Biochemical and Biophysical Research Communications 411 (2011) 162–167

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of epigenetically silenced genes in human pancreatic cancer by a novel method "microarray coupled with methyl-CpG targeted transcriptional activation" (MeTA-array)

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ARTICLE INFO

Article history:
Received 16 June 2011
Available online 23 June 2011

Keywords:
Epigenetics
DNA methylation
Transcriptional repression
MeTA-array
Pancreatic cancer
Tumor biomarkers

ABSTRACT

Identification and characterization of epigenetically silenced genes is important for cancer research, because information from hypermethylated genes provides clues to understand roles of epigenetics in tumorigeneses and genes frequently methylated in a tumor-specific manner can be used as tumor markers. Here, we describe the identification of transcriptionally silenced hypermethylated genes in pancreatic cancer cells by using a novel method called "microarray coupled with methyl-CpG targeted transcriptional activation" (MeTA-array for short), which can effectively reactivate genes containing the stringent criteria of CpG islands at promoter regions. Three representative pancreatic cancer cell lines, AsPC-1, MIA PaCa-2 and PANC-1, with a normal pancreatic ductal epithelial cell line HPDE as a control, were examined with this method, and 19 genes were upregulated twofold or more in all the three cancer cell lines after MeTA; 16 of these 19 genes have not been detected previously when using a conventional DNA demethylating agent, 5-aza-2′-deoxycytidine. Among these 16 genes, *CSMD2*, *SLC32A1*, *TMEM204* and *TRH* were further analyzed by methylation-specific PCR, and we found that 90% (19/21) of *CSMD2*, 100% (21/21) of *SLC32A1*, 95% (20/21) of *TMEM204* and 100% (21/21) of *TRH* were methylated in our series of pancreatic cancer cell lines. Furthermore, *CSMD2*, *SLC32A1* and *TRH* were also hypermethylated in primary pancreatic cancers in a tumor-specific manner. These results suggest that MeTA-array is a highly efficient method for identifying methylation-mediated transcriptionally silenced genes in human pancreatic cancer and that this method can be applied to other types of human cancer.

1. Introduction

In human cancer, abnormalities of cancer-related genes can occur through both genetic and epigenetic mechanisms [1]. Sjöblom et al. sequenced 13,023 genes in 11 colorectal and 11 breast cancers and estimated an average of 11 significant mutations per tumor [2], suggesting that a limited number of genetic mutations may be driving the tumorigenic pathway. Nonetheless, the full spectrum of epigenetic alterations is not currently well understood. The best-defined epigenetic alteration of cancer-related genes involves DNA hypermethylation of CpG islands (CGIs) in promoter regions associated with the transcriptional inactivation of the affected genes [3]. Schuebel et al. have used a gene expression microarray to identify genes affected by promoter CGI hypermethylation and transcriptional silencing in colorectal cancer [4]. They validated tumor specific hypermethylation in primary colorectal cancer specimens and estimated that nearly 5% of all known genes may be promoter hypermethylated in an individual tumor; these should include candidate driver genes for carcinogeneses. It is now thought that the epigenetic alterations in tumors through changes in chromatin or DNA methylation are more common events for a subset of cancer-related genes than mutational alterations during human carcinogeneses. Detailed elucidation of these genes is valuable in understanding the molecular pathways driving tumorigeneses and in providing promising biomarkers for monitoring cancer risk assessments, early diagnoses, prediction of prognoses; possibly, yet-to-be identified gene re-expression method(s) can contribute to cancer prevention and/or therapeutic strategies [5].

Methyl-CpG binding domain proteins (MBPs) mediate transcriptional repression through hypermethylated promoters and

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doi:10.1016/j.bbrc.2011.06.121

harbor two functional domains, a methyl-CpG binding domain (MBD) and a transcription repression domain (TRD) [6]. We previously found that cancer-related genes transcriptionally silenced by promoter hypermethylation can be reactivated by the introduction of the plasmid which carries a fusion gene comprised of MBD and transcription activation domain (TAD) instead of TRD into cancer cells [7]. We named this method "methyl-CpG targeted transcriptional activation", MeTA for short. We found that the NFκB transcriptional activation domain (AD) recruits p300/CREB binding protein (CBP) and that the surrounding histone H3 lysine 9/14 is acetylated [8].

In this report, we show that microarray coupled with MeTA, termed MeTA-array, is highly useful in identifying candidate hypermethylation-mediated silenced genes in primary cancers. Because MeTA-array searches for hypermethylated genes by a completely different mechanism than DNA demethylating agent-based microarray, this method enables identification of hypermethylated genes that were difficult to find by the previous conventional methods. Furthermore, MeTA-array provides not only information about promoter hypermethylation, but also that about transcriptional repression in a genome-wide fashion at the same time.

2. Materials and methods

2.1. Cell culture

Twenty-two human cell lines were used in this study: an immortal cell line derived from normal pancreatic ductal epithelium, HPDE6-E6E7 (HPDE hereinafter) and 21 pancreatic cancer cell lines, AsPC-1, MIA PaCa-2, PANC-1, BxPC-3, PAN09JCK, PCI-6, PCI-19, PCI-24, PCI-35, PCI-43, PCI-55, PK-1, PK-8, PK-9, PK-12, PK-14, PK-36, PK-45P, PK-45H, PK-47 and PK-59. HPDE was a kind gift from Dr. M-S Tsao (University of Toronto, Canada) and was grown in HuMedia-KG2 (Kurabo). AsPC-1, MIA PaCa-2, PANC-1 and BxPC-3 were purchased from American Type Culture Collection (ATCC), and the remaining 17 cell lines have been described previously [9]. The pancreatic cancer cell lines were grown in RPMI-1640 medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum (Invitrogen), except for MIA PaCa-2 and PANC-1; these were grown in Dulbecco's modified Eagle's medium (Sigma–Aldrich) supplemented with 10% fetal bovine serum.

2.2. Tissue specimens

Primary pancreatic tumors and their corresponding normal tissue counterparts were obtained at surgery from a total of 22 patients with pancreatic cancer at Tohoku University Hospital (Sendai, Miyagi, Japan). These specimens were collected between July 2006 and December 2008, and their UICC stages were IIA or IIB. The tissue specimens were frozen in liquid nitrogen immediately after resection and stored at −80 °C. Written informed consent was obtained from all patients. The study was approved by the Ethics Committee of Tohoku University School of Medicine.

2.3. Plasmids

The plasmid to induce MeTA, pcDNA6-3xFLAG-NFκB (AD)-MBD, was constructed previously [7]. In pcDNA6-3xFLAG-NFκB (AD)-MBD, three DNA fragments, 3xFLAG from p3xFLAG-CMV-10 (Sigma–Aldrich), MBD from pcDNA-MBD2 [10] (codons 144 through 230), and NFκB (AD) from pCMV-AD (Stratagene), were cloned into the pcDNA6/myc-His vector (Invitrogen).

2.4. Transfection and immunoblotting

AsPC-1, MIA PaCa-2, PANC-1, and HPDE were seeded on 6-well tissue culture dishes and transfected with an aliquot of 4 μg of pcDNA6/myc-His vector or pcDNA6-3xFLAG-NFκB (AD)-MBD using lipofectamine reagents (Invitrogen) according to the supplier's recommendations. The cells were harvested 48 h after transfection, and immunoblotting experiments were performed as described previously [11].

2.5. 5-Aza-2′-deoxycytidine and trichostatin A treatments

AsPC-1, MIA PaCa-2, PANC-1, and HPDE were treated with 5-aza-2′-deoxycytidine (DAC, Sigma–Aldrich) and trichostatin A (TSA, Wako). Cells were exposed to DAC (1 μM) for 96 h or to TSA (1 μM) for 24 h. We used these conditions because Sato et al. had previously reported that treatment of these cell lines with DAC (1 μM) for 96 h resulted in marked induction of several genes silenced by aberrant methylation without evidence for cell death [12]. We also treated cells with the equivalent volume of PBS alone. For combined treatment, these cells were cultured in the presence of DAC (1 μM) for 72 h and then treated for another 24 h with TSA (0.5 μM).

2.6. RT-PCR and Southern blotting

Total RNAs were extracted from cells and the single stranded cDNA was synthesized as described previously [11]. RT-PCR amplifications using intron-spanning primers were performed as described [13] for the analysis of the following genes: *NEFH* (GenBank ID: NM_021076), *NPTX2* (GenBank ID: NM_002523), *TIMP3* (GenBank ID: NM_000362), *UCHL1* (GenBank ID: NM_004181), *ANKRD35* (GenBank ID: NM_144698), *CSMD2* (GenBank ID: NM_052896), *HOXA7* (GenBank ID: NM_006896), *SLC32A1* (GenBank ID: NM_080552), *TMEM204* (GenBank ID: NM_024600), *TNXB* (GenBank ID: NM_019105), *TRH* (GenBank ID: NM_007117). *B2M* (GenBank ID: NM_004048) was used as the internal control [14]. The PCR products were run in 3% agarose gels and then Southern hybridization was performed as described previously [13]. Nucleotide sequences of the primers used in RT-PCR and Southern hybridization are described in Supplementary Table S1.

2.7. Microarray analysis

Microarray analyses were performed according to methods described previously [11]. AsPC-1, MIA PaCa-2, PANC-1 and HPDE were transfected with the plasmid containing NFκB (AD)-MBD or the vector alone, and the RNAs were prepared, amplified, and labeled using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). Aliquots of 1.65 μg Cy-3 labeled cRNAs were assembled and hybridized to Agilent whole human genome microarray (4 × 44 K), and microarray slides were scanned with the Agilent G2565BA microarray scanner. Intensity data from microarray images were extracted with Feature Extraction Software 9.5.1 (Agilent Technologies) at the Biomedical Research Core of Tohoku University School of Medicine. The cut-off value for two-fold upregulation was employed for selection of the genes. High-throughput microarray data are available in the Gene Expression Omnibus database (http://www.ncbi.nlom.nih.gov/geo) under the Accession number: GSE26215.

2.8. Methylation-specific PCR (MSP)

Methylation status of each gene was determined by methylation-specific PCR (MSP) [15]. Genomic DNAs were extracted using DNeasy Blood & Tissue Kit (Qiagen) and were bisulfite-treated

using EpiTect Bisulfite Kit (Qiagen). Primers used for MSP analyses are listed in Supplementary Table S2.

2.9. CGI analysis

For CGI identification, we analyzed DNA sequences from 1-kb upstream from the transcription start site (TSS) to 1-kb downstream (CGI TSS ± 1000-bp). The CpG island searcher (http://cpgislands.usc.edu/) was used for Takai and Jones' criteria: %GC ⩾ 55, length ⩾ 500-bp and the observed CpG/expected CpG value ⩾ 0.65 [16].

2.10. Statistical analysis

Statistical analysis was performed using Wilcoxon signed-ranks test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. NFκB (AD) linked to MBD reactivates hypermethylated genes previously identified by DNA demethylating agent in pancreatic cancer

In order to assess whether the MeTA-array can identify hypermethylated genes very efficiently in cancer cells, we used three pancreatic cancer cell lines, AsPC-1, MIA PaCa-2, and PANC-1 along with a non-neoplastic pancreatic ductal epithelial cell line, HPDE. These four cell lines have been previously analyzed to identify hypermethylated genes in pancreatic cancer by using the combination between conventional DAC treatment and gene expression microarray [12]. Therefore we thought it would be easy to compare both methods. We first transfected the plasmid containing NFκB (AD)-MBD (MeTA) or the vector alone (Vec) into four cell lines and confirmed the expression of the NFκB (AD)-MBD fusion protein only in MeTA-transfected cell lines by Western blot analysis (Supplementary Fig. S1A). To determine whether MeTA in fact can reactivate hypermethylated genes as described in the literature, we extracted total RNAs from the vector or MeTA-transfected samples and performed RT-PCR followed by Southern hybridization. The four genes, *NEFH, NPTX2*, *TIMP3*, and *UCHL1*, were upregulated by the DAC treatment in at least one of these three pancreatic cancer cell lines and were selected for further characterization. As shown in Supplementary Fig. S1B, these four genes were also all reactivated by the NFκB (AD)-MBD transfection.

3.2. MeTA-array identifies genes commonly upregulated in three pancreatic cancer cell lines but not the nonneoplastic pancreatic ductal cell line

Total RNAs from the vector or MeTA-transfected samples in four cell lines were also subjected to gene expression microarray to see whether the MeTA-array can detect hypermethylated and transcriptionally silenced genes in pancreatic cancer cells. A number of genes were upregulated twofold or more in these pancreatic cancer cell lines (AsPC-1, 846 genes; MIA PaCa-2, 478 genes; PANC-1, 977 genes) as well as a non-neoplastic pancreatic ductal cell line (HPDE, 591 genes). Among these, 19 genes were selected because these genes were commonly upregulated twofold or more by MeTA in all three pancreatic cancer cell lines, but not in HPDE (Table 1). Interestingly, these 19 genes contained CGIs defined by Takai and Jones at promoter regions at extremely high frequency (17/19, 89.5%, see Table 1); these genes may possibly be hypermethylated at these regions only in pancreatic cancer cell lines. Surprisingly, among the 19 genes, only *NEFH*, *HOXA9* and *CLDN5* have been previously reported by the combination between DAC treatment and microarray analysis [12]; the remaining 16 genes had never been observed without MeTA-array. Seven of these 16 genes were selected for further analysis by RT-PCR followed by Southern hybridization (see Fig. 1); *ANKRD35*, *HOXA7*, *SLC32A1*, *TMEM204* and *TNXB* showed downregulation in all three pancreatic cancer cell lines when compared to normal HPDE (Table 1) and were selected. The other two genes, *CSMD2* and *TRH*, were highly upregulated by MeTA in all three pancreatic cancer cell lines but not in HPDE; these were possible candidates for tumor-specific markers although the expression levels of these two genes were similarly very low in all four cell lines (Table 1). RT-PCR and Southern hybridization analyses showed that five genes, *ANKRD35*, *CSMD2*, *SLC32A1*, *TMEM204*, and *TRH*, were clearly reactivated by MeTA, but the other two genes, *HOXA7* and *TNXB*, were hardly reactivated by MeTA (Fig. 1). All five genes reactivated by MeTA contained CGIs defined by Takai and Jones at promoter regions. In addition, none of three pancreatic cancer cell lines were methylated in the promoter CGI of *ANKRD35*, suggesting that DNA methylation in other genome regions such as distant enhancers or in other genes such as transcription factors may be important for *ANKRD35* expression (data not shown). Therefore, using MSP, we further analyzed the DNA methylation status of four genes, *CSMD2*, *SLC32A1*, *TMEM204*, and *TRH*.

3.3. The promoter CGIs of genes selected by MeTA-array are highly methylated in pancreatic cancer cell lines

To assess whether four genes selected by MeTA-array are methylated specifically in pancreatic cancer cell lines, but not in a non-neoplastic pancreatic ductal cell line HPDE, we designed MSP primers in the promoter CGIs in each gene and applied them to 21 pancreatic cancer cell lines, including AsPC-1, MIA PaCa-2 and PANC-1, as well as HPDE (Fig. 2). All four genes were unmethylated in HPDE, and aberrant methylation of *CSMD2*, *SLC32A1, TMEM204* and *TRH* was detected in 19 (90%), 21 (100%), 20 (95%) and 21 (100%) of these 21 pancreatic cancer cell lines. Among these, *CSMD2* was partially methylated in many pancreatic cancer cell lines (13/21: 62%). Except *TMEM204* in the BxPC-3 cell, the three other genes were completely methylated only in cancer cells. These results clearly indicate that MeTA-array can identify genes hypermethylated and silenced specifically in pancreatic cancer cell lines.

3.4. Genes selected by MeTA-array are also hypermethylated in primary pancreatic cancers

To examine whether the aberrant methylation of four genes identified by MeTA-array was also present in primary pancreatic cancers, we analyzed methylation statuses of 22 paired DNAs from primary resected pancreatic cancer and corresponding normal pancreatic ductal epithelium (Fig. 3). Unfortunately, these samples were not microdissected; thus all primary tumors were contaminated with extensive amounts of normal tissue, and MSP analyses using unmethyl primer sets produced clear amplification bands in all samples (see Fig. 3 *CSMD2*-U). Aberrant methylation was detected in a tumor-specific manner in 36.4% (8/22; $P = 0.008$) for *CSMD2*, 72.7% (16/22; $P = 0.0003$) for *SLC32A1*, 4.5% (1/22) for *TMEM204*, and 81.8% (18/22; $P = 0.0009$) for *TRH*. These results confirm the abnormal methylation patterns of three genes, *CSMD2*, *SLC32A1*, and *TRH*, but not *TMEM204*, in primary pancreatic carcinomas as well as in pancreatic cancer cell lines.

4. Discussion

In a previous study, we compared gene expression profiles by microarray between MeTA and a treatment with a conventional DNA demethylating agent, 5-aza-cytidine (Aza-CR), using a human

Table 1
Nineteen genes reactivated after MeTA.

Gene name	CGI	Fold change (MeTA/Vec)				Signal intensity[a]			
	TSS ± 1000 bp	HPDE	AsPC-1	MIA PaCa-2	PANC-1	HPDE	AsPC-1	MIA PaCa-2	PANC-1
TRH	P	0.9	96.9	15.8	293.9	1.2	2.6	2.4	0.3
HOXA9	P	1.2	2.1	10.8	24.7	12.3	780.4	0.1	63.8
CYP26A1	P	1.7	70.7	9.5	6.1	76.6	4.6	11.6	38.8
TMEM204	P	1.0	2.4	9.1	28.2	24088.1	148.7	0.1	12.8
GAD1	P	1.7	17.1	9.0	10.4	3.1	5.5	7.0	14.9
CSMD2	P	1.7	14.1	8.8	9.9	0.1	0.1	0.1	0.1
NEFH	P	0.9	60.5	6.0	15.1	37173.6	4.1	0.1	406.9
FRG2	A	1.6	120.0	5.9	24.2	15.6	14.4	3.3	16.5
ARC	P	0.8	3.2	4.8	7.7	11.4	75.1	16.9	47.6
CLDN5	P	1.4	4.3	4.4	64.7	1528.5	46.0	9.5	14.5
SLC32A1	P	0.9	102.6	4.2	422.6	22.3	5.3	5.0	3.5
FOXJ1	P	1.2	4.1	4.1	10.9	6.6	60.1	8.5	48.5
TBX21	P	1.6	27.6	3.5	30.4	8.6	11.4	12.0	16.9
HOXA7	P	0.9	5.6	3.0	13.9	452.0	39.3	19.6	17.5
ANKRD35	P	1.1	34.7	2.7	14.4	163.7	11.7	20.3	59.2
HBA2	P	0.9	6.0	2.5	7.0	86.5	29.0	16.0	37.3
SP5	P	0.8	24.2	2.3	3.8	18.0	10.4	14.1	157.1
TNXB	A	0.8	3.4	2.3	7.3	1863.2	116.2	219.2	122.0
GRASP	P	1.6	7.5	2.0	17.1	118.3	169.2	114.2	228.0

P: Presence, A: Absence.
[a] The values of signal intensity represent ones without DNA transfection in four cell lines.

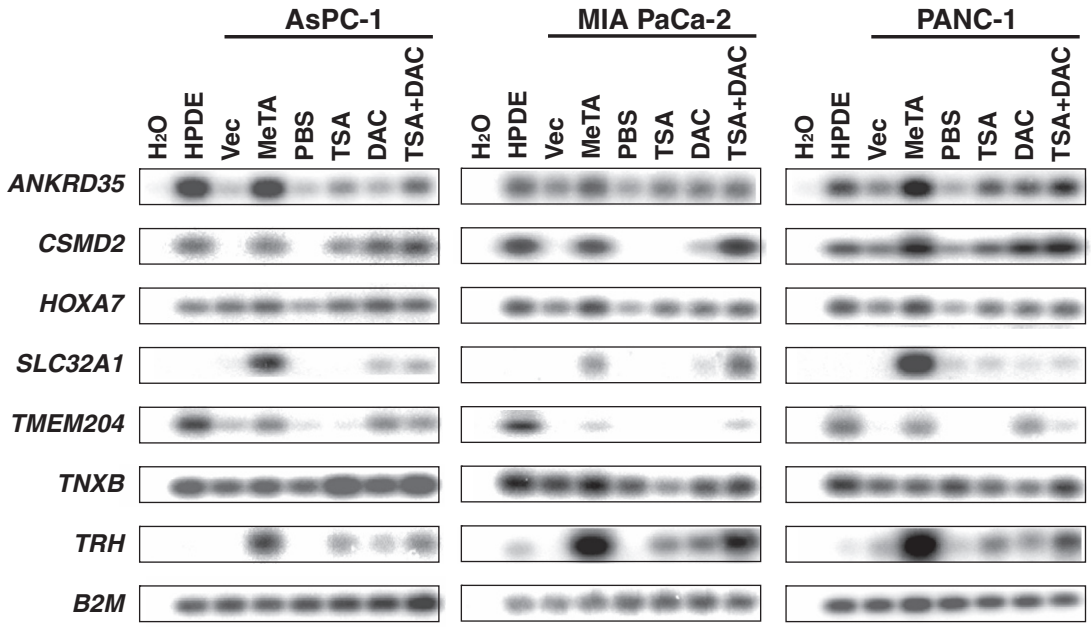

Fig. 1. Expression analyses of seven genes selected by MeTA-array in pancreatic cancer cell lines. In addition to DNA transfection of the vector or the MeTA-inducing plasmid, AsPC-1, MIA PaCa-2, and PANC-1 were treated with PBS, TSA, DAC or TSA + DAC. The expression levels of *ANKRD35*, *CSMD2*, *HOXA7*, *SLC32A1*, *TMEM204*, *TNXB*, and *TRH* were analyzed by RT-PCR and Southern hybridization. Expression of a nonneoplastic pancreatic ductal epithelial cell line, HPDE, was also examined. Note that five of the seven genes, but not *HOXA7* or *TNXB*, were strongly reactivated by MeTA. Expression of *B2M* was monitored as the control.

embryonic kidney cell line 293T and found that MeTA can reactivate silenced genes with CGIs under stringent criteria (defined by Takai and Jones within ±1000-bp of TSS) at higher frequency [11]; 109 of 138 (79.0%) genes in MeTA vs. 121 of 202 (59.9%) genes in Aza-CR. The 19 genes identified in the present study also showed extremely high densities of CpG sites at promoter regions (17/19; 89.5%, see Table 1). This is probably due to the property of MBD derived from MBD2 protein. MBD2 has been demonstrated to recognize a single methylated CpG pair *in vitro* [17]. However, it may prefer more densely methylated DNAs *in vivo*; early literature suggested that the MeCP1 complex, which contains MBD2 as a mediator of methyl-CpG information, requires at least 12 consecutive methyl-CpG sequences for binding [18,19]. Recently Nair et al. compared two commonly used approaches to enrich methylated DNA regions of the genome [20], methylated DNA immunoprecipitation (MeDIP) [21] using antibodies specific for 5-methylcytosine and methyl-CpG binding domain-based capture (MBD capture) [22] using a MBD of MBD2 protein. They found that both enrichment techniques preferentially identified different CpG rich regions of the genome; MeDIP commonly enriching methylated regions with a low CpG density and MBD capture favoring regions of higher CpG density and identifying the greatest proportion of CGIs. Therefore, all of these results indicate that the genome-wide approaches using MBD preferentially identify genes meeting

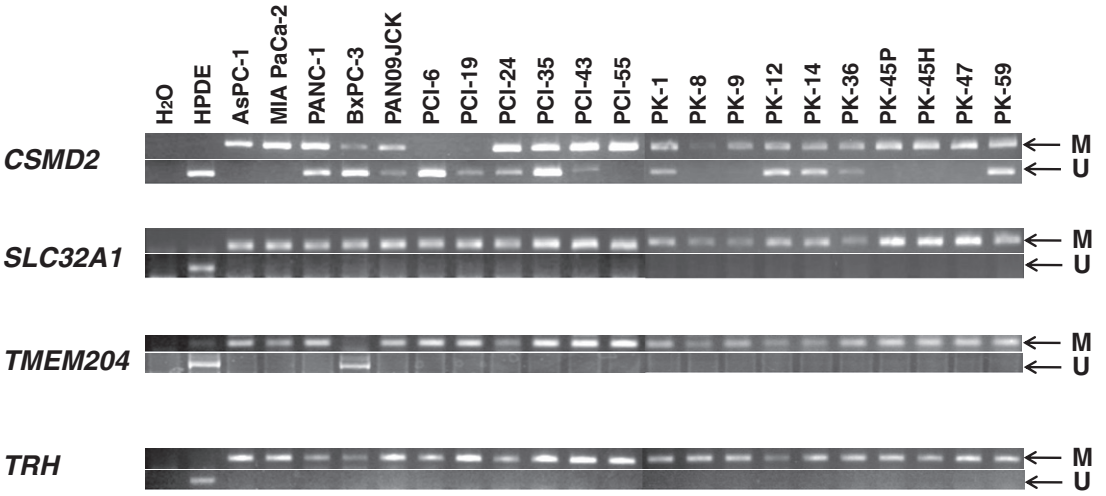

Fig. 2. MSP analyses of four genes selected by MeTA-array in 21 pancreatic cancer cell lines and a nonneoplastic pancreatic ductal epithelial cell line, HPDE. *CSMD2*, *SLC32A1*, *TMEM204*, and *TRH* were highly methylated in 90% (19/21), 100% (21/21), 95% (20/21), and 100% (21/21) of pancreatic cancer cell lines, respectively, and were unmethylated in the nonneoplastic HPDE cell. The arrows M and U indicate methylated- and unmethylated-specific PCR products, respectively.

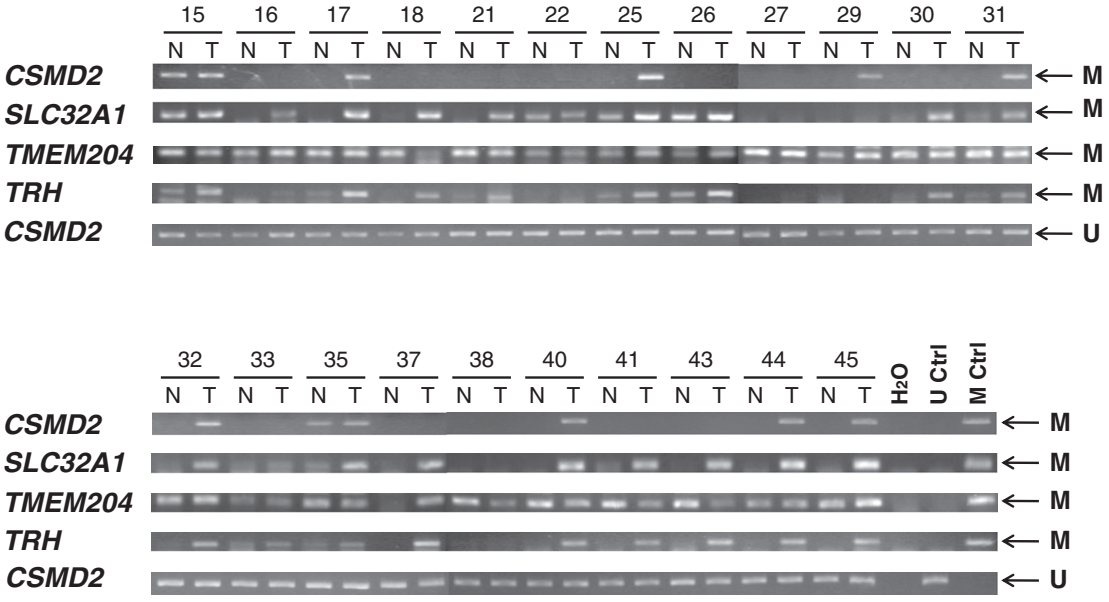

Fig. 3. MSP analyses of four genes selected by MeTA-array in 22 pairs of primary pancreatic tumors (T) and normal pancreatic ductal epithelia (N). U Ctrl indicates unmethylated DNA controls in which HPDE was used for *CSMD2*, *SLC32A1* and *TRH* genes; BxPC3 was used for *TMEM204* gene. MIAPaCA-2 was used as a methylated DNA control (M Ctrl). The arrows M and U indicate methylated- and unmethylated-specific PCR products, respectively. All primary tumors include amplification with the U primer set, a result of the presence of normal contaminating tissue. Note that *CSMD2*, *SLC32A1*, and *TRH*, but not *TMEM204*, were highly methylated in primary pancreatic carcinomas.

the stringent criteria of CGIs both *in vitro* and *in vivo*; this property should be useful to identify hypermethylated genes as candidates for tumor-specific biomarkers.

What kinds of methods are useful for detecting transcriptionally silenced genes by DNA hypermethylation in cancer cells? Although MeDIP and MBD capture are useful techniques for detecting hypermethylated promoters in cancer cells, it requires further analyses to clarify whether the methylated DNAs are involved in transcriptional repression. Because MeTA-array can screen hypermethylated and transcriptionally silenced genes at the same time, it is more advantageous to eliminate many laborious tasks in comparison with MeDIP and MBD capture. At present, DNA demethylating agent-based microarray is the most commonly used method to identify hypermethylated and/or transcriptionally silenced genes. A major obstacle of this method is that DNA demethylating agents reactivate many genes which lack CGIs at promoter regions [23,24]. This is caused in part by inhibition of DNA methyltransferases (DNMTs), which have the potential to repress transcription independently of their methylating activities both directly and through interaction with histone deacetylases and other corepressor proteins [25–27]. MeTA-array can eliminate this obstacle by using MBD not associated with DNMTs.

Although only three pancreatic cancer cell lines were analyzed in this study, three commonly silenced genes by hypermethylation in primary pancreatic cancers were identified, *CSMD2*, *SLC32A1*, and *TRH*. Among these, *CSMD2* (CUB and Sushi multiple domains 2) is a member of *CSMD* gene family and is mapped to human chromosome band 1p34.3 [28]. One of the other members, *CSMD1*, has been characterized more in detail and is thought to be a putative tumor suppressor of head and neck squamous cell carcinoma [29,30]. These two CSMD proteins have very similar structures and it is possible that both proteins function as either

transmembrane receptors or adhesion molecules. Our previous comparative genomic hybridization (CGH) analysis showed that chromosome 1p34.3 was deleted in 50% (6/12) pancreatic cancer cell lines, including PK-1, PK-8, PK-9, PK-45P, and PK-59 [31], and LOH at 32% (6/19) was also detected in this region [32]. These results may suggest that *CSMD2* is a candidate tumor suppressor in pancreatic cancer, and further analyses will clarify this possibility. On the other hand, although the expression levels of *SLC32A1* and *TRH* are extremely low in nonneoplastic HPDE cells (Fig. 1), and, therefore, both genes are unlikely to be associated with tumorigenesis, they can be possible tumor markers, because they are methylated in a tumor-specific manner at very high frequency. Because it is known that DNA methylation generally occurs early in tumorigenesis, further methylation analysis of microdissected samples may uncover the exact time of methylation in a multi-step tumor developmental model. Given all these results, if this method is applied to more tumors, MeTA-array should become an intriguing method to search for transcriptionally silenced genes by hypermethylation, which will be useful in the analyses of tumorigenesis and the production of tumor markers.

Acknowledgments

We are grateful to Dr. B.L.S. Pierce (University of Maryland University College, USA) for editorial work in the preparation of this manuscript and to Biomedical Research Core (Tohoku University School of Medicine) for technical support. This work was supported in part by Grants-in-Aid (Grant # 17015003, 21590425, 22591512), by the Academic Frontier Project for Private Universities: matching fund subsidy 2006–2010 from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a Grant-in-Aid for Cancer Research (Grant # 18–19) from the Ministry of Health, Labour and Welfare of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.06.121.

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