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Abrogation of *DUSP6* by hypermethylation in human pancreatic cancer

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Abstract Our previous study indicated that DUSP6/MKP-3/PYST1 could act as a tumor suppressor in human pancreatic cancer. DUSP6 was frequently underexpressed in primary pancreatic cancer tissues by an unknown mechanism. In this study, we demonstrated that hypermethylation of the expressional control region of *DUSP6* could account for its abrogation in cultured human pancreatic cancer cells and in primary pancreatic cancer tissues. First, we checked intrinsic transcriptional expression levels of *DUSP6* by a quantitative real time PCR assay in 16 cultured pancreatic cancer cell lines and found that the cells could be classified into four groups: very-low-level expression, low-level expression, high-level expression, and very-high-level expression. We observed restored expression of *DUSP6* after treatment with 5-azacytidine and trichostatin A, a DNA methyltransferase inhibitor and a histone deacetylase inhibitor, respectively, in cells with intrinsically very-low-level and low-level expression of *DUSP6*. Using a sodium-bisulfite-modification assay, we found that CpG sequences in intron 1 of *DUSP6* were heavily methylated in MIA PaCa-2 and PAN07JCK, both showing the very low level of intrinsic expression of the gene. On the other hand, no methylation in this region was detected in 14 other cell lines. We checked the methylation state of this region by a methylation-specific PCR method in 12 primary pancreatic cancer tissues and compared it with the expression state of DUSP6 investigated by immunohistochemistry. Methylation was detected in five of eight cases with abolished expressions of DUSP6, four of which were poorly differentiated adenocarcinoma. On the other hand, none of the four cases with preserved expression of DUSP6 showed methylation. The methylation state significantly correlated with both the abolishment of protein expression ($p = 0.038$) and the histological subtype of adenocarcinoma ($p = 0.023$) by chi-square test. These results indicate that hypermethylation of the CpG islands in intron 1 may account for the strong suppression of *DUSP6* expression. Other mechanism(s) and/or other CpG sites outside of our investigation may have some influence upon expressional suppression. Our combined results suggest that hypermethylation with modification of histone deacetylation play an important role in transcriptional suppression of *DUSP6* in human pancreatic cancer.

Keywords Acetylation · CpG island · DUSP6 · Methylation · Pancreatic cancer

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Introduction

DUSP6/MKP-3/PYST1 is located on 12q21–q22 (Furukawa et al. 1998), a region showing common and frequent loss of heterozygosity (LOH) in human pancreatic cancer (Kimura et al. 1996, 1998). Loss of this region has also frequently been observed cytogenetically (Fukushige et al. 1997). *DUSP6* is a dual-specificity phosphatase that intrinsically binds and inactivates ERK2/MAPK1 in a feedback loop manner, and a number of studies have indicated that DUSP6 plays an important role in physiological regulation of the RAS-MAPK signaling pathway (Groom et al. 1996; Muda et al. 1996; Kawakami et al. 2003; Tsang et al. 2004). In a previous study, we found immunohistochemically that expression of DUSP6 was reduced or abolished in cells of invasive ductal carcinoma in contrast to its increased expression in

the dysplastic ductal cells in primary pancreatic cancer tissues (Furukawa et al. 2003). Cultured human pancreatic cancer cells lacking the expression of DUSP6 tended to show a constitutively active ERK, and the adenovirus-mediated introduction of *DUSP6* into such *DUSP6*-inactivated cancer cells induced inactivation of ERK, resulting in marked growth suppression and eventual apoptosis (Furukawa et al. 2003). Because a vast majority of pancreatic cancer cells harbor the gain-of-function mutation of *KRAS2*, which constitutively activates several downstream signal cascade components including RAF-MEK-ERK, these results indicate that the abrogation of DUSP6 synergistically contributes to hyperactivation of ERK, which may eventually result in development and progression of the invasive carcinoma of the pancreas (Furukawa and Horii 2004). All the lines of evidence suggest that *DUSP6* acts as a tumor suppressor gene and that there may be some epigenetic mechanisms working for the abrogation of *DUSP6* in pancreatic cancer because no mutation contributing to loss of function had been detected in this gene in our previous analysis (Furukawa et al. 1998). Hypermethylation is a major epigenetic mechanism for silencing gene expression in physiological as well as pathological patterns, especially in carcinogenesis among the latter (Herman 1999; Egger et al. 2004). Herein we report the results of our efforts to elucidate the epigenetic mechanisms involved in abrogation of *DUSP6* in pancreatic cancer cells.

Materials and methods

Cell culture

Human pancreatic cancer cell lines PK-1, PK-8, PK-9, PK45H, PCI-10, PCI-35, PCI-43, PCI-55, PCI-66, PAN03JCK, PAN07JCK, MIA PaCa-2, SU.86.86, AsPC-1, BxPC-3, and PANC-1 were cultured, as previously described (Furukawa et al. 1998). The immortalized normal human pancreatic ductal cells (HPDE) were kindly provided by Dr. Ming S. Tsao and cultured as previously described (Furukawa et al. 1996).

Treatment with 5-azacytidine and trichostatin A

Cells were seeded at a density of $1–2\times10^5$ /10 cm (in diameter) culture dish. The cells were maintained for 96 h while replacing the culture medium with the appropriate medium containing 5-azacytidine (Sigma, St Lois, MO, USA) at 0.2 or 2 μM every 24 h. At the last replacement, trichostatin A (Wako, Osaka, Japan) was added at 300 ng/ml optionally. These cells were harvested for further investigation.

Quantitative real time RT-PCR

Total RNAs were extracted from the harvested cells using RNeasy mini kit (QIAGEN, Tokyo, Japan) according to the supplier's instructions. Each purified RNA was dissolved in RNase-free water, and its concentration was measured by optical absorbance at A_{260}. First-strand cDNA was synthesized using an aliquot of 10 μg total RNA and Super Script II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) by methods described previously (Mori et al. 1997). The synthesized cDNA was used for a quantitative real time PCR analysis using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Specific primers and a common probe to monitor the two distinct transcripts of *DUSP6*, the full-length transcript (*DUSP6-FULL*) and the exon 2 lacking alternative transcript (*DUSP6-ALT*) (Furukawa et al. 1998), were designed by using the Primer Express software (Applied Biosystems), and their nucleotide sequences are listed in Table 1. The primers were purchased from Nihon Gene Research Laboratories (Sendai, Japan), and the common FAM-TAMURA-labeled probe was purchased from Sigma Genosys (Ishikari, Japan). Expression of the ß2-microglobulin (*B2M*) was monitored as an internal control, and nucleotide sequences of the primers and a FAM-TAMURA-labeled probe are listed in Table 1. Amplifications were carried out in the reaction mixture in 25 μl containing 5 μl of cDNA samples and 12.5 μl of 2×ABsolute QPCR ROX Mix (ABgene, Epsom, UK), and the final concentration of 0.2 μM of each primer pair and 0.4 μM of the probe were added in a program comprised of 2 min at 50°C, 15 min. at 95°C, followed by 40 cycles consisting of 15 s at 95°C and 1 min at 60°C. Expression ratio of *DUSP6-FULL/B2M* or *DUSP6-ALT/B2M* was calculated and used. Each experimental reaction was performed in triplicate.

Rapid amplification of cDNA ends (RACE)

To determine the 5′ end of *DUSP6* transcript, we performed a 5′-RACE experiment with total RNA extracted from NTI-4 cells, the normal human lung fibroblast, using a Smart RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. Nucleotide sequences of the primers are shown in Table 1. The PCR-amplified 5′-RACE product was cloned into λZapII vector (Stratagene, La Jolla, CA, USA) and purified as described (Horii et al. 1993), and the nucleotide sequences of the clones were determined using an ABI PRIZM BigDye Terminator Cycle Sequencing FS Ready Reaction Kit and an ABI PRIZM 310 DNA Analyzer according to the manufacturer's instructions (Applied Biosystems).

Pancreatic cancer tissues, immunohistochemistry, microdissection, and DNA extraction

Formalin-fixed and paraffin-embedded pancreatic tissues with invasive ductal carcinoma from 12 patients

Table 1 Nucleotide sequences of primers and probes

	Forward primer (5′–3′)	Reverse primer (5′–3′)	Size of product	Annealing temp. (°C)
5′ RACE				
1st primer (−198 to −218)		CGCTGGCTCTTAGTGTCAAT		
Nested primer[a] (−267 to −239)		AATGAATTCCAATTAATTCGGACTCCGT		
Adaptor primer	AAGCAGTGGTAACAACGCAGAGTACGCGGG			
Real-time PCR				
DUSP6-FULL	CAGTGGTGCTCTACGACGAG	GCAATGCAGGGAGAACTCGGC	161-bp	60
DUSP6-ALT	the same as above	GCCCCGGGCTTCATCTTCCA	132-bp	60
DUSP6-probe	AATACGGGCGGCGAGTCGGTGCT			
B2M	GTGACTTTGTCACAGCCCAAGA	AACCTCCATGATGCTGCTTACA	65-bp	60
B2M-probe	AGTTAAGTGGGATCGAGAC			
Genomic bisulfite-modification specific PCR and sequencing for *DUSP6*				
Region 1	TAGTAAGGGATATTTTTTAAAGT	CAAAACTCTATTATTATAAAAATAC	55	
Region 2	GAGTTGGGTTTTTAAAAGTGGTAAATA	CAAATAATTTTTATTCCTCCCCAA	60	
Region 3	TTGGGGAGGAATAAAAATTATTTG	CTTAAACTTCTTAAACAACAACC	58	
Region 4	GGTATTGATATAGTGGTGTTTTA	AAACAAAAATATTTTCAATCCAC	55	
Region 5	GTGGATTGAAAATATTTTTTGTTT	CTAAAATATACCAATTTACATCC	53	
Methylation specific PCR for *DUSP6*				
Methylated	GTAGGGGTCGCGAATCGCGC	ACCGCCGATACCCGCAACCG	84-bp	65
Unmethylated	GTAGGGGTTGTGAATTGTGT	ACCACCAATACCCCACAACCA	84-bp	58

[a]Nested primer for 5′ RACE harbor *Eco*RI site to facilitate cloning

operated at Tohoku University Hospital were employed for this study. Of these tissues, five were with poorly differentiated adenocarcinoma, and the remaining seven were with moderately differentiated ductal adenocarcinoma. Immunohistochemical staining of DUSP6 was done, as described previously (Furukawa et al. 2003). Carcinoma cells were microdissected from sections 10 μm thick using the LM100 Laser Capture Microdissection system, according to the manufacturer's instructions (Arcturus, Mountain View, CA, USA). DNA was extracted by using the QIAamp DNA Micro Kit (QIAGEN). This study was approved by the Ethical Committee of Tohoku University School of Medicine.

Genomic sodium bisulfite sequencing analysis and methylation-specific PCR assays

Each aliquot of 1 μg of genomic DNA from cultured cancer cells or primary tumor cells was modified with sodium bisulfite by using CpGenome DNA Modification Kit (Chemicon, Temecula, CA, USA) according to the supplier's instructions, and their nucleotide sequences were determined by the method described previously (Sakurada et al. 1997). The modified DNA was used as a template for PCR amplification, and we analyzed five regions: region 1, between −2250 and −2016; region 2, between −996 and −241; region 3, between −265 and +369; region 4, between +270 and +487; and region 5, between +465 and +769 where the adenine residue at the translation initiation codon was numbered as +1. Nucleotide sequences and PCR conditions are summarized in Table 1.

The methylation-specific PCR assay (MSP) was carried out based on the methods described elsewhere (Sato et al. 1998). For MSP analysis, we designed primer sets for both unmethylated and methylated sequences at the highest methylated region in the intron 1 of *DUSP6*, and the nucleotide sequences of the primers and PCR conditions are also shown in Table 1.

Statistics

All experiments were performed at least three times independently. Statistical calculation was done using Statview software (SAS Institute Inc., Cary, NC, USA).

Results

Quantification of the intrinsic expression levels of *DUSP6* in cultured pancreatic cancer cells

First, we determined the precise intrinsic expression levels of *DUSP6-FULL* in cultured pancreatic cancer cells by the quantitative real-time PCR method. As shown in Fig. 1, our results indicated that it was possible to divide the cell lines into four groups, depending on the expressional levels of the cell lines and that of HPDE,

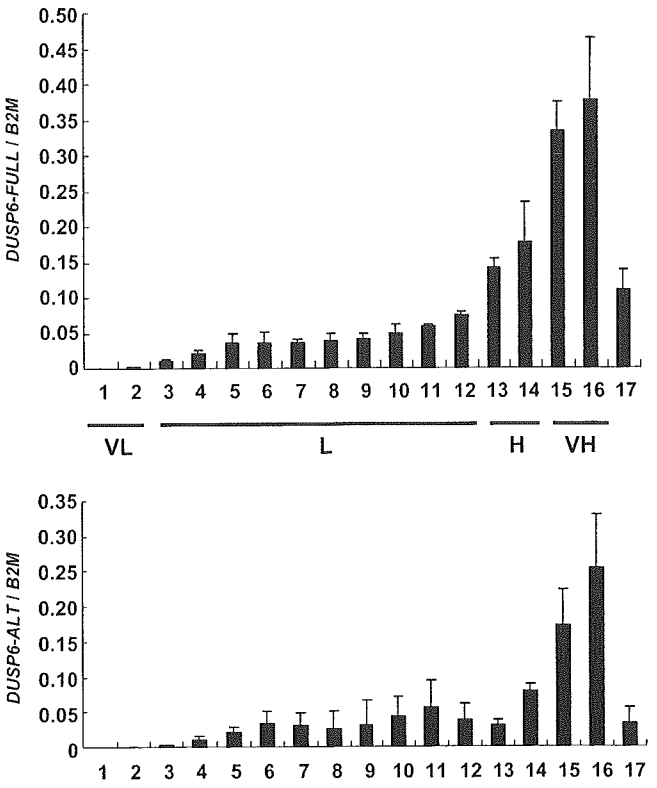

Fig. 1 *DUSP6* expression levels in pancreatic cancer cell lines determined by the quantitative real-time PCR assay. Relative expression levels of *DUSP6-FULL* (*upper column*) or *DUSP6-ALT* (*lower column*) to β 2-microglobulin(*B2M*) are presented. A total of 16 pancreatic cancer cell lines were analyzed and run on gels in *lanes 1–16* in the order of PAN07JCK, MIA PaCa-2, PK-45H, PK-9, PCI-35, PK-1, PCI-43, PCI-66, PANC-1, PCI-55, PCI-10, SU.86.86, PAN03JCK, PK-8, AsPC-1, and BxPC-3. The immortalized normal pancreatic ductal cell line human pancreatic ductal cells (HPDE) is on *lane 17*. *Horizontal bars under the lane numbers* indicate four groups: very-low-level expression (*VL*), low-level expression (*L*), high-level expression (*H*), and very-high-level expression (*VH*), as described in text. *Error bars* denote the standard deviations

the immortalized normal human pancreatic ductal cell line: cells in which expression levels were very low or high (beyond the range of average *pm*3SD) were classified as very-low-level expression group (PAN07JCK and MIA PaCa-2) or very-high-level expression group (AsPC-1 and BxPC-3), respectively. The remaining cells were further divided into two groups: cells in which expression levels were lower or higher than that of HPDE were classified as low-level expression group (PK-45H, PK-9, PCI-35, PK-1, PCI-43, PCI-66, PANC-1, PCI-55, PCI-10, and SU.86.86) or high-level expression group (PAN03JCK and PK-8), respectively. We also assessed the expressions of *DUSP6-ALT*, the alternative transcript we identified previously (Furukawa et al. 1998), in these cells and found almost in parallel level expressions to those of the *DUSP6-FULL* transcript in most of the cell lines, as shown in Fig. 1, suggesting that both transcripts were expressed under the same transcriptional controlling machinery.

Restoration of *DUSP6* by treatments with 5-azacytidine and trichostatin A

To determine whether the transcriptional silencing of *DUSP6* can be attributed to a hypermethylation and/or a modulation of histone deacetylation, we determined the intrinsic expression levels of *DUSP6* in cells with different intrinsic expression levels, including MIAPaCa-2, PAN07JCK, PCI-35, PCI-55, PCI-66, PK-8, and AsPC-1 after treatment with 5-azacytidine and trichostatin A to prevent DNA methylation and histone deacetylation, respectively (Ghoshal et al. 2002). As shown in Fig. 2a, expression of *DUSP6-FULL* increased by 7.4 fold in MIA PaCa-2 cells by 5-azacytidine treatment alone and increased 34 fold in PAN07JCK with 5-azacytidine and trichostatin A treatment. Both of the cells showed intrinsically very-low-level expression. The cell lines with intrinsically low-level expression showed increased expression by cotreatment with 5-azacytidine and trichostatin; 9.4, 4.3, and 1.9 fold in PCI-35, PCI-66, and PCI-55, respectively. No obvious increase was observed in PK-8 or AsPC-1, the cells with high-level and very-high-level expression, respectively. We also observed parallel restorations of *DUSP6-ALT* (see Fig. 2b). The results suggested that the hypermethylation with or without histone deacetylation played an important role in the transcriptional suppression of *DUSP6* in pancreatic cancer cells.

In search for the transcriptional control region(s) of *DUSP6*

To determine whether or not hypermethylation actually occurs in the expressional regulatory regions of *DUSP6*, we moved on to DNA analysis beginning with searching for the transcriptional control regions of the gene. Because the transcriptional start site of *DUSP6* was not certain, we first determined it by the 5′-RACE method; the position at 462-nt upstream from the translation initiation codon seemed to be the major transcription start site. The nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession number AB189400. Comparing it with the human genome sequence data, we identified several CpG islands, one between −600 and −320 in the predicted promotor region and 5′ portion of exon 1 and another between −189 and +674 in exon 1 and intron 1; both regions were considered as potential transcriptional regulatory regions. We found several potential binding sequences for transcription factors in and around these regions using the Match program (http://www.gene-regulation.com), including the consensus-binding motif for ELK1 (CCGGAARY), CCGGAAGG, in the upstream region between −2109 and −2116 from the translation initiation site (with one mismatch) in the antisense strand, and CCGGAAGC in intron 1 between +610 and +603 from the translation initiation site in the antisense strand. Other motifs such as AG, AP-1,

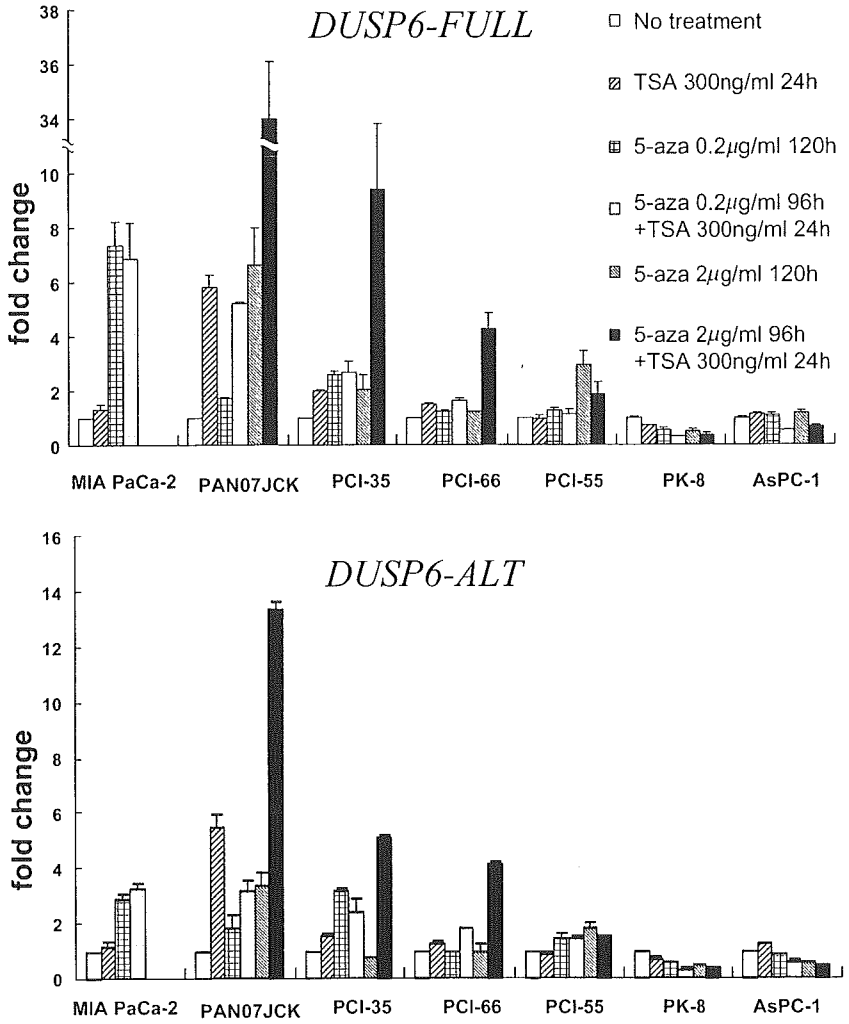

Fig. 2 Restoration of *DUSP6* by treatments with 5-azacytidine (*5-aza*) and trichostatin A (*TSA*). The cells were treated with 5-azacytidine at different concentrations for 96 h followed by trichostatin A treatment or 5-azacytidine for 24 h or trichostatin A only for 24 h, and the relative mRNA levels of *DUSP6-FULL*/ß2-microglobulin (B2M) (*panel A*) and *DUSP6-ALT*/B2M (*panel B*) were measured by the quantitative real-time RT-PCR assay. In each case, results are shown in fold changes relative to the no treatment cell, as indicated by the *open box*. Each experiment was performed in triplicate, and *error bars* denote the standard deviations

Oct-1, HNF-4, myogenin/NF-1, NF-Y, HNF-1, SOX-9, FOXD3, v-Myb, and CCAAT box were found in the upstream region; COMP1, Barbie Box and Hand1/E47 were found in exon1; and Oct-1 was found in intron 1 of *DUSP6* with the help of this program. Because ELK1 is one of major target transcription factors of ERK, we performed nucleotide sequencing analyses for the regions covering the binding sites (between −2500 and −2003 in the upstream region and between +477 and +686 in intron 1) in the pancreatic cancer cell lines analyzed in this study, but no alterations were observed.

Identification of methylated CpG sites in the putative regulatory regions of *DUSP6*

We employed the sodium bisulfite-modification method to identify methylated sequences in the putative expressional control regions of *DUSP6*, the putative promotor region, and the 5′ region in intron 1, in MIAPaCa-2, PAN07JCK, PCI-35, PCI-55, PCI-66, PK-8, and AsPC-1. We found that the CpG sites in intron 1 of *DUSP 6* were highly methylated in MIA PaCa-2 and PAN07JCK (see Figs. 3, 4); the cells showed intrinsically very-low-level expression and restoration by the demethylating treatment. We found no methylated sequences in PCI-35, PCI-55, or PCI-66, cells with intrinsically low-level expression with modest response to the demethylating treatment, or in PK-8 or AsPC-1, cells with intrinsically high or very-high-level expression without any response to the demethylating treatment. Some of the methylated CpG sequences involved potential binding sites for transcription factors, including ELK1 in intron 1, as described above. In this region, we observed almost complete methylation at the CpG sites in MIA PaCa-2 and a partially methylated CpG site in PAN07JCK but not in other cells (see Fig. 4). We found a partial methylation in the upstream region in MIA PaCa-2 (data not shown). Although this region was not hypermethylated, there is a possibility of some association between methylation and transcriptional suppression. We found no methylation in this upstream region in any other cell lines.

We designed a primer set for methylation-specific PCR analysis targeting the most methylated region between +544 and +627, the' portion of intron 1, to reveal the methylation status in all pancreatic cancer cell lines analyzed in this study. We observed methylation-specific products in MIA PaCa-2 and

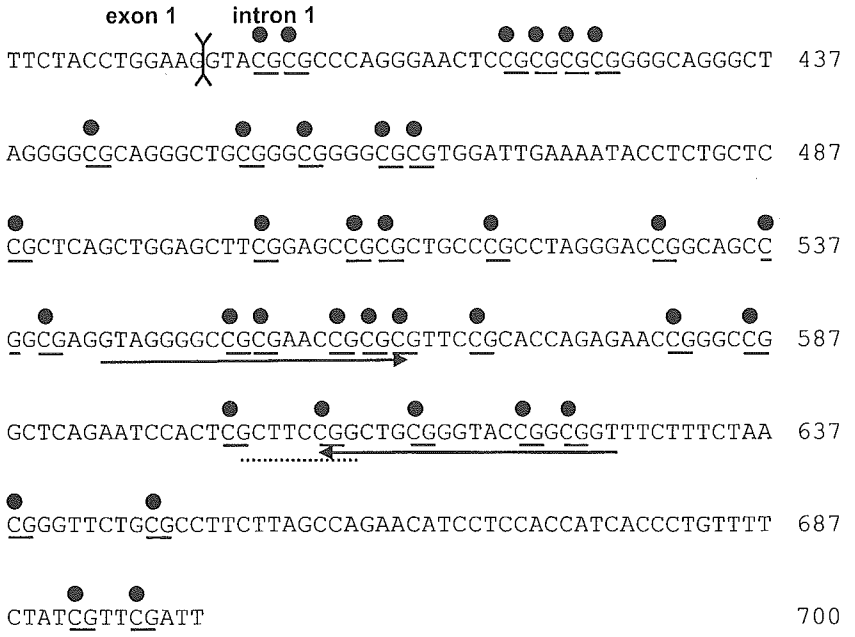

Fig. 3 Hypermethylation of the CpG islands in intron 1 of *DUSP6*. Nucleotide sequences of intron 1 of *DUSP6* are shown, and the CpG sites are *underlined*. Methylated cytosine residues identified by the sequencing analysis after sodium bisulfite treatment in MIA PaCa-2 and PAN07JCK, either completely or partially, are indicated by *closed circles*. *Arrows* indicate positions of primers used in the methylation-specific PCR assay. The consensus-binding motif for ELK1 is indicated by a *dotted bar*

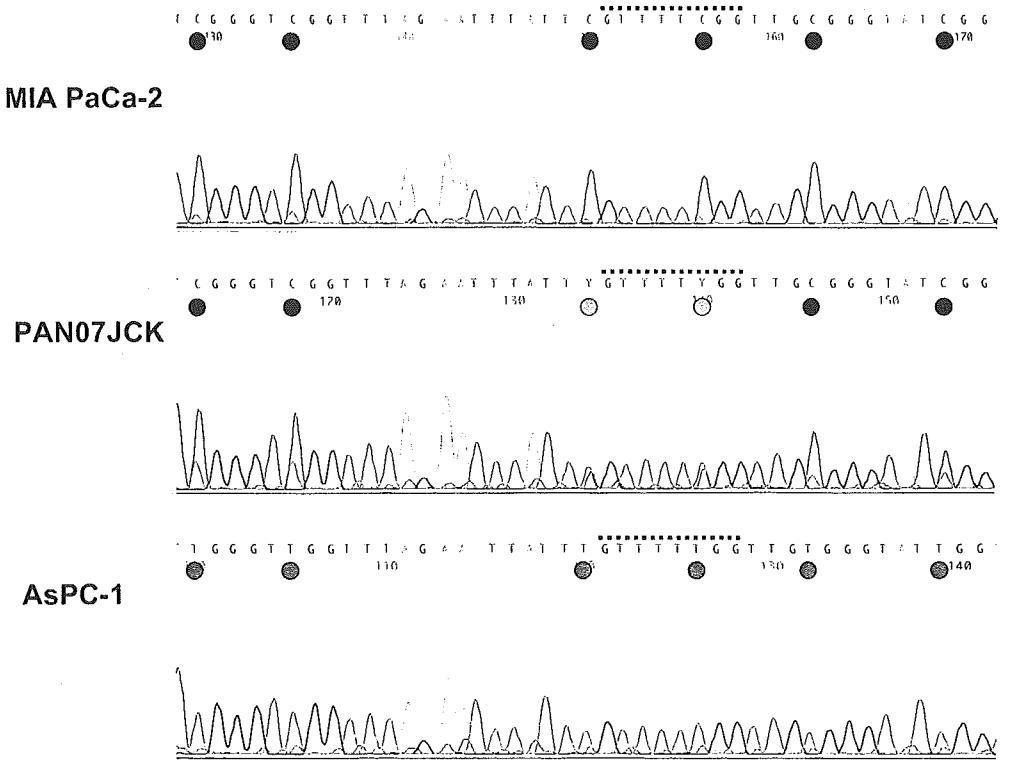

Fig. 4 Representative results of the genomic sequencing analysis of intron 1 of *DUSP6* after sodium bisulfite modification. *Circles in blue* indicate the cytosine residues in CpG sequences with complete methylation; only a single peak for C is observed. *Circles in green* indicate partial methylation; double peaks for C and T are observed. *Circles in red* indicate no methylation; only a single peak for T is observed. The consensus-binding motif for ELK1 is indicated by the *dotted bar*

PAN07JCK, as expected, but not in any other cell lines (Fig. 5). In order to further determine whether or not the methylation is observed in primary pancreatic cancer tissues, we performed methylation-specific PCR analyses in 12 cases with primary invasive ductal carcinoma of the pancreas. By taking advantage of the previous immunohistochemical analysis (Furukawa et al. 2003), we selected eight cases with almost completely abolished expression of DUSP6 (four poorly differentiated type and four moderately differentiated type) and four with preserved expression of DUSP6 (one poorly differentiated type and three moderately differentiated type) (see Fig. 6). The methylation was detected in five of the eight cases with abolished expressions of DUSP6: four of them were poorly differentiated adenocarcinoma while none of the four cases with preserved expression of DUSP6 showed methylation (see Fig. 5). The methylation status significantly correlated with both the abolishment of protein expression ($p = 0.038$) and the histological type of adenocarcinoma ($p = 0.023$) by chi-

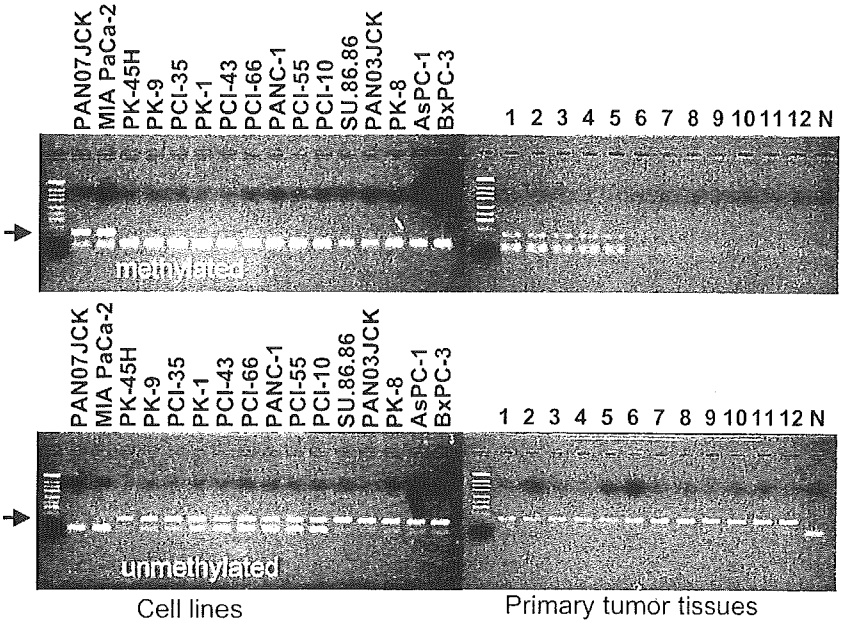

Fig. 5 Methylation-specific PCR assay for the CpG islands in intron 1 of *DUSP6*. Localization of the primers used is shown in Fig. 3. *Arrows* indicate methylation-specific PCR products in the *upper panel* and unmethylated PCR products in the *lower panel*. Cell lines are indicated by the *name*, and primary tumors are indicated by the *numbers*. *Lanes 1– 4*: poorly differentiated adenocarcinoma with abolished DUSP6 expression (cases 8590, 8625, 1839–2, and 7668); *lanes 5– 8*: moderately differentiated adenocarcinoma with abolished DUSP6 expression (cases 8199, 8657, 7326, and 239–290); *lane 9*: poorly differentiated adenocarcinoma with preserved DUSP6 expression (case 5537); *lanes 10– 12*: moderately differentiated adenocarcinoma with preserved DUSP6 expression (cases 7975, 7936, and 8596). *N* denotes the negative control (PCR without template DNA)

square test. These results indicated that hypermethylation of CpG islands in intron 1 might account for the expressional suppression of DUSP6 in pancreatic cancers, particularly in poorly differentiated types.

Discussion

Expression of DUSP6 is frequently suppressed in human pancreatic cancer. We found that hypermethylation was one of the important players in transcriptional suppression of *DUSP6* in some of the pancreatic cancer cell lines and primary pancreatic cancer tissues. To our best knowledge, this is the first report identifying a mechanism for abrogation of *DUSP6* in human cancers.

The pancreatic cancer cell lines we tested could be classified into four groups by their intrinsic expression levels of *DUSP6*; a very-low-level expression group, a low-level expression group, a high-level expression group, and a very-high-level expression group. In our previous investigations, we hypothesized that DUSP6 is overexpressed in a feedback loop manner in cells harboring the gain-of-function mutation of *KRAS2* to suppress hyperactivated ERK driven by the activated RAS (Furukawa et al. 2003; Furukawa and Horii 2004). This hypothesis led us to think that the cells in the groups of very-low-level expression, and possibly some of the cells in low-level expression, harbor oncogenic phenotypes through downregulation of DUSP6.

In analyses to determine the mechanisms of underexpression of DUSP6, the cells in the group of very-low-level expression and low-level expression responded to the demethylation and modifications of histone deacetylation treatments. We detected hypermethylation of CpG islands in the potential transcriptional control region of *DUSP6* in the cells in the former group but not in the latter group or the groups with high-level and very-high-level expression. These observations suggest that the hypermethylation of the CpG islands is responsible for the strong suppression of *DUSP6* while those outside of our investigation may account for the moderate suppression. Our results also suggest that the modification of histone deacetylation plays a synergistic role with hypermethylation in the transcriptional suppression; this needs further investigation.

Our present results indicate that hypermethylation of the CpG islands in intron 1 leads to a strong suppression of *DUSP6* expression. Interestingly, this region harbors the consensus-binding sequence for ELK1 that contains CpG in the core sequence CCGGAARY. As noted, ELK1 is one of the major targets for ERK, the activity of which is controlled by DUSP6. These results suggest that ELK1 binding plays an important role in regulation of *DUSP6* expression, which fits the concept of a feedback loop relationship between ERK and DUSP6.

The methylation in intron 1 is associated with abolished protein expression of DUSP6 in primary pancreatic cancer tissues, especially in those with invasive ductal adenocarcinoma of poorly differentiated type. Our previous immunohistochemical analysis indicated that abolishment of DUSP6 expression was significantly associated with invasive ductal adenocarcinoma of

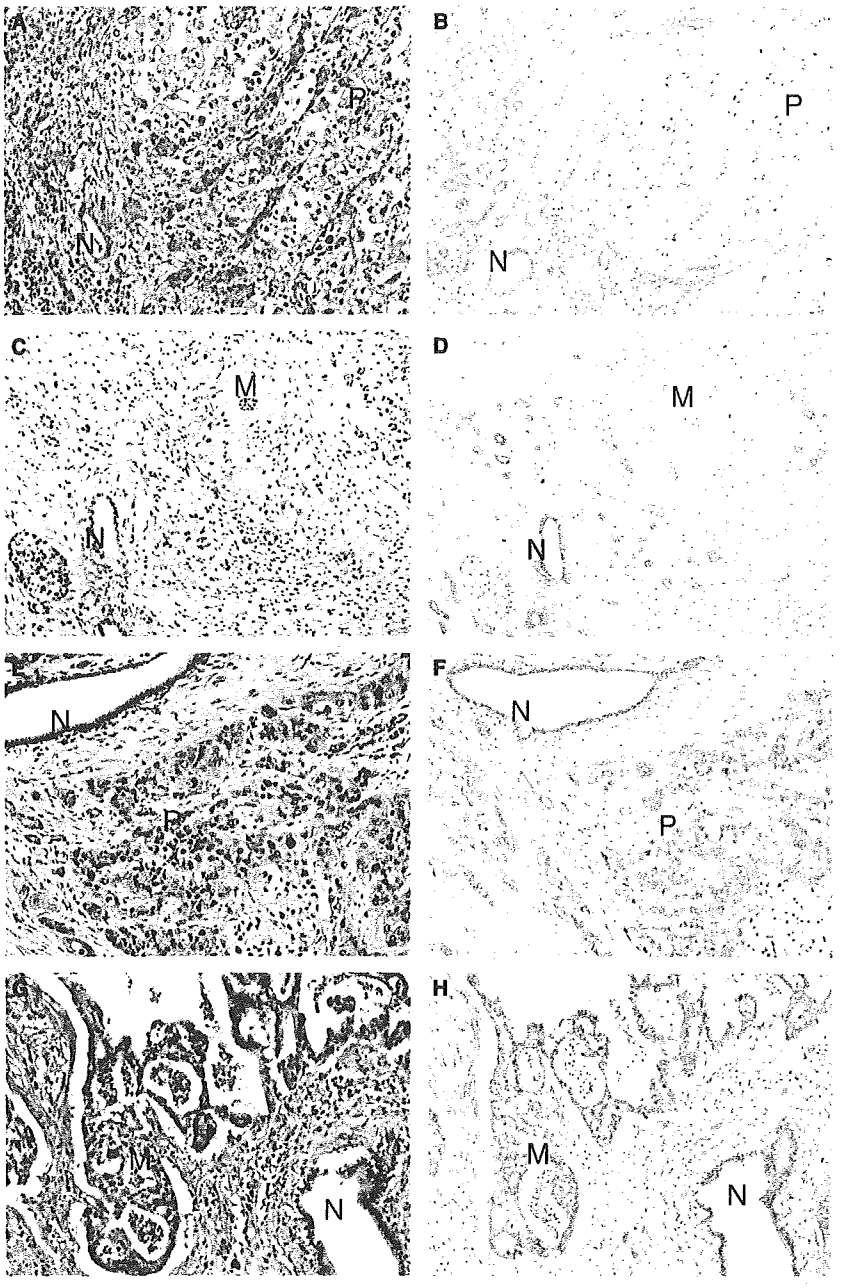

Fig. 6 Representative immunohistochemical images of analyzed samples of primary pancreatic cancer tissues. Hematoxylin-eosin (*HE*)-stained tissues (**a, c, e, g**, and immunohistochemical (*IH*)-stained tissues with anti-DUSP6 antibody (**b, d, f, h**). **a, b** : case 8590 (*lane 1* in Fig. 5); **c, d**: case 8199 (*lane 5* in Fig. 5); **e, f**, case 5537 (*lane 9* in Fig. 5); **g, h**, case 8596 (*lane 12* in Fig. 5). *N* normal pancreatic duct, *P* invasive ductal carcinoma of poorly differentiated type, *M* invasive ductal carcinoma of moderately differentiated type

poorly differentiated type (Furukawa et al. 2003). These results suggest that the abolishment of DUSP6 by hypermethylation may lead to development of invasive ductal adenocarcinoma, particularly of poorly differentiated type.

As demonstrated, treatment with 5-azacytidine and trichostatin A could restore the endogenous expression of *DUSP6* in pancreatic cancer cells with high suppression. We previously demonstrated that the exogenous expression of *DUSP6* induced marked growth suppression and eventual apoptosis of the pancreatic cancer cells. It is of great interest to determine whether or not such 5-azacytidine and trichostatin A treatment can lead to modify pancreatic cancer cell growth although their broad effects on gene expression could lead to unexpected side effects.

Taking our results together, hypermethylation with modification of histone deacetylation is suggested to play an important role in transcriptional control of *DUSP6*. Our results may shed light on understanding those mechanisms of pancreatic carcinogenesis, which could be particularly important in the progression of dysplastic ductal cells/pancreatic intraepithelial neoplasia (PanIN) to invasive ductal carcinoma. This is the step with which the abrogation of DUSP6 is strongly associated, and it is critical for development of pancreatic cancer (Furukawa et al. 2003), one of the most fatal diseases of any human malignancy.

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Distinct progression pathways involving the dysfunction of DUSP6/MKP-3 in pancreatic intraepithelial neoplasia and intraductal papillary-mucinous neoplasms of the pancreas

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DUSP6/MKP-3 **is identified as a candidate tumor suppressor gene for pancreatic cancer. The aim of this study was to elucidate the roles of DUSP6 in the pancreatic carcinogenesis through the pancreatic intraepithelial neoplasia and/or intraductal papillary-mucinous neoplasms, both of which are considered to be precursor lesions of invasive carcinoma of the pancreas, by comparing with involvements of other major tumor suppressive pathways. Expressions of DUSP6, CDKN2A, TP53, and SMAD4 were investigated by immunohistochemistry in a total of 206 lesions of dysplastic ductal precursors and carcinomas retrieved from 52 pancreata with invasive ductal carcinomas and 51 of those with intraductal papillary-mucinous neoplasms. The intensity of staining was evaluated in lesions at different atypical grades and statistically compared among them. Mutations of *KRAS2* were analyzed by methods of the allele-specific oligonucleotide hybridization and nucleotide sequencing. In pancreata with invasive ductal carcinomas, expressions of DUSP6 were abrogated exclusively in the invasive carcinoma cells in contrast to its fairly preserved expressions in pancreatic intraepithelial neoplasia. In pancreata with intraductal papillary-mucinous neoplasms, abrogated expressions of DUSP6 were observed in a relatively small fraction of intraductal adenoma/borderlines and intraductal carcinomas. Most of the intraductal adenoma/borderline lesions with abrogation of DUSP6 harbored mutations of *KRAS2*. None of the molecules was associated with each other in any grade of lesions. Morphological variations of papillae of the intraductal papillary-mucinous neoplasms were evaluated and analyzed for their associations with abrogations of the molecules, which resulted in finding of no significant associations. Our results suggest that the abrogation of DUSP6 is associated exclusively with progression from pancreatic intraepithelial neoplasia to the invasive ductal carcinoma while it is potentially associated with initiation of intraductal papillary-mucinous neoplasms with mutated *KRAS2*, which is independent of other major tumor suppressive pathways in both types of neoplasms.**
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Keywords: intraductal papillary-mucinous neoplasm; pancreatic intraepithelial neoplasia; DUSP6; p16; p53; SMAD4

Genomic analysis of invasive ductal carcinoma of the pancreas has revealed frequent loss of heterozygosities in several specific chromosomal regions, including 1p36, 6q21–q24, 9p21, 12q21–q23.1, 17p13, and 18q21.[1–3] These regions potentially harbor tumor suppressor genes involved in development and progression of the pancreatic cancer. To date, several candidate tumor suppressor genes are identified in these regions, and frequent inactivating mutations have been reported in the pancreatic cancer; *CDKN2A/INK4/p16* at 9p21, *TP53/p53* at 17p13, and *SMAD4/MADH4/DPC4* at 18q21.[4–6] Recently, we identified *DUSP6/MKP-3* at 12q21–q22 as a candidate tumor suppressor gene.[7,8] DUSP6 is a dual specificity phosphatase that intrinsically

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binds and inactivates MAPK1/ERK2 in a feedback loop manner[9] and plays an important role in physiological regulation of the signaling pathway of RAS and mitogen-activated protein kinases (MAPK).[10–13] In a previous report, we demonstrated that expressions of DUSP6 were reduced or abolished in cells of invasive ductal carcinoma of the pancreas in contrast to its overexpressions in dysplastic cells of pancreatic ducts.[8] Cultured human pancreatic cancer cells lacking an expression of DUSP6 tended to show constitutively active MAPK1, and an adenovirus-mediated introduction of *DUSP6* into such DUSP6-inactivated cells induced inactivation of MAPK1, resulting in a marked growth suppression and eventual apoptosis of the cells.[8] Since a vast majority of pancreatic cancer cells harbors the gain-of-function-mutation of *KRAS2*, which constitutively activates several downstream signal cascades including RAF1-MAP2K1-MAPK1, these results indicated that the abrogation of DUSP6 synergistically contributes to hyperactivation of MAPK1, which may eventually result in the development and progression of invasive ductal carcinoma of the pancreas.[9]

Dysplastic cells in pancreatic ducts are thought to be precursor lesions of the invasive ductal carcinoma, not only because of the classic findings of their frequent distributions in pancreata involved with invasive ductal carcinomas but also because of the similarities in their molecular backgrounds that have been discovered by recent molecular studies.[14–19] The recently proposed progression model based on the definition of the pancreatic intraepithelial neoplasia facilitates recognition of these dysplastic ductal regions as major precursor lesions and investigation of their molecular phenotypes.[20,21] Intraductal papillary-mucinous neoplasm was first reported in 1982 as a special type of pancreatic neoplasm exhibiting a mucinous ectatic duct with dysplastic papillary epithelia.[22] Intraductal papillary-mucinous neoplasms are considered to be one of the precursor lesions of the pancreatic cancer, because some patients with intraductal papillary-mucinous neoplasms eventually develop an invasive papillary-mucinous carcinoma/invasive carcinoma associated with intraductal papillary-mucinous neoplasm, which is comprised of either invasive colloid mucinous carcinoma or invasive ductal carcinoma.[23,24] Although there are some morphological similarities between the pancreatic intraepithelial neoplasia and intraductal papillary-mucinous neoplasms, different clinicopathological features between them suggest that the pancreatic intraepithelial neoplasia and intraductal papillary-mucinous neoplasms would develop and progress through distinct pathways involving different genetic and molecular alterations, a hypothesis which seems to be endorsed by some molecular studies.[25–27]

The aim of this study was to elucidate the roles of DUSP6 in the development and progression of pancreatic intraepithelial neoplasia and the intraductal papillary-mucinous neoplasm in association with other major tumor suppressive pathways. We investigated abrogations of DUSP6, CDKN2A, TP53, and SMAD4, and mutations of *KRAS2* in the dysplastic ductal precursor lesions and carcinoma cells in pancreata afflicted by invasive ductal carcinomas or intraductal papillary-mucinous neoplasms.

Materials and methods

Pancreatic Tissues

Pancreatic tissues from 103 patients were analyzed in this study; 52 cases with invasive ductal carcinomas and 51 cases with intraductal papillary-mucinous neoplasms were employed in this study. Of the 52 cases with invasive ductal carcinomas, 42 were classified as tubular adenocarcinomas of the well or moderately differentiated type and 10 as adenocarcinomas of the poorly differentiated type. Of the 51 cases with intraductal papillary-mucinous neoplasms, 16 tumors were intraductal papillary-mucinous adenoma/borderlines, 27 were intraductal papillary-mucinous carcinomas, five were invasive colloid mucinous carcinomas associated with intraductal papillary-mucinous neoplasms, and the remaining three were invasive ductal carcinomas associated with intraductal papillary-mucinous neoplasms. All these tissues had been surgically resected at the Department of Gastroenterological Surgery, Tohoku University Hospital. All the tissues were fixed in 10% buffered formalin and embedded in paraffin. Consecutive sections at 4 μm thick were prepared; one section was stained with hematoxylin and eosin, and the others were used for immunohistochemical studies. Atypical epithelial lesions were evaluated in the sections according to the criteria published previously.[19,21,27] From 52 pancreata with invasive ductal carcinoma, 38 lesions of low-grade dysplasia/pancreatic intraepithelial neoplasia-1 and -2, 24 lesions of high-grade dysplasia/pancreatic intraepithelial neoplasia-3, and 57 lesions of invasive carcinoma (47 of those of the well or moderately differentiated type and 10 of those of the poorly differentiated type) were retrieved (two or more lesions were retrieved independently from most of the patients). From 51 patients with intraductal papillary-mucinous neoplasms, 47 lesions of intraductal papillary-mucinous adenoma or borderline, 32 lesions of intraductal papillary-mucinous carcinoma, five lesions of invasive colloid mucinous carcinoma associated with intraductal papillary-mucinous neoplasm, and three lesions of invasive ductal carcinoma associated with intraductal papillary-mucinous neoplasm were retrieved. Multiple kinds of lesions were retrieved independently from each case. This study was approved by the Ethical

Committee of the Tohoku University School of Medicine.

Immunohistochemistry

Immunohistochemistry was performed by the indirect biotin–streptavidin–peroxidase method as described previously.[8] The antigen retrieval method employed for staining of all molecules but DUSP6 were as described.[28] The antibodies employed were a polyclonal anti-DUSP6 antibody, C-20 (Santa Cruz Biotechnology Lab Inc., Santa Cruz, CA, USA), monoclonal anti-CDKN2A antibodies, G175-405 (BD Biosciences, San Jose, CA, USA) and DCS-50 (SIGMA, St Louis, MO, USA), a monoclonal anti-TP53 antibody, DO-7 (Dako Cytomation Co. Ltd, Glostrup, Denmark), a monoclonal anti-SMAD4 antibody, B8 (Santa Cruz Biotechnology Lab Inc.), a monoclonal anti-MUC1-CORE antibody, Ma552 (Novocastra Laboratories Ltd. Newcastle upon Tyne, UK), a monoclonal anti-MUC2 antibody, Ccp58 (Santa Cruz Biotechnology Lab Inc.), and a monoclonal anti-MUC5AC antibody, CLH2 (Chemicon International Inc., Temecula, CA, USA) for primary antibody reactions and biotin-conjugated anti-goat or mouse IgG (H+L) antibodies (Vector Lab. Inc., Burlingham, CA, USA) for secondary antibody reactions. The streptavidine solution (Nichirei, Tokyo, Japan) was used for a streptavidine–biotin reaction. DAB (3,3′-diaminobenzidine tetrahydrochloride) was used as a chromogen, and hematoxylin was used for counterstaining. Immunoreactivities for DUSP6, CDKN2A, TP53, and SMAD4 were graded and scored in the retrieved atypical lesions as negative (0+), focally or weakly positive (1+), consistently positive (2+), and intensely positive (3+), as described previously.[8] In the evaluated expressions, 0+ and 1+ for DUSP6, 0+ and 1+ for CDKN2A, 0+ and 3+ for TP53, and 0+ for SMAD4 were considered as abnormal expressions, all of them suggesting abrogation of their proper functions.

Analysis of *KRAS2* Mutation

Lesions were microdissected using LM100 Laser Capture Microdissection system according to the manufacturer's instructions (Arcturus, Mountain View, CA, USA). DNA was extracted by using the Pico-Pure DNA Extraction Kit according to the manufacturer's instructions (Arcturus). The allele-specific oligonucleotide hybridization method employed for analysis of mutations at cordon 12 of *KRAS2* was based on the procedure described previously.[29] In brief, the region harboring codon 12 of *KRAS2* was amplified by PCR using specific primers reported previously,[30] and the amplified product was electrophoresed in 3% agarose gel and Southern-blotted on a nitrocellulose membrane. Oligonucleotides corresponding to all the mutations of codon 12 of *KRAS2* were synthesized according to the literature,[29] and γ-[^{32}P] labeled probes were prepared by T4 polynucleotide kinase. Hybridization at 50°C in 7% PEG/10% SDS and subsequent stringent wash in 6 × SSC at 58–65°C (temperature depending on probes) was performed according to methods described previously.[31] Signals were visualized by BAS-1500 according to the manufacturer's instructions (Fujifilm Co. Ltd, Minamiashigara, Japan). A control for normal *KRAS2* gene (GGT/Gly at the codon 12) was prepared by PCR amplification of a normal human genomic DNA, and specific mutational patterns for the codon 12 were prepared by PCR amplification of genomic DNAs derived from cancer cell lines of PK-8 (CGT/Arg), MIA PaCa-2 (TGT/Cys), SU 86.86. (GAT/Asp), PAN03JCK (GTT/Val), and A549 (AGT/Ser) based on the information reported previously.[30,32] In lesions that did not show any mutation by the allele-specific oligonucleotide hybridization at codon 12, the direct sequencing of PCR-amplified products was performed to analyze genetic alterations around codons 12, 13, and 61 of *KRAS2* gene as described previously.[30,33] Primers used for amplifying the genetic region around codons 12 and 13 were the same as used in the allele-specific oligonucleotide analysis. Primers used for amplifying the genetic region around codon 61 were 5′-CCTTCTCAG GATTCCTACAGG-3′ and 5′-AAGAAAGCCCTCCC CAGTCCT-3′.

Statistics

The results of immunohistochemistry were compared statistically by the χ^2 method. Significant difference was considered as $P<0.05$ in Fisher's exact test. Calculations were performed using Statview software (SAS Institute Inc., Cary, NC, USA).

Results

We first investigated the expression of DUSP6 along with CDKN2A, TP53, and SMAD4 by immunohistochemistry in a total of 206 lesions of dysplastic ductal precursors and carcinomas retrieved from 52 pancreata with invasive ductal carcinomas and 51 pancreata with intraductal papillary-mucinous neoplasms. In the retrieved lesions, abrogations of expressions of the molecules in pancreatic intraepithelial neoplasia and invasive ductal carcinomas are summarized in Table 1. Representative images of the immunohistochemistry are shown in Figure 1. To elucidate critical and susceptible stages for inactivations of the molecules, the frequencies of abnormal expressions were compared among the atypical grades statistically by the χ^2 method and the difference was represented by Fisher's exact *P*-value. As indicated in Table 1 and Figure 2a, significant differences were observed in comparison

Table 1 Abnormal expressions of DUSP6, CDKN2A, TP53, and SMAD4 in atypical ductal lesions/pancreatic intraepithelial neoplasia and invasive ductal carcinoma of the pancreas

	LD/PanIN-1 and -2	*HD/PanIN-3*	*IDC*	*LD/PanIN-1 and -2* vs *HD/PanIN-3*[a]	*HD/PanIN-3* vs *IDC*[a]
DUSP6	0/38 (0%)	0/24 (0%)	30/57 (53%)	NA[b]	<0.0001
CDKN2A	12/38 (32%)	20/24 (83%)	47/57 (83%)	<0.0001	>0.9999
TP53	0/38 (0%)	10/24 (42%)	39/57 (68%)	<0.0001	0.0451
SMAD4	1/38 (3%)	13/24 (54%)	52/57 (91%)	<0.0001	0.0003

[a]Fisher's exact *P*-value by χ^2-test.

[b]NA, not applicable.

LD, low-grade dysplasia; HD, high-grade dysplasia; PanIN, pancreatic intraepithelial neoplasia; IDC, invasive ductal carcinoma.

between low-grade dysplasia/pancreatic intraepithelial neoplasia-1 and -2 and high-grade dysplasia/pancreatic intraepithelial neoplasia-3 for CDKN2A, TP53, and SMAD4 ($P<0.0001$ for all). DUSP6 is not applicable for this comparison because none of them revealed abnormality in both of the lesions. In comparison between high-grade dysplasia/pancreatic intraepithelial neoplasia-3 and the invasive carcinoma, significant differences were observed in DUSP6 ($P<0.0001$) as well as in SMAD4 ($P=0.0003$). None of the molecules statistically correlated with each other in any grade. These results indicated that gradual accumulation of inactivations of the molecules were associated with progression of the pathway through pancreatic intraepithelial neoplasia to invasive ductal carcinoma along the atypical grades; CDKN2A, TP53, and SMAD4 were associated with the progression from low-grade dysplasia/pancreatic intraepithelial neoplasia-1 and -2 to high-grade dysplasia/pancreatic intraepithelial neoplasia-3, while the inactivation of DUSP6 was strongly associated with the progression from high-grade dysplaisa/pancreatic intraepithelial neoplasia-3 to invasive carcinoma.

In the retrieved lesions from pancreata with intraductal papillary-mucinous neoplasms, abnormal expressions of DUSP6 as well as CDKN2A, TP53, SMAD4 were analyzed; results are summarized in Table 2 and Figure 1b. In comparisons among atypical grades, significant differences were observed between intraductal papillary-mucinous adenoma/borderlines and intraductal papillary-mucinous carcinomas for CDKN2A ($P=0.0188$) and TP53 ($P=0.0159$) and between intraductal papillary-mucinous carcinomas and the invasive components of invasive ductal carcinoma associated with intraductal papillary-mucinous neoplasm for SMAD4 ($P=0.0148$). None of the molecules statistically correlated with each other in any grade.

Morphological variations of papillae in the intraductal papillary-mucinous neoplasms were evaluated and classified according to criteria based on morphologies and expressions of mucin proteins including MUC1-CORE, MUC2, and MUC5AC, as described elsewhere,[34] as follows: 21 cases of the gastric (null) type, 26 cases of the intestinal type, three cases of the pancreatobiliary type, and one case of the oncocytic type. The evaluated types and abrogations of DUSP6, CDKN2A, TP53, and SMAD4 were statistically compared, but no significant associations were observed.

Since the underexpression of DUSP6 was observed in a relatively small fraction of intraductal papillary-mucinous adenoma/borderlines (8/47, 17%), we investigated mutations of *KRAS2* in those lesions to determine whether or not the underexpression would be pathologic. First, we investigated the mutation of codon 12 of *KRAS2* by the allele-specific oligonucleotide hybridization analysis in six of the eight of intraductal papillary-mucinous adenoma/borderlines with underexpressions of DUSP6. We found that five of these 6 lesions harbored *KRAS2* mutations. The samples revealing no mutation by the allele-specific oligonucleotide hybridization were submitted for additional analyses of codons 12, 13, and 61 by the direct nucleotide sequencing, which resulted in no additional finding of mutations. These results indicated that most of the lesions of intraductal papillary-mucinous adenoma/borderlines with underexpressions of DUSP6 were pathologic in phenotypes (Figure 3, Table 3).

In order to understand the differences between the molecular pathways involved in pancreatic intraepithelial neoplasia and intraductal papillary-mucinous neoplasms, the frequencies of abnormal expressions were compared. As shown in Table 4, the abnormal expression of DUSP6 was significantly less frequently observed in low-grade dysplasia/pancreatic intraepithelial neoplasia-1 and -2 than in intraductal papillary-mucinous adenoma/borderlines ($P=0.0076$). The abnormal expression of SMAD4 was significantly more frequent in high-grade dysplasia/pancreatic intraepithelial neoplasia-3 than in intraductal papillary-mucinous carcinomas ($P<0.0001$). The abnormal expressions of SMAD4 and TP53 were significantly more frequent in invasive ductal carcinomas than in the invasive components of colloid mucinous carcinoma associated with intraductal papillary-mucinous neoplasms ($P=0.0011$ and 0.0493, respectively). The abnormal expressions of TP53 were significantly more frequent in invasive ductal carcinoma than in the invasive ductal components in invasive

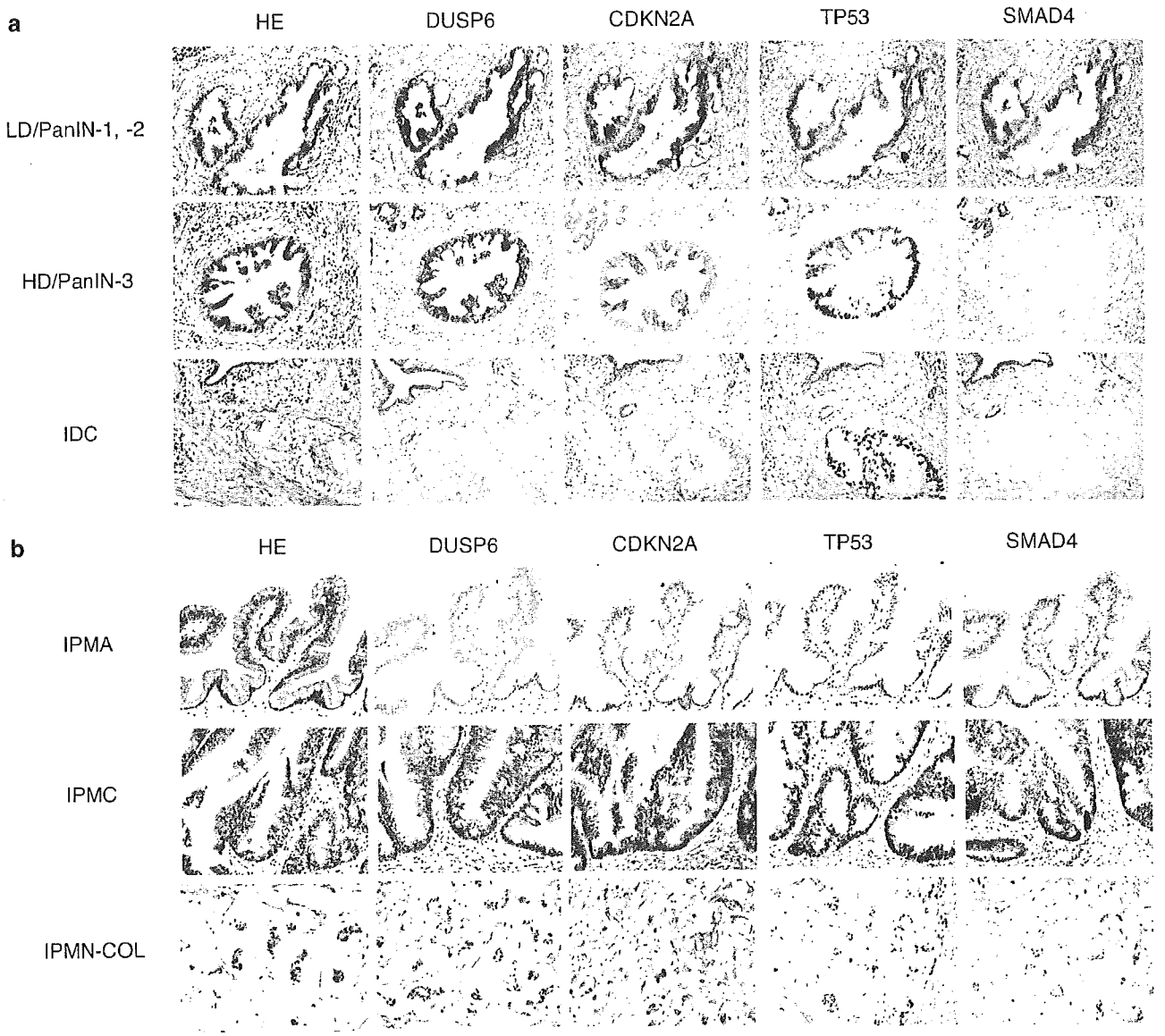

Figure 1 Representative images of immunohistochemistry for DUSP6, CDKN2A, TP53, and SMAD4 in low-grade dysplasia/pancreatic intraepithelial neoplasia-1 and -2 (LD/PanIN-1, -2), high-grade dysplasia/pancreatic intraepithelial neoplasia-3 (HD/PanIN-3), and invasive ductal carcinoma (IDC) (**a**) and in intraductal papillary-mucinous adenoma (IPMA), intraductal papillary-mucinous carcinoma (IPMC), and invasive colloid mucinous carcinoma associated with intraductal papillary-mucinous neoplasm (IPMN-COL) (**b**). Panel **a** shows overexpression of DUSP6 in both low-grade dysplasia/pancreatic intraepithelial neoplasia-1 and -2 and high-grade dysplasia/pancreatic intraepithelial neoplasia-3 in contrast with its underexpression in invasive ductal carcinoma along with loss of intranuclear expression of CDKN2A throughout the lesions, abnormal intranuclear accumulation of TP53 and loss of expression of SMAD4 in high-grade dysplasia/pancreatic intraepithelial neoplasia-3 and invasive ductal carcinoma. Panel **b** shows underexpression of DUSP6 in intraductal papillary-mucinous adenoma, intraductal papillary-mucinous carcinoma, and invasive colloid mucinous carcinoma associated with intraductal papillary-mucinous neoplasm along with loss of intranuclear expression of CDKN2A in all the lesions, abnormal intranuclear accumulation of TP53 in intraductal papillary-mucinous carcinoma, and constitutive expressions of SMAD4 throughout the lesions.

ductal carcinoma associated with intraductal papillary-mucinous neoplasms ($P = 0.0389$), although the number of examined cases for the latter was quite small (three cases).

Discussion

In this study, we found that abrogation of DUSP6 was significantly associated with progression from pancreatic intraepithelial neoplasia to the invasive ductal carcinoma, which was quite characteristic and independent of abrogations of other major tumor suppressor genes; the abrogation of CDKN2A was associated with the development of low-grade dysplasia/pancreatic intraepithelial neoplasia-1 and -2, and abrogations of TP53 and SMAD4 were associated with the development of high-grade dysplasia/pancreatic intraepithelial neoplasia-3. The abrogation of SMAD4 was also significantly

associated with the invasive phenotype, which was consistent with our previous results demonstrating the suppression of invasiveness after introduction of *SMAD4* in *SMAD4*-null pancreatic cancer cells.[35]

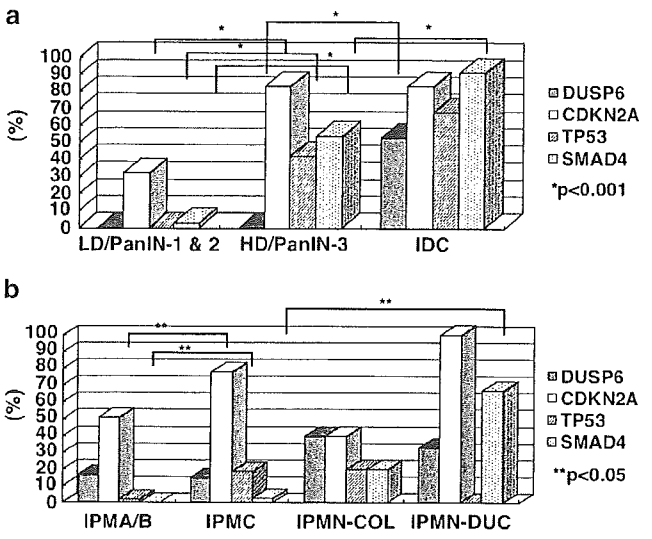

Figure 2 Frequency of inactivation of DUSP6, CDKN2A, TP53, and SMAD4 in pancreata with ductal dysplasia/pancreatic intraepithelial neoplasia (PanIN) and invasive ductal carcinoma (IDC) (**a**) and those with intraductal papillary-mucinous adenoma/borderline (IPMA/B), intraductal papillary-mucinous carcinoma (IPMC), invasive colloid mucinous carcinoma associated with intraductal papillary-mucinous neoplasm (IPMN-COL), and invasive ductal carcinoma associated with intraductal papillary-mucinous neoplasm (IPMN-DUC) (**b**). Asterisks denote significant differences between the groups statistically evaluated by χ^2 test.

However, no significant association was observed between the abrogation of DUSP6 and the abrogation of SMAD4 in spite of our previous results showing the frequent concordant losses of 12q and 18q in

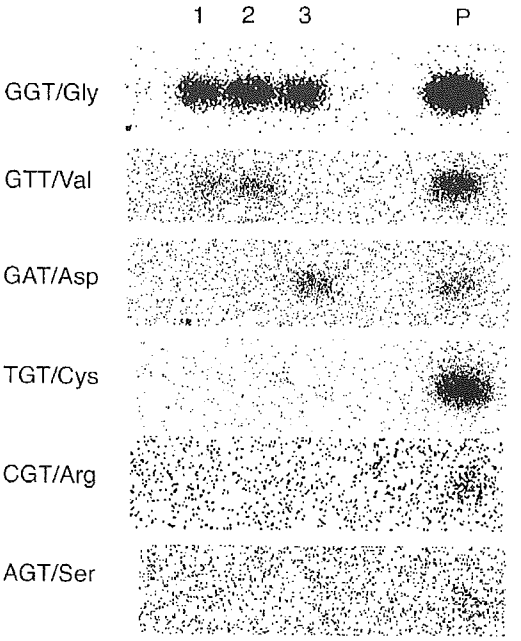

Figure 3 Representative examples of allele-specific oligonucleotide hybridization for analysis of *KRAS2* mutation. Lanes 1–3 correspond to cases 4597, 4868, and 6973, respectively. P denotes positive controls for each hybridization; PCR products prepared from genomic DNA of a normal human individual for GGT/Gly, PAN03JCK for GTT/Val, SU 86.86 for GAT/Asp, MIA PaCa-2 for TGT/Cys, PK-8 for CGT/Arg, and A549 for AGT/Ser.

Table 2 Abnormal expressions of DUSP6, CDKN2A, TP53, and SMAD4 in components of intraductal papillary-mucinous neoplasms

	IPMA/B	*IPMC*	*IPMN-COL*	*IPMN-DUC*	*IPMA/B* vs *IPMC*[a]	*IPMC* vs *IPMN-COL*[a]	*IPMC* vs *IPMN-DUC*[a]
DUSP6	8/47 (17%)	5/32 (15%)	2/5 (40%)	1/3 (33%)	>0.9999	0.2330	0.4417
CDKN2A	24/47 (51%)	25/32 (78%)	2/5 (40%)	3/3 (100%)	0.0188	0.1102	>0.9999
TP53	1/47 (2%)	6/32 (19%)	1/5 (20%)	0/3 (0%)	0.0159	>0.9999	>0.9999
SMAD4	0/47 (0%)	1/32 (3%)	1/5 (20%)	2/3 (67%)	0.1571	0.2553	0.0148

[a]Fisher's exact *P*-value by χ^2-test.
IPMA/B, intraductal papillary-mucinous adenoma/borderline; IPMC, intraductal papillary-mucinous carcinoma; IPMN-COL, invasive colloid mucinous carcinoma associated with intraductal papillary-mucinous neoplasms; IPMN-DUC, invasive ductal carcinoma associated with intraductal papillary-mucinous neoplasms.

Table 3 *KRAS2* Mutation in intraductal papillary-mucinous adenoma/borderlines with abrogation of DUSP6

IPMA/B Sample#	*KRAS2*			*DUSP6 expression*
	Codon 12	*Codon 13*	*Codon 61*	
179 166	GAT/Asp			Faint/reduced
291	GGT/Gly (wild type)	GGC/Gly (wild type)	CAA/Gln (wild type)	Faint/reduced
4597	GTT/Val			Faint/reduced
6973	GAT/Asp			Faint/reduced
4868	GTT/Val			Faint/reduced
132 115	GTT/Val			Faint/reduced

IPMA/B, intraductal papillary-mucinous adenoma/borderlines.

Table 4 Comparison of abnormal expressions of DUSP6, CDKN2A, TP53, and SMAD4 between pancreatic intraepithelial neoplasia-invasive ductal carcinoma and intraductal papillary-mucinous neoplasms

	LD/PanIN-1 and -2 vs *IPMA/B*	*HD/PanIN-3* vs *IPMC*	*IDC* vs *IPMN-COL*	*IDC* vs *IPMN-DUC*
DUSP6	0/38 *vs* 8/47 (0.0076)	0/24 *vs* 5/32 (0.0638)	30/57 *vs* 2/5 (0.6666)	30/57 *vs* 1/3 (0.6059)
CDKN2A	12/38 *vs* 27/47 (0.0815)	20/24 *vs* 25/32 (0.7413)	47/57 *vs* 2/5 (0.0576)	47/57 *vs* 3/3 (>0.9999)
TP53	0/38 *vs* 1/47 (>0.9999)	10/24 *vs* 6/32 (0.0777)	39/57 *vs* 1/5 (0.0493)	39/57 *vs* 0/3 (0.0389)
SMAD4	1/38 *vs* 0/47 (0.4270)	13/24 *vs* 1/32 (<0.0001)	52/57 *vs* 1/5 (0.0011)	52/57 *vs* 2/3 (0.2752)

Numbers in parentheses denote Fisher's exact *P*-value by χ^2-test.
LD, low-grade dysplasia; HD, high-grade dysplasia; PanIN, pancreatic intraepithelial neoplasia; IDC, invasive ductal carcinoma; IPMA/B, intraductal papillary-mucinous adenoma/borderline; IPMC, intraductal papillary-mucinous carcinoma; IPMN-COL, invasive colloid mucinous carcinoma associated with intraductal papillary-mucinous neoplasms; IPMN-DUC, invasive ductal carcinoma associated with intraductal papillary-mucinous neoplasms.

invasive pancreatic cancer.[36] These results suggest that both molecules may associate with the invasive phenotype independently, that is, RAS-MAPK pathway *vs* TGFß-SMAD pathway, and another molecules might be involved concordantly in invasive phenotypes. The significant association between the abrogation of DUSP6 and the invasive phenotype suggests that DUSP6 serves as a gatekeeper for the progression from pancreatic intraepithelial neoplasia to invasive ductal carcinomas. Fairly preserved/overexpressions of DUSP6 in pancreatic intraepithelial neoplasia are suggested to be a result of accelerating feedback mechanism of suppression of hyperactivated/mutated RAS. Breakdown of the feedback loop indicated by the abrogated expression of DUSP6 may have contributed to progression from pancreatic intraepithelial neoplasia to invasive ductal carcinoma. As we reported previously in the experiment employing cultured pancreatic cancer cells, the abrogation of DUSP6 seems to be associated with the constitutive activation/phosphorylation of MAPK1 synergistically under a gain-of-function mutation of *KRAS2*,[8] which may play a major role in the establishment of invasive ductal carcinoma of the pancreas. Our results indicated that inactivations of CDKN2A, TP53, and SMAD4 were associated with progression of pancreatic intraepithelial neoplasia from low-grade dysplasia to high-grade dysplasia, which was consistent with reports by others.[18,37,38]

We found that expressions of DUSP6 were abrogated in a relatively small fraction of intraductal papillary-mucinous adenoma/borderline lesions, most of which harbored the mutated *KRAS2*. As we described in the Introduction, we hypothesized that the inactivation of DUSP6 under mutated *KRAS2* would induce a pathologic phenotype for the pancreatic carcinogenesis. We can assume that the hypothesis can be applied to initiation of some of intraductal papillary-mucinous neoplasms, although its frequency may be relatively low. However, the potential association of DUSP6 abrogation with an initiation of the intraductal papillary-mucinous neoplasm is of quite different character from that in the pathway through pancreatic intraepithelial neoplasia to the invasive ductal carcinoma, in which DUSP6 abrogation was associated exclusively with the invasive phenotype. Our results suggest that DUSP6 may play a different role in the progression of intraductal papillary-mucinous neoplasms from that of pancreatic intraepithelial neoplasia to the invasive ductal carcinoma.

We also found that the abrogation of SMAD4 was significantly associated with the ductal invasive phenotype but not with the colloid mucinous invasive phenotype of intraductal papillary-mucinous neoplasms. Although the number of analyzed cases with invasive carcinomas associated with intraductal papillary-mucinous neoplasms was not large, our present results suggest that the invasive ductal phenotype and the invasive colloid mucinous phenotype may be results of progressions through distinct molecular pathways involving SMAD4.

Our recent results indicated that epigenetic mechanisms suppressed expression of *DUSP6*.[39] However, upstream mechanism(s) for this suppression still remains in an open question.

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Immune responses to DNA mismatch repair enzymes hMSH2 and hPMS1 in patients with pancreatic cancer, dermatomyositis and polymyositis

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To identify tumor antigens useful for diagnosis and immunotherapy of patients with pancreatic ductal adenocarcinoma, we applied a SEREX approach with a cDNA library made from 5 pancreatic cancer cell lines and sera obtained from 8 patients with pancreatic cancer, and isolated total 32 genes, including 14 previously characterized genes and 18 genes with unknown functions. Among these isolated antigens, serum IgG antibodies for 2 isolated DNA mismatch repair enzymes, Homo sapiens *mutS* homolog 2 (*hMSH2*) and Homo sapiens postmeiotic segregation increased 1 (*hPMS1*), were detected in patients with pancreatic ductal adenocarcinoma and dermatomyositis (DM), and polymyositis (PM), but not in sera from healthy individuals. Immunohistochemical study demonstrated that hMSH2 and hPMS1 were over-expressed in pancreatic ductal adenocarcinoma compared to normal pancreatic ducts. These results suggested that hMSH2 and hPMS1 may be useful as CD4+ helper T cell antigens for immunotherapy of pancreatic cancer patients and that serum IgG antibodies may be useful for diagnosis of patients with pancreatic ductal adenocarcinoma and DM/PM.

Key words: SEREX; tumor antigens; pancreatic cancer; DNA mismatch repair enzyme

Early diagnosis and curative treatment are still difficult for pancreatic ductal adenocarcinoma despite of increased incidence rate.[1] New diagnostic and therapeutic methods, including new chemotherapy, intra-operative irradiation and cytology of pancreatic juice for detection of minimally infiltrated carcinoma *in situ*, are being applied. However, local recurrences or remote metastases, particularly in liver, often result in poor prognosis even in the patients for whom curative resection was performed.[2,3] Immunotherapy may be one of the additional therapeutic modalities for patients with pancreatic cancer since immunotherapies previously conducted on the patients with pancreatic cancer appeared to demonstrate some anti-tumor effects. However, it has not been easy to improve the immunotherapies, since mechanisms for the immunological tumor rejection has not been analyzed well in patients with pancreatic cancer partly due to the limited number of identified pancreatic cancer antigens, including MUC-1, K-ras, Her2/neu and p53.

Human tumor antigens recognized by T cells have recently been identified using various methods, including cDNA expression cloning with tumor reactive T cells[4–6] or patients' serum IgG antibodies (Ab) and various systematic genetic analysis along with currently well established gene databases. Since generation of tumor reactive T cells are relatively difficult in many cancers in the exception of melanoma, we have applied cDNA expression cloning with sera from cancer patients, called SEREX (serological identification of tumor antigens by cDNA expression cloning), for the identification of tumor antigens in various cancers including melanoma,[7] glioma,[8] colon cancer[9] and bladder cancer.[10]

In our study, we isolated tumor antigens using SEREX with sera from patients with pancreatic ductal adenocarcinoma. Among the identified antigens, 2 DNA mismatch repair enzymes, hMSH2 and hPMS1, were found to over-express in pancreatic ductal adenocarcinoma by immunohistochemical analysis, and their antibodies were detected in sera from patients with various cancers, particularly with pancreatic ductal adenocarcinoma, and in sera from patients with dermatomyositis/polymyositis (DM/PM), but not sera from healthy individuals. Therefore, hMSH2 and hPMS1 are immunogenic antigens in multiple patients with pancreatic ductal adenocarcinoma, indicating that they may be useful for immunotherapy of pancreatic cancer. Furthermore, serum Ab may be useful for diagnosis of pancreatic cancer and DM/PM.

Material and methods

Cell lines and tissues

Pancreatic ductal adenocarcinoma cell lines, PK1, PK8, PK9, PK45P and PK59, were established in Tohoku University, cultured in RPMI1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 6 μg/l epidermal growth factor, 150 units/l insulin, 0.5 mg/l hydrocortisone, 10 mg/l transferrin, 100 IU/ml penicillin and 100 μg/ml streptomycin. Pancreatic ductal adenocarcinoma cell lines, Panc-1, MIAPaCa-2, AsPC-1 and BxPC-3, and colon cancer cell lines, Colo201, DLD-1 and Nota, were cultured in 10%FBS RPMI1640. Colon carcinoma cell lines, Colo320, SW837 and LoVo, were cultured in 10%FBS DMEM. Tumor specimens were obtained from patients who had undergone surgical resection at Tohoku university hospital or Keio university hospital with informed consent according to institution guidelines. These specimens were stored in −80°C.

Profiles of patients with pancreatic ductal adenocarcinoma used for immunoscreening of the cDNA library and of patients with DM/PM

Sera from 8 patients with advanced pancreatic ductal adenocarcinoma patients (Table I) admitted in Tohoku University hospital or Keio University hospital were used for immunoscreening. Sera from patients with 119 DM/PM, 10 rheumatoid arthritis, 10 systemic lupus erythematodes (SLE), 10 sclerosis, 10 tuberculosis and 2 brain abscesses in Keio University hospital were used for serum screening.

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TABLE 1 – PROFILES OF THE PATIENTS AND NUMBERS OF THE ISOLATED CLONES BY SEREX

Patient	Age	Sex	Clinical stage	Pathological feature	Number of screened clones	Positive clones	Number of total antigens	Known antigens	Uncharacterized antigens
PA1	70	F	IVb	Moderately differentiated adenocarcinoma					
PA2	66	F	IVb	Poorly differentiated adenocarcinoma	7.0×10^5	18	11	10	1
PA3	61	M	IVb	N.D.					
PA4	54	F	III	Adenosquamous carcinoma					
PA5	64	F	IVb	Moderately differentiated adenocarcinoma					
PA6	49	M	III	Intraductal-papillary mucinous carcinoma	6.4×10^5	12	7	4	3
PA7	63	M	III	Moderately differentiated adenocarcinoma					
PA8	83	M	IVb	N.D.					
Total					1.3×10^6	30	18	14	4

Construction of the cDNA library from human pancreatic ductal adenocarcinoma cell lines and immunoscreening with patients' sera (SEREX)

Total RNA was extracted from human pancreatic ductal adenocarcinoma cell line PK1, PK8, PK9, PK45P and PK59, by cesium gradient ultracentrifugation. Poly(A)+ RNA was purified twice from a mixture of 5 pancreatic cancer total RNA with latex beads coated with oligo-dT (Oligotex-dT 30 super mRNA Purification kit; Takara, Kyoto, Japan). A lambda phage cDNA library was constructed using lambda ZAP II (Stratagene, La Jolla, CA) as previously described.[7] The primary size of the library was 3.1×10^6 pfu. For immunoscreening, 2 mixtures of sera from 4 patients with pancreatic ductal adenocarcinoma were diluted 1:15 in 5% skim milk supplemented Tris-buffered saline (TBS) with 0.01% sodium azide. To remove anti-*E.coli* and anti-lambda phage antibodies, they were first absorbed with *E. coli* lysates overnight and then the 1:100 diluted solution in 5% skim milk TBS was absorbed with no insert lambda phages on nitrocellulose membrane. The cDNA library was screened with the serum mixtures as previously described.[11] Briefly, cDNA library was plated at the 1.5×10^4/dish and transferred to nitrocellulose membranes with IPTG. The membranes were incubated with the serum mixtures and then clones recognized by serum IgG Ab were detected by enzymatic dye reaction with alkaline phosphatase conjugated anti-human IgG-Fc antibody. Positive clones were further purified by additional immunoscreening. The isolated cDNAs were amplified by PCR with T3 sense and T7 antisense primers and sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction kit and ABI Prism 310 or 3100 automated sequencer (Foster City, CA). The isolated cDNAs were analyzed by comparing genetic databases.

Real-time RT-PCR and Northern blot analysis

Total RNA was extracted from various cell lines and pancreatic ductal adenocarcinoma tissues by cesium gradient ultracentrifugation. Total RNAs of various normal tissues were purchased from CLONTEC (Palo Alto, CA), BioChain Institute (Hayward, CA), Cell Applications (SanDiego, CA), CHEMICON International (Temecula, CA) and Stratagene. cDNAs were synthesized from total RNA by reverse transcriptase (M-MLV Reverse Transcriptase, Promega Corp., Madison, WI). The mRNAs for hMSH2 and hPMS1 were quantitatively measured by TaqMan real-time RT-PCR with the specific probes (Hs00179857 and Hs00153333) and the TaqMan Universal PCR Master Mix Kit (Applied Biosystems) and the ABI Prism 7900 Sequence Detection System. GAPDH was used as an internal control and amplified in a same tube to normalize variance of the input RNA. The level of target mRNA in various samples was estimated by the 2[-Delta Delta C(T)] relative quantification method. A ratio of the mRNA in various samples to that of colon tissue was determined.

Northern blot analysis was performed using 10 μg of total RNAs as previously described.[7] Briefly, total RNA was electrophoresed in a 1.0% formaldehyde agarose gel and transferred to a nylon membrane (Hybond-XL; Amersham Biosciences, Piscataway, NJ). The membranes were incubated in QuickHyb Hybridization Solution (Stratagene) with P-32 labeled cDNA probes prepared using the High Prime DNA Labeling kit (Boehringer Mannheim), washed in 0.1% SDS 0.1×SSC solution and radioactivity was detected with BAS-2500 or 5000 (Fujifilm, Tokyo, Japan).

Immunohistochemistry

Three micrometer thick slices were prepared from formalin-fixed and paraffin-embedded tissues, deparaffinized in xylene and soaked in 100% ethanol. The slides were processed in 0.3% hydrogen peroxide aqua/99% methanol for 30 min to block the endogenous peroxidase and dipped into 10 mM citrate buffer, and heat-treated at 120°C for 10 min for antigen retrieval. Blocking was performed by incubation with 10% mouse serum or rabbit serum for 30 min. The tissues were then treated with 200-fold diluted anti-human MSH2 monoclonal antibody G219-1129 (PharMingen, San Diego, CA) or 1,000-fold diluted anti-human PMS1 polyclonal antibody sc-615 (Santa Cruz, Santa Cruz) overnight at 4°C. The tissues were reacted with the secondary antibody, either Histofine Simple Stain PO (M) or (R) (Nichirei, Tokyo, Japan), at room temperature for 30 min. Detection was performed by dipping into 150 ml of 10 mM DAB (WAKO, Osaka, Japan). Sections were counterstained with methylgreen.

In vitro *transcription/translation of hPMS1 and immunoprecipitation assay*

In vitro transcription/translation was performed using Single Tube Protein System 3, T7 (Takara Shuzo). Briefly, isolated *hPMS1* cDNA clone was amplified by 30 cycles of PCR using the Pyrobest kit (Takara Shuzo) with the sense primer (5′-AATGAAACAATTGCCTGCGGC-3′) and the antisense primer (5′-TGCTGTTTTATGACAGAACCA-3′). It was mixed with the PCR products and incubated with End Conversion mix at 22°C for 5 min and then incubated with 4 U of T4 DNA ligase, pT7 Blue-2 Blunt Vector at 22°C for 1 hr. This cDNA template was amplified by 30 cycles of PCR using Ex Taq kit (Takara Shuzo) with the R20mer (5′-CAGCTATGACCATGATTACG-3′) and the *hPMS1*-antisense primer. Amplified cDNA template was used for *in vitro* transcription and translation reaction with the S-35-labeled methionine. Translated proteins were separated and identified in 10% SDS-PAGE. Radioisotope was detected in BAS 2500 or 5000 (Fujifilm).

Immunoprecipitation was performed with the *in vitro* translated S-35-labeled hPMS1 protein. Ten microliters of sera was mixed with 2 mg of Protein A- Sepharose CL-4B (Pharmacia, Inc., Piscataway, NJ) in TBS buffer (10 mM Tris-HCl, pH 7.4, and 140 mM NaCl) and incubated overnight at 4°C. Sepharose absorbed in IgG was washed in immunoprecipitation (IPP) buffer (10 mM Tris-HCl, pH 8.0, 500 mM NaCl and 0.1%Nonidet P-40) 3 times. Absorbed Sepharose was mixed with 5 μl of the *in vitro* translated protein and incubated at 4°C for 2 hr. The Sepharose was washed by IPP buffer 5 times, dissolved in 1×SDS gel-loading buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and heat-treated for 100°C