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Shanhai Xu · Toru Furukawa · Naomi Kanai
Makoto Sunamura · Akira Horii

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

S. Xu · T. Furukawa · N. Kanai · A. Horii (✉)
Department of Molecular Pathology,
Tohoku University School of Medicine, 2-1 Seiryo-machi,
Aoba-ku, Sendai 980-8575, Japan
E-mail: horii@mail.tains.tohoku.ac.jp
Tel.: +81-22-7178042
Fax: +81-22-7178047

M. Sunamura
Gastroenterological Surgery,
Tohoku University School of Medicine,
2-1 Seiryo-machi, Aoba-ku,
Sendai 980-8575, Japan

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	TAGTAAGGGATATTTTTTTAAAGT	CAAAACTCTATTATTATATAAAATAC		55
Region 2	GAGTTGGGTTTTTTAAAAGTGGTAAATA	CAAATAATTTTTTATTCCTCCCCAA		60
Region 3	TTGGGGAGGAATAAAAATTATTTG	CTTAAACTTCTTAAACAACAACC		58
Region 4	GGTATTGATATAGTGGTGTTTTTA	AAACAAAAATATTTTCAATCCAC		55
Region 5	GTGGATTGAAAATATTTTTTGTTT	CTAAAATATACCAATTTACATCC		53
Methylation specific PCR for *DUSP6*				
Methylated	GTAGGGGTCGCGAATCGCGC	ACCGCCGATACCCGCAACCG	84-bp	65
Unmethylated	GTAGGGGTTGTGAATTGTGT	ACCACCAATACCCACAACCA	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,

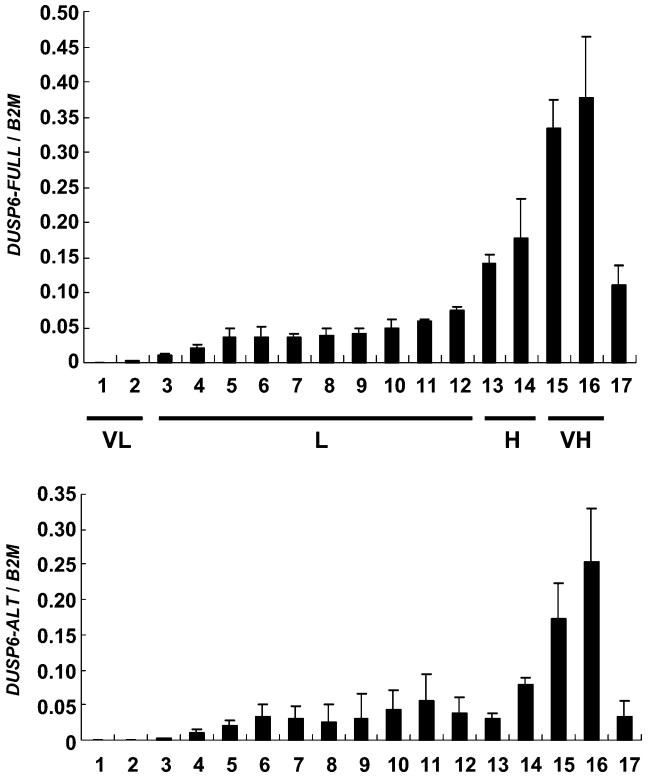

Fig. 1 *DUSP6* expression levels in pancreatic cancer cell lines determined by the quantitative real-time PCR assay. Relative expression levels of *DUSP6-FULL* (*upper column*) or *DUSP6-ALT* (*lower column*) to β 2-microglobulin(*B2M*) are presented. A total of 16 pancreatic cancer cell lines were analyzed 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,

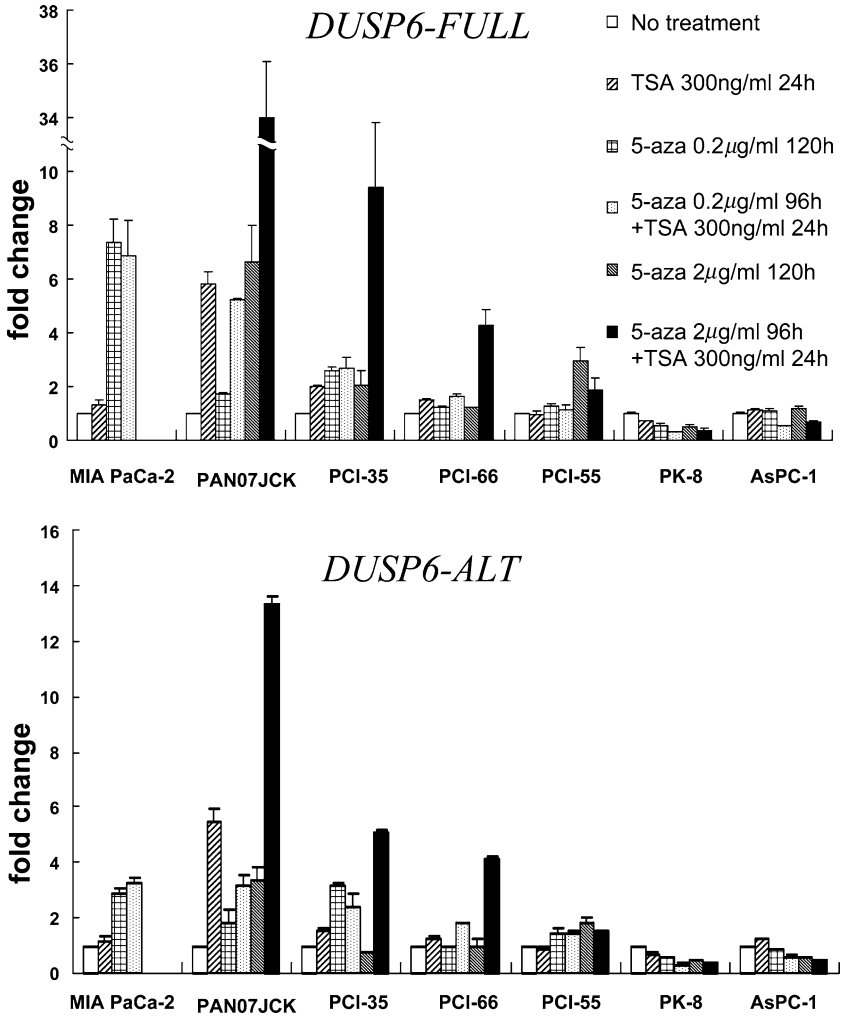

Fig. 2 Restoration of *DUSP6* by treatments with 5-azacytidine (*5-aza*) and trichostatin A (*TSA*). The cells were treated with 5-azacytidine at different concentrations for 96 h followed by trichostatin A treatment or 5-azacytidine for 24 h or trichostatin A only for 24 h, and the relative mRNA levels 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

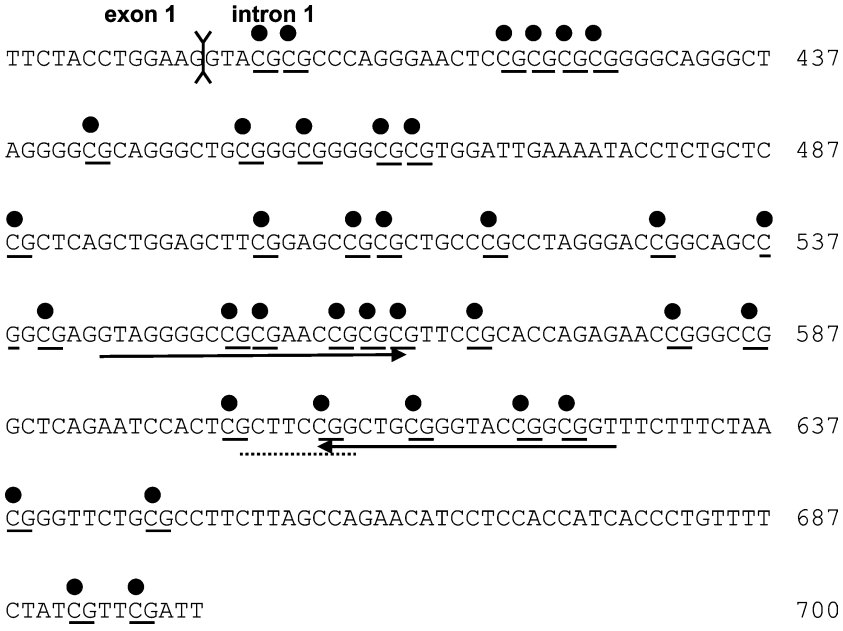

Fig. 3 Hypermethylation of the CpG islands in intron 1 of *DUSP6*. Nucleotide sequences of intron 1 of *DUSP6* are shown, and the CpG sites are *underlined*. Methylated cytosine residues identified by the sequencing analysis after sodium bisulfite treatment in MIA PaCa-2 and PAN07JCK, either completely 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*

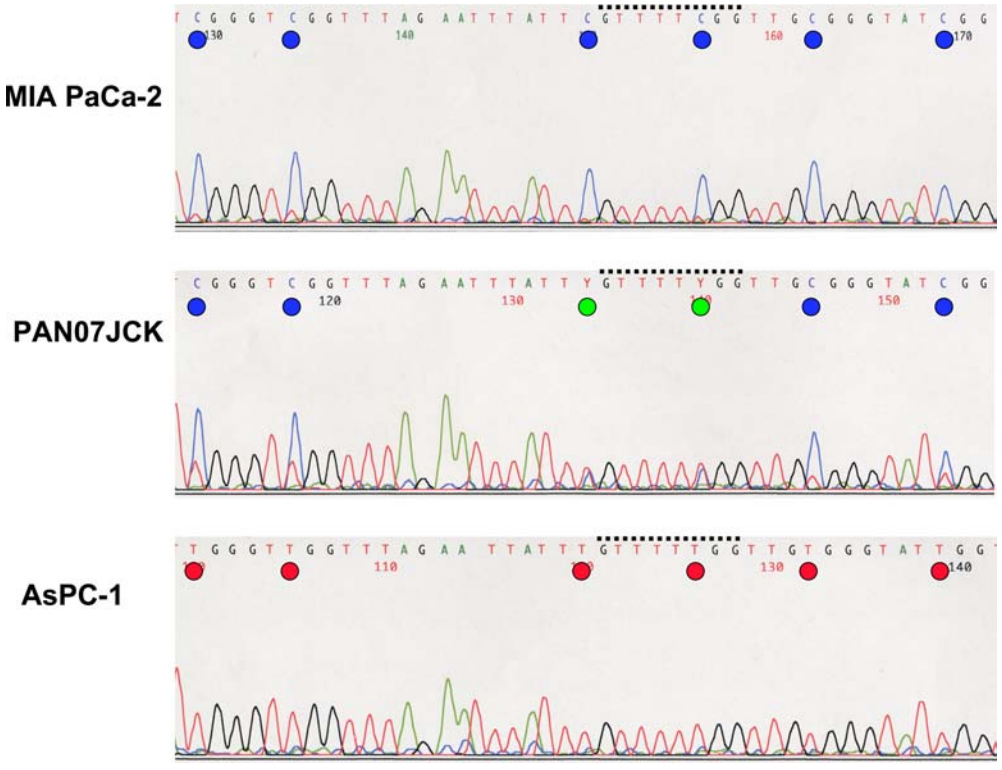

Fig. 4 Representative results of the genomic sequencing analysis of intron 1 of *DUSP6* after sodium bisulfite modification. *Circles in blue* indicate the cytosine residues in CpG sequences with complete methylation; only a single peak for C is observed. *Circles in green* indicate partial methylation; 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-

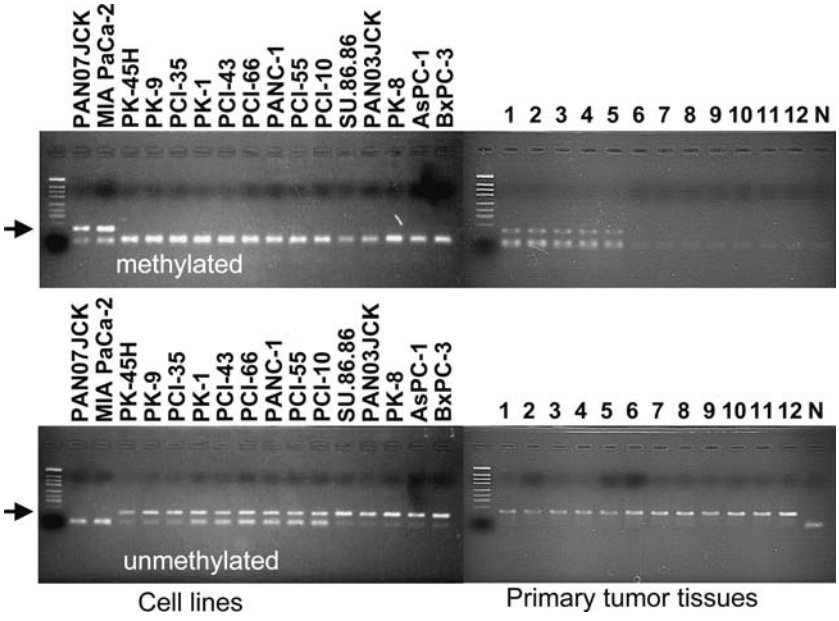

Fig. 5 Methylation-specific PCR assay for the CpG islands in intron 1 of *DUSP6*. Localization of the primers used is shown in Fig. 3. *Arrows* indicate methylation-specific PCR products in the *upper panel* and unmethylated PCR products in the *lower panel*. Cell lines are indicated by the *name*, and 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

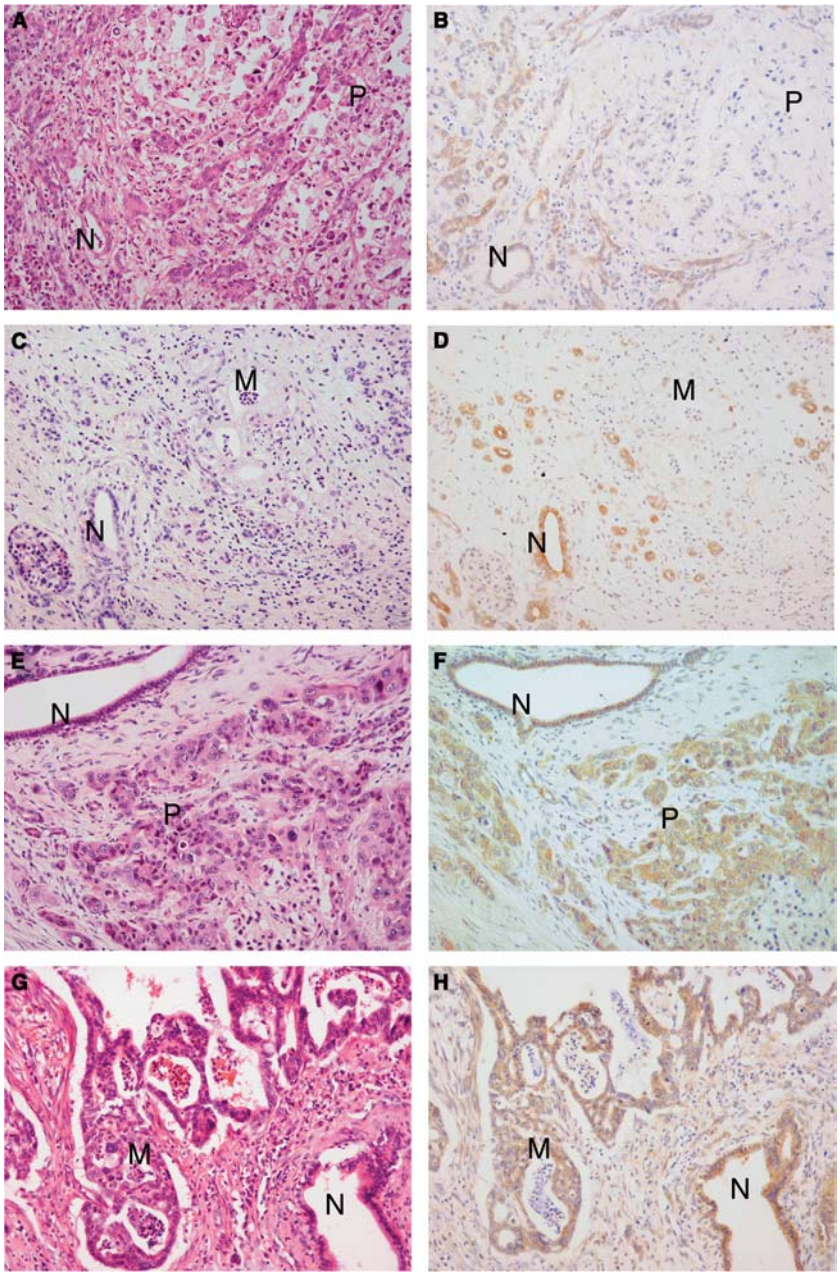

Fig. 6 Representative immunohistochemical images of analyzed samples of primary pancreatic cancer tissues. Hematoxylin-eosin (*HE*)-stained tissues (**a, c, e, g**, and immunohistochemical (*IH*)-stained tissues with anti-DUSP6 antibody (**b, d, f, h**). **a, b** : case 8590 (*lane 1* in Fig. 5); **c, 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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