoxylin and eosin stain (C).