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# DNA methylation and histone H3-K9 modifications contribute to *MUC17* expression

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**MUC17 glycoprotein is a membrane-associated mucin that is mainly expressed in the digestive tract. It has been suggested that MUC17 expression is correlated with the malignancy potential of pancreatic ductal adenocarcinomas (PDACs). In the present study, we provided the first report of the *MUC17* gene expression through epigenetic regulation such as promoter methylation, histone modification and microRNA (miRNA) expression. Near the transcriptional start site, the DNA methylation level of *MUC17*-negative cancer cell lines (e.g. PANC1) was high, whereas that of *MUC17*-positive cells (e.g. AsPC-1) was low. Histone H3-K9 (H3-K9) modification status was also closely related to *MUC17* expression. Our results indicate that DNA methylation and histone H3-K9 modification in the 5′ flanking region play a critical role in *MUC17* expression. Furthermore, the hypomethylation status was observed in patients with PDAC. This indicates that the hypomethylation status in the *MUC17* promoter could be a novel epigenetic marker for the diagnosis of PDAC. In addition, the result of miRNA microarray analysis showed that five potential miRNA candidates existed. It is also possible that the *MUC17* might be post-transcriptionally regulated by miRNA targeting to the 3′-untranslated region of its mRNA. These understandings of the epigenetic changes of *MUC17* may be of importance for the diagnosis of carcinogenic risk and the prediction of outcomes for cancer patients.**

*Keywords:* DNA methylation/epigenetics/MUC17/mucin/pancreatic cancer

## Introduction

Pancreatic ductal adenocarcinomas (PDACs) are highly malignant neoplasms that are difficult to detect at an early stage (Bardeesy and DePinho 2002; Yonezawa et al. 2009). However, early detection of these neoplasms is important for the improvement of outcome and effective tools are needed for this purpose. Mucins are heavily O-glycosylated proteins found in the mucus layer and differentially expressed at the cell surface of many epithelia. They are responsible for the physical properties of mucus gels and are involved in epithelial cell protection and maintain the local molecular microenvironment (Bhaskar et al. 1992; Linden et al. 2004, 2008; Ho et al. 2006). There is also increasing evidence that mucin members are aberrantly expressed and contributes to the pathogenesis of cancer (Hollingsworth and Swanson 2004; Yonezawa et al. 2008). We have demonstrated that MUC1 and MUC4 expression is a poor prognostic factor, whereas MUC2 expression is related to a favorable outcome in various human neoplasms including PDACs and have stressed that they are indicators of potential for malignancy (Yonezawa and Sato 1997; Yonezawa et al. 2008) since our first report (Osako et al. 1993). In addition, we have reported that MUC5AC expression is also an effective tool for the early detection of the pancreatic neoplasms (Kim et al. 2002; Yonezawa et al. 2010).

MUC17 glycoprotein was identified and mapped to a mucin cluster at chromosome 7q22, along with MUC3A/B, MUC11 and MUC12 mucins, and it was categorized as a membrane-associated mucin (Williams et al. 1999; Gum et al. 2002). RNA blot analysis indicated that *MUC17* is primarily expressed in the digestive tract, including the duodenum, ileum and the transverse (Gum et al. 2002; Moehle et al. 2006). Although the physiological function of MUC17 is still unclear, it may serve as a physical barrier molecule against microorganisms and cell-surface sensors. MUC17 may also conduct signals in response to external stimuli that lead to cellular responses, including proliferation, differentiation, apoptosis or secretion of cellular products such as other membrane-bound mucin members. Involvement of MUC17 expression in cancer pathogenesis, Moniaux et al. (2006) reported that aberrant MUC17 expression is observed in PDACs compared to no expression in the normal pancreas or pancreatitis. Recently, Hirono et al. (2010) revealed that MUC17 is an independent prognostic factor associated with lymph node metastasis in PDACs.

It has been reported that 5′ flanking region (1167 bp) of *MUC17* possessing promoter activity harbors various transcriptional factor-binding elements, including GATA, VDR/RXR,

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Cdx-2, NFkB and Pdx-1 (Moniaux et al. 2006). However, the exact regulatory mechanism is still not fully understood. We have described the mechanisms of epigenetic regulation of *MUC1*, *MUC2*, *MUC3A*, *MUC4* and *MUC5AC* expression (Yamada et al. 2006, 2008, 2009, 2010; Kitamoto et al. 2010), and thus we hypothesized that *MUC17* may also be regulated epigenetically. In the present study, we mapped the CpG methylation status of *MUC17* from −620 to +209 using MassARRAY analysis in various human cancer cell lines including pancreatic cancers.

Modification of histone tails also plays a critical role in epigenetic silencing (Nguyen et al. 2002). Therefore, to investigate the relationship between DNA methylation and histone modification, chromatin immunoprecipitation (ChIP) assays were performed in 10 cancer cell lines. Moreover, to examine whether *MUC17* mRNA expression is regulated by the DNA methylation status and histone H3 modification, we also treated MUC17-negative cells or cells with low MUC17 expression (MUC17-negative/low cells) with a DNA methylation inhibitor, 5-aza-2′-deoxycytidine (5-AzadC) and a histone deacetylase inhibitor, trichostatin A (TSA).

In the present study, we describe that the tightly related combination of DNA methylation and histone H3 lysine 9 (H3-K9) modifications contribute to *MUC17* gene expression. In addition, from the results of miRNA microarray analysis, we found five candidates of microRNAs (miRNAs) that might regulate MUC17 protein expression.

## Results

### *Influence of DNA methylation and histone deacetylation on the MUC17 expression induced by epigenetic-modifying agents*

Experiments were performed using the 10 cancer cell lines derived from different tissues. Expression levels of *MUC17* mRNA and MUC17 protein in the cell lines were examined by reverse transcription–PCR (RT–PCR) analysis and immunohistochemical staining (Figure 1A and B). HPAFII, AsPC-1 and LS174T cells expressed MUC17, but the other cells did not. To investigate the possible epigenetic regulation of *MUC17* expression, quantitative RT–PCR analysis was performed in MUC17-negative/low cells treated with a DNA demethylating agent, 5-AzadC, a histone deacetylase inhibitor, TSA, or 5-AzadC/TSA in combination. The obvious tendency that the expression of *MUC17* mRNA was restored by drug treatment was observed in MUC17-negative/low cells (Figure 1C). Consistent with the mRNA level, the MUC17 expression is also restored by the drug treatments (Supplementary data, Figure S1).

Next, to rule out the contribution of single nucleotide polymorphisms (SNPs) in control of *MUC17* expression, we sequenced a 1.2 kb putative promoter region of the human *MUC17* gene from 10 cell lines. There are no differences from the sequence with GenBank accession number NT_007933 (Table I, Supplementary data, Figure S2).

### *Quantification of DNA methylation status within the* MUC17 *gene promoter*

Quantification of *MUC17* promoter CpG methylation levels in 10 cancer cell lines was examined by the MassARRAY compact system and mapped the efficacious data (Figure 2A). Near the transcriptional start site (−179 to +52), a high level of CpG methylation was observed in MUC17-negative/low cells (MCF-7, MDA-MB-453, NCI-H292, A427, BxPC-3, PANC1 and Caco2) compared with MUC17-positive cell lines (HPAFII, AsPC-1 and LS174T). These results indicate that hypomethylation near the transcriptional start site (corresponding to CpG site numbers 10–14) contributes to *MUC17* expression. To confirm the MassARRAY analysis, methylation-specific-PCR (MSP) was done with primers targeting to five CpG sites on the 10 cell lines (Figure 2B, Table I). An unmethylated band (lanes indicated by *U*; Figure 2B) was clearly obtained in MUC17-positive HPAFII, AsPC-1 and LS174T cells, and a methylation band (lanes indicated by *M*; Figure 2B) was observed in MUC17-negative/low cells. These results were consistent with those from MassARRAY analysis.

Moreover, we examined the methylation status of normal and tumoral pancreas tissues from patients with PDAC. First, we confirmed the mRNA and protein expression of MUC17 in tissues with PDAC. In tumor tissue, MUC17 was overexpressed compared with the normal pancreas (Figure 2C and D), which is in accordance with the previous study reported by Moniaux et al. (2006). Although islets of Langerhans were stained by rabbit anti-MUC17 polyclonal antibody, the unmethylated band was obtained in tumoral tissues with PDAC in six out of the eight cases we tested (Figure 2C and D, Table II).

### *Correlation between CpG methylation and histone H3-K9 modification in the MUC17 regulation*

Furthermore, we investigated the possibility of another form of epigenetic regulation of *MUC17* using a ChIP assay targeting to H3-K9 modification. To examine the relationship of *MUC17* expression with DNA methylation and histone modification, ChIP primers were designed for the region containing CpG site numbers 10–14 (Figure 3A, Table I). The results showed that the dimethylation of histone H3-K9 was detected in cells with no or low MUC17 expression (MCF-7, MDA-MB-453, NCI-H292, A427, BxPC-3, PANC1 and Caco2). In contrast, histone H3-K9 acetylation was predominantly found in MUC17 high expression cells (HPAFII, AsPC-1 and LS174T). These results suggest that DNA methylation status and histone H3-K9 modification in its 5′ flanking region contribute to *MUC17* expression (Figure 3B).

### *Identification of miRNA candidates in MUC17 expression in cancer cells*

miRNAs are a class of noncoding RNA gene, product sizes are ∼22 nt sequences, that play important roles in the regulation of translation and degradation of mRNAs through imprecise base pairing in the untranslated regions of the message. The important roles of miRNA in cancer are intensively reported. Therefore, their regulatory impact has become more pronounced and impossible to overlook (Calin et al. 2002, 2005; Marcucci et al. 2008; Lujambio and Esteller 2009). We have previously performed miRNA microarray on 11 cancer cell lines (Yamada et al. 2010). The analysis has been carried out using an miRNA Complete Labeling and

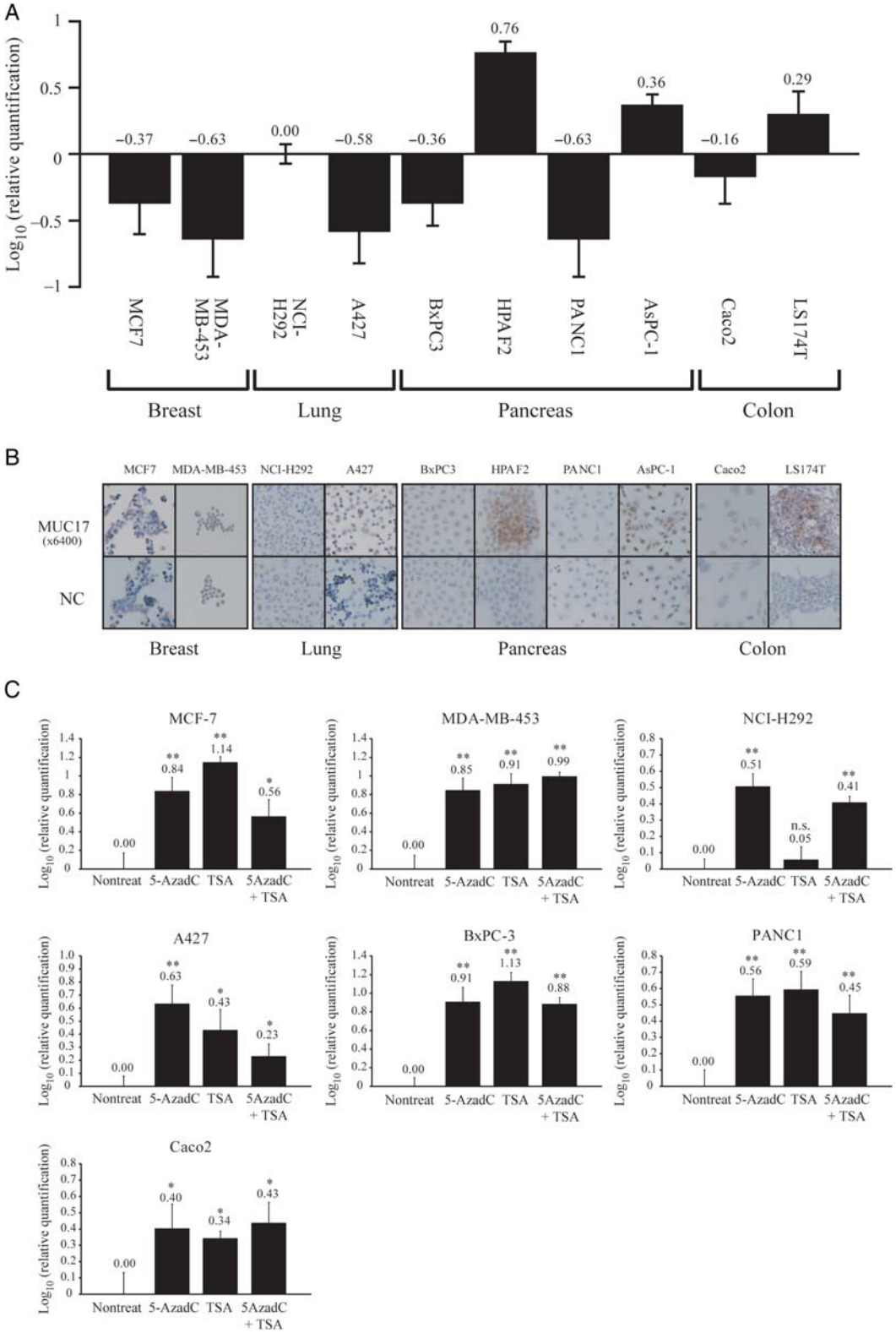


**Fig. 1.** Expression of the *MU17* gene examined by quantitative RT–PCR and immunohistochemistry. (**A**) Quantitative RT–PCR results in 10 cell lines. Bars, gene expression levels relative to those in NCI-H292 cells. HPAFII, AsPC1 and LS174T cells showed a high mRNA expression level in *MUC17* gene, whereas MCF-7, MDA-MB-453, NCI-H292, A427, PANC1, BxPC-3 and Caco2 cells had no or low expression. (**B**) *MUC17* immunoreactivity in 10 cancer cell lines. *MUC17* expression was consistent with the results of quantitative RT–PCR. (**C**) Quantitative RT–PCR results before and after treatment with 5-AzadC, TSA and 5AzadC/TSA in combination in cells with little or no *MUC17* expression. After drug treatments, these cells showed significant restoration of *MUC17* expression. $*P < 0.05$, $**P < 0.005$, when compared with nontreated cells; Student's *t*-test; n.s., not significant.

**Table I.** Synthetic oligonucleotides used in this study

| Name | Primer sequence | Position |
|---|---|---|
| MUC17 promoter sequencing primers | | |
| MUC17-S1F | CACTCTGGAATTTCCCTACCAA | −1190 to −1169 |
| MUC17-S1R | CCTGTAATCCCAGCTACTCAGG | −583 to −562 |
| MUC17-S2F | GGCACGATCTTGGCTCTC | −647 to −630 |
| MUC17-S2R | GACCTTTGGTCCCTGAGACA | −62 to −43 |
| MUC17-S3F | GTCATGAGGGAAGCTTCCAG | −117 to −98 |
| MUC17-S3R | TATGGGTTGAACACCAGTCTTG | +529 to +550 |
| 10mer-tagged or T7-tagged primers | | |
| MUC17-1F | AGGAAGAGAGTTTTAGATGGTTATGGGAGTTGTGT | −825 to −801 |
| MUC17-1R | CAGTAATACGACTCACTATAGGGAGAAGGCTTTTAACCCTAACCCCTAACAAAAAC | −372 to −348 |
| MUC17-2F | AGGAAGAGAGAGAGATAAAGGGGGTGTTTTTGTTA | −387 to −363 |
| MUC17-2R | CAGTAATACGACTCACTATAGGGAGAAGGCTCAAAAACCAACCTAAACCTAAATCA | −95 to −71 |
| MUC17-3F | AGGAAGAGAGGTTTTTGTTAGGGGTTAGGGTTAAA | −372 to −348 |
| MUC17-3R | CAGTAATACGACTCACTATAGGGAGAAGGCTAAAAACCAAAATCAACAAACACAAC | +77 to +101 |
| MUC17-4F | AGGAAGAGAGTTTGGTTTTTATAGGTTTTTTGGGT | −255 to −231 |
| MUC17-4R | CAGTAATACGACTCACTATAGGGAGAAGGCTAAACAAACAAAACAAACTAACCCCT | +179 to +203 |
| MUC17-5F | AGGAAGAGAGTGTTTTAGGGATTAAAGGTTTTTGG | −62 to −38 |
| MUC17-5R | CAGTAATACGACTCACTATAGGGAGAAGGCTCTAAACTCCAAACAAAAAACACACC | +241 to +265 |
| MSP primers | | |
| MUC17-UF[a] | GTTGATAATTTTTATGTTTATGGGTTGTT | −207 to −179 |
| MUC17-UR[a] | CTAACCTTAACATCAAAACTCTAAACACA | +38 to +66 |
| MUC17-MF[b] | GTTGATAATTTTTATGTTTATGGGTTGTC | −207 to −179 |
| MUC17-MR[b] | CCTTAACATCGAAACTCTAAACACG | +38 to +62 |
| Semiquantitative PCR primers | | |
| MUC17-F1 | GCTGTGTCTGCTGACCTTGG | Exon 1 |
| MUC17-R1 | TGGCACTGACGGTTCAAGAC | Exon 2 |
| ChIP primers | | |
| MUC17-CF1 | CCAGTGTCTCAGGGACCAAAG | −66 to −46 |
| MUC17-CR1 | CCAAGGTCAGCAGACACAGC | +77 to +96 |

Synthetic oligonucleotides listed with the position number with respect to the transcriptional start site, respectively.
[a]The U primer for unmethylated alleles in MSP analysis.
[b]The M primer for methylated alleles in MSP analysis.

Hyb Kit (Human miRNA Microarray v.2, which contains probes for 723 human and 76 human viral miRNAs from the Sanger database v.10.1). In this analysis, 148 miRNAs with a maximum or minimum signal that varied by more than twice among the 11 cell lines were identified. A total of 18 of the 148 miRNAs had potential for MUC17 regulation based on target prediction using the miRBase. The comparison of these results with *MUC17* expression revealed a significant relationship with five candidates to regulate *MUC17* expression (miR-17, miR-20a, miR-20b, miR-30c, miR-30e; target prediction based on miRBase). Hence, these miRNAs might participate in the post-transcriptional regulation of *MUC17* expression (Figure 4).

## Discussion

It has been reported that the human *MUC17* gene is predominantly expressed in PDACs and identified as an independent prognostic factor of the patients with PDAC carrying lymph node metastasis (Hirono et al. 2010). However, little is known about the functional role of MUC17 in cancer pathology. Understanding the expression mechanism of *MUC17* can be a key step in developing new strategies for cancer diagnosis and treatment.

To examine the possible epigenetic regulation of *MUC17* expression, we treated MUC17-negative/low cells with 5-AzadC and/or TSA. In MCF-7, A427, BxPC3 and PANC1 cells the restoration level of *MUC17* mRNA decreased in 5-AzadC/TSA treatments rather than in treatment with 5-AzadC or TSA alone. Yet, it is unclear whether 5-AzadC and TSA caused an antagonistic effect in three cells. We showed that treatment with TSA did not restore the *MUC17* mRNA in NCI-H292 cells. Kondo et al. (2003) showed that 5-AzadC or a combination of 5-AzadC and TSA, but not TSA alone, reactivates tumor suppressor gene expression at the silenced loci (e.g. *p16*). Our results in NCI-H292 cells are in agreement with their observation. Although there were differences in the restoration level of *MUC17* mRNA among treated cell lines, our overall results suggested the possibility that *MUC17* expression is regulated by epigenetic mechanisms.

Before beginning the epigenetic analysis, we performed a sequence analysis of the *MUC17* promoter in 10 cancer cells to examine the possible regulatory SNPs (rSNPs). rSNPs are usually promoter region mutations that cause variation in gene expression levels (Knight 2005). Our results were congruent in the 10 cell lines and matched the data for GenBank accession number NT_007933. Therefore, we examined the DNA methylation status of the *MUC17* promoter in 10 cancer cell lines.

In this study, we used MassARRAY analysis to examine the methylation status of 19 CpG sites in the promoter of the *MUC17* gene and identified five CpG sites (site numbers 10–14) involved in MUC17 expression. We also examined the methylation status of normal and tumoral pancreatic tissues

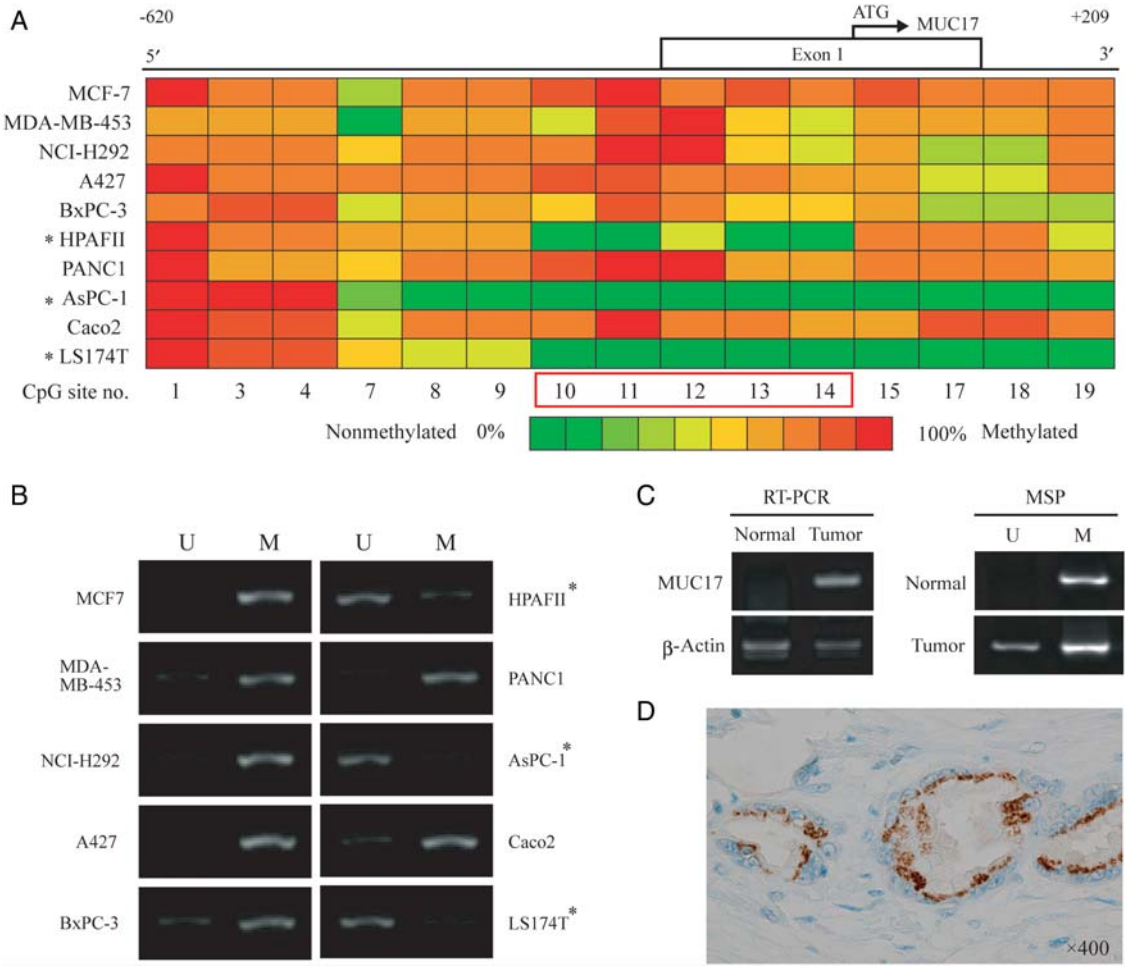


**Fig. 2.** Methylation pattern of *MUC17* promoter and MSP analysis in cell lines and biological samples. (**A**) Quantitative methylation analysis of CpG sites located in the *MUC17* promoter using a MassARRAY compact system. Different colors display relative methylation changes in 10% increments (green = 0%, red = 100% methylated). CpG sites that correlated well with *MUC17* expression are boxed in red. **MUC17*-positive cell lines. (**B**) MSP analysis of 10 cancer cell lines. The PCR products labeled M (methylated) were generated by methylation-specific primers and those labeled U (unmethylated) were generated by primers specific for unmethylated DNA. (**C**) Correlation between the mRNA expression level and methylation status in the 5′-flanking region in the normal pancreas (N) and tumoral pancreatic tissues (T) from patients with PDAC. Semi-quantitative RT–PCR and MSP analysis were carried out, respectively. (**D**) Immunohistochemical staining for MUC17 in a patient with PDAC. The carcinoma cells show granular staining in the supranuclear areas (×400, original magnification).

**Table II.** Methylation status in biological samples from patients with PDAC

| | Unmethyl (U) | *P*-value |
|---|---|---|
| Tissue/normal | 2/8 | 0.046 |
| Tissue/PDAC | 6/8 | |

MSP analysis of eight normal and tumoral tissues from patients with PDAC.

from patients with PDAC. As a result of the MSP analysis, the methylation status in the *MUC17* promoter was consistent with the expression of MUC17 in both mRNA and protein levels (Figure 2C and D, Table II). These results raise the possibility that human pancreatic tissue might be regulated with the epigenetic mechanism similar to cell lines. Although further studies are needed to clarify the relationship, these findings indicate that the methylation status in the *MUC17* promoter could be a novel epigenetic marker for the diagnosis of PDAC.

We also examine the relationship between DNA methylation and histone modification in *MUC17* expression by ChIP assay, resulting in the presence of dimethyl-H3-K9 in cells with no or low MUC17 confirmed, whereas histone H3-K9 was more highly acetylated in MUC17-positive cells than in cells with no or low MUC17 (Figure 3B). These results suggest that the combination of DNA methylation and histone H3-K9 modification contribute to MUC17 expression.

Epigenetic regulation of small non-coding RNAs plays a critical role in the modulation of mammalian gene expression (Baek et al. 2008; Siomi H and Siomi MC 2009). In addition, miRNAs have been reported to regulate both tumor suppressor genes and oncogenes. We have previously examined the expression profiles of miRNAs in 11 cancer cell lines (Yamada et al. 2010). The result of miRNA microarray analysis showed 148 miRNAs with a maximum or a minimum signal that varied by more than twice among the 11 cells lines that were identified. Furthermore, the comparison of these

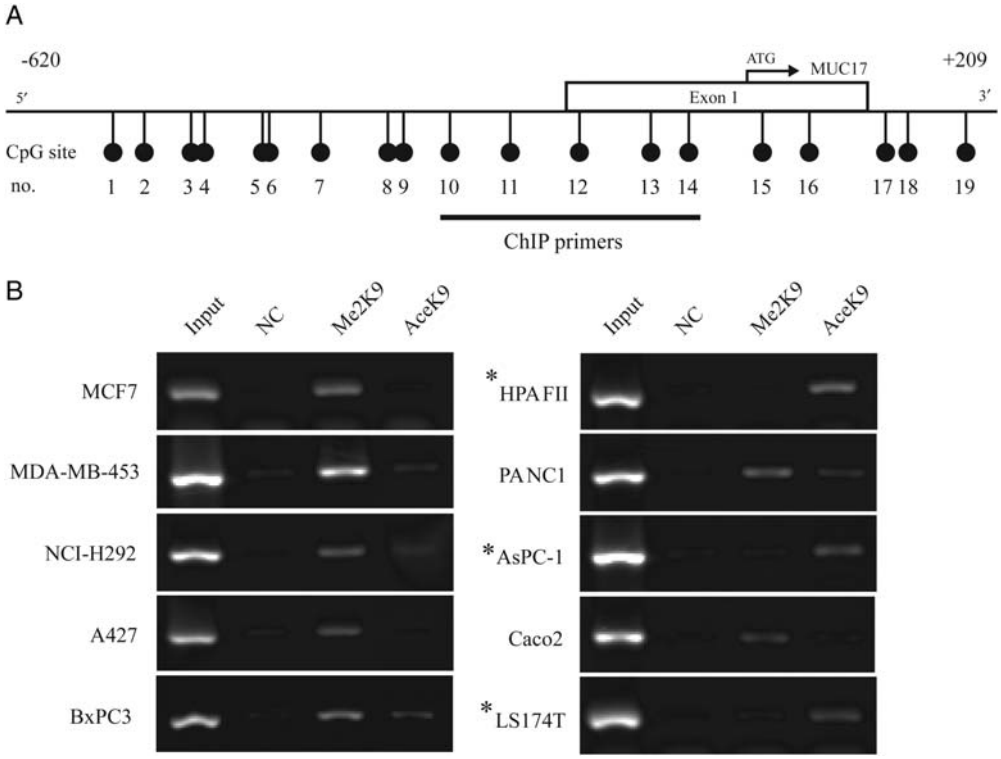


**Fig. 3.** Histone modification status of MUC17 proximal promoter region in 10 cancer cell lines. (**A**) Schematic representation of the *MUC17* gene promoter region. The relative positions of CpG sites and the ChIP primers used in this experiment are indicated. (**B**) ChIP analysis in the *MUC17* promoter region was performed using antibodies against dimethylated H3-K9 (Me2-K9) and acetylated H3-K9 (Ace-K9). Input DNA was used as a positive control. NC indicates ChIP performed using rabbit IgGs as an isotype antibody control. Asterisks indicate *MUC17*-positive cell lines. Histone H3-K9 dimethylation was preferentially observed in cells with little or no *MUC17* expression, whereas acetylation of histone H3-K9 was detected in all *MUC17*-positive cells. **MUC17*-positive cell lines.

results with *MUC17* expression revealed a significant relationship with five candidates to regulate *MUC17* expression (miR-17, miR-20a, miR-20b, miR-30c, miR-30e; target prediction based on miRBase). Of all the potential candidates of a given mRNA of *MUC17*, it is still unclear which ones are authentic miRNA in vivo. Our data suggest that the *MUC17* gene may be regulated post-transcriptionally by miRNAs (Figure 4).

In this study, our data suggested that the epigenetic regulation of the *MUC17* gene in cancer as follows: (1) open chromatin can be characterized by unmethylated status in the proximal *MUC17* promoter region and histones modification with acetylated H3-K9. This allows the assembly of transcription factors and transcription by RNA polymerase. DNA methylation and histone deacetylation result in the condensation of chromatin into a compact state that is inaccessible by transcription factors. (2) The methylation of the proximal *MUC17* promoter region directly blocks binding of transcription factors and prevents transcription. It may also recruit methyl-CpG-binding domain proteins such as MeCP2 and MBD1 that have associated histone deacetylases. (3) Post-transcriptional regulation by miRNA might also participate in MUC17 expression.

Although the functional roles of MUC17 in cancer development are still unknown, there are some common structural features in other mucins. First, MUC17 is similar to MUC1 in the aspect of possessing SEA (sperm protein, enterokinase and agrin) domain, hypothesized to be the proteolytic cleavage site. Mahanta et al. (2008) showed that MUC1 cleavage by an SEA domain leads to activation of the MAP kinase signaling and stimulates tumor cell growth. MUC17 also has two EGF-like domains separated by the SEA domain. Other experimental data have shown that the extracellular EGF-like motif of MUC4 can interact with ERBB2 (Jepson et al. 2002; Hollingsworth and Swanson 2004). Additionally, the soluble EGF-ligand on a mucin might serve as a mediator of inflammatory or immune responses (Agrawal et al. 1998; Correa et al. 2003). These findings indicate that MUC17 might have multiple functions, including cell growth, differentiation or immunoregulatory mediation. Our results indicate that *MUC17* is regulated tightly through epigenetic manners including DNA methylation, histone modification and miRNA regulation. These findings suggest the possible functional importance of MUC17 in both physiological and pathological conditions. The understanding of these intimately correlated epigenetic changes for the *MUC17* gene expression may also be of importance for the diagnosis of carcinogenic risk and the prediction of outcomes of patients with PDAC.

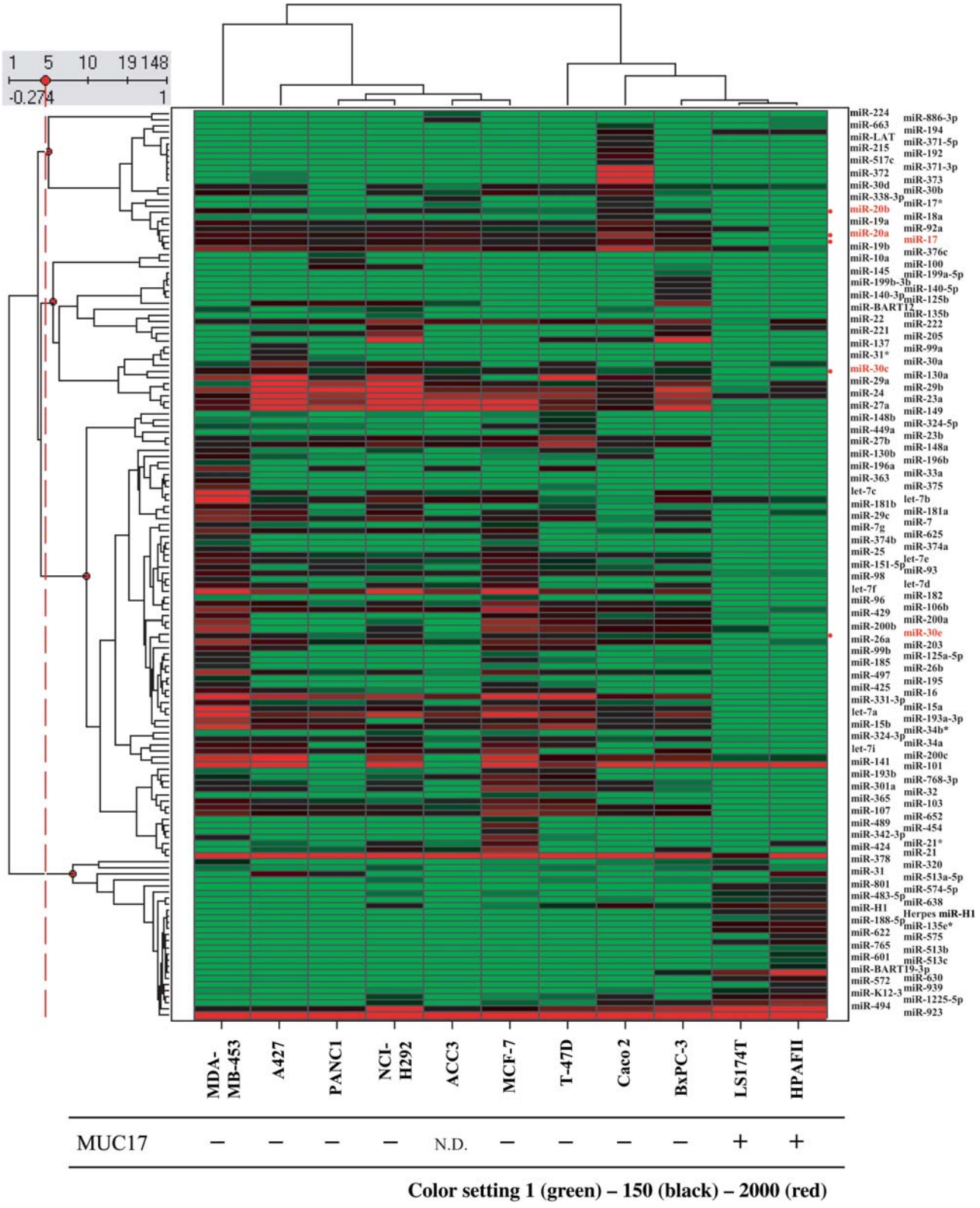


**Fig. 4.** miRNA expression profiles of 11 human cancer cell lines. The heat map shows the 148 miRNAs for which the maximum or minimum signal differed by more than twice among the 11 cancer cells. The cluster shows correlated groups of miRNAs and cell lines. The five miRNAs with the potential for *MUC17* regulation are highlighted in red. The MUC17 protein expression pattern is shown below. Reprinted, with permission and modification, from Yamada et al. (2010).

## Materials and methods

### *Cells and treatment*

Human pancreatic carcinoma cell lines HPAFII, BxPC-3, PANC1 and AsPC-1, human breast cancer cell lines MCF-7 and MDA-MB-453, human lung cancer cell lines NCI-H292 and A427 and human colon adenocarcinoma cell lines Caco2 and LS174T were obtained from the American Type Culture Collection. MCF-7, A427, HPAFII, Caco2 and LS174T cells were cultured in Eagle's minimum essential medium (Sigma, St. Louis, MO); PANC1 cells were cultured in D-MEM (Sigma); NCI-H292, BxPC3, AsPC-1 and ACC3 cells were cultured in RPMI-1640 medium (Sigma) and MDA-MB-453 cells were cultured in Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA). All media were supplemented with 10% fetal bovine serum (Invitrogen) and 100 U/mL of penicillin/100 μg/mL of streptomycin (Sigma). MUC17-negative/low cells were split 24 h before treatment. BxPC-3, NCI-H292 cells were incubated with 100 μM 5-AzadC (Sigma) and/or 100 nM TSA (Sigma) for 5 days; MCF7, PANC1 and Caco2 cells were incubated with 100 μM 5-AzadC and/or 50 nM TSA for 5 days; MDA-MB-453 cells were incubated with 100 μM 5-AzadC and/or 10 nM TSA for 5 days and A427 cells were incubated with 1 μM 5-AzadC and/or 50 nM TSA for 5 days. Media were changed every 24 h.

### *Patients and biological samples*

This study was approved by the ethical committee of Kagoshima University Hospital. Fresh pancreatic tumor tissues were obtained from the surgically resected pancreatic specimens from eight patients with PDAC. The normal pancreatic tissues from the same resected specimens were obtained from the areas apart from the tumor in each patient. To conduct the following study, all tissue samples were stored at –80°C. The adjacent areas of cancerous and normal tissues were fixed in buffered formalin (pH 7.4) and were examined histologically and immunohistochemically.

### *Quantitative RT–PCR analysis*

Total RNA from cells that did or did not undergo 5-AzadC, TSA or 5-AzadC + TSA treatment was purified with an RNeasy Mini kit (Qiagen, Valencia, CA). Total RNA (20 μL) was reverse-transcribed with a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA) as described previously (Yamada et al. 2008). The primers and probes were designed and synthesized by Applied Biosystems. The product number of the Target Assay Mix used for MUC17 was Hs00959753_s1. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; product number 4310884E) was used to calibrate the original concentration of mRNA; i.e. the concentration of mRNA in the cell was defined as the ratio of target mRNA copies versus GAPDH mRNA copies. In this analysis, data from three separate experiments were averaged.

### *Immunohistochemical staining*

MUC17 protein expression levels were assessed by immunohistochemistry. For cultured cells, MUC17 was detected by rabbit anti-MUC17 polyclonal antibody (generated by one of us, by S.K.B., University of Nebraska Medical Center, Omaha; dilution rate at 1:6400 for overnight at 4°C). Immunohistochemical staining was performed by an immunoperoxidase method using a Vectastain *Elite* ABC kit (Vector Laboratories), as described previously (Yonezawa et al. 1997; 2008). For tissue staining, antigen retrieval was performed using CC1 antigen retrieval buffer (Ventana Medical Systems, Tucson, AZ) for all sections. Following incubation with the primary antibody (dilution rate at 1:20,000), sections were stained on the Ventana automated slide stainer (Benchmark XT) using the Ventana diaminobenzidine detection kit (Ventana Medical Systems).

### MUC17 *gene promoter sequencing*

Genomic DNA was extracted from the 10 cell lines using a DNeasy Tissue System (Qiagen) according to the manufacturer's instructions. Sequencing was carried out using three pairs of forward and reverse primers (Table I) in *MUC17* promoter.

### *Quantitative methylation analysis*

Quantitative methylation analysis of the *MUC17* promoter (GenBank accession number: NT_007933) was performed using the MassARRAY Compact system (Sequenom), as described previously (Ehrich et al. 2005). The target regions were amplified using the primer pairs shown in Table I. Each forward primer is tagged with a 10mer (5′-AGG AAG AGA G-3′) to balance the PCR and a reverse primer (5′-CAG TAA TAC GAC TCA CTA TAG GGA GAA GGC T-3′) with a T7-promoter tag for *in vitro* transcription. The resultant methylation cells were analyzed with EpiTyper software v1.0 (Sequenom) to generate quantitative results for each CpG site or an aggregate of multiple CpG sites.

### *DNA extraction and MSP analysis*

DNA from cell lines was extracted using a DNeasy Tissue System (Qiagen), according to the manufacturer's instructions. Bisulfite modification of the genomic DNA was carried out using an Epitect Bisulfite kit (Qiagen), and the modified DNA was amplified by PCR using AmpliTaq Gold (Applied Biosystems). The target regions were amplified using the primer pairs shown in Table I. The PCR conditions were 95°C for 10 min, 40 cycles at 96°C for 5 s, 59°C for 5 s and 68°C for 5 s, with a final extension reaction at 72°C for 1 min. The amplified products were subjected to 1.0% agarose gel electrophoresis.

### *ChIP assay*

ChIP assay was performed using a Shearing-ChIP kit and a 1 day ChIP kit (Diagenode, Philadelphia, PA), according to the manufacturer's instructions. Briefly, the nucleoprotein complexes were sonicated to reduce the sizes of DNA fragments from 300 to 500 bp using a Bioruptor (Cosmo Bio). One microgram of normal mouse immunoglobulin was used as the negative control (NC), and anti-dimethyl histone H3-K9 antibody (Abcam) and anti-acetyl histone H3-K9 antibody (Abcam) were used for each immunoprecipitation. Immunoprecipitated DNA was amplified by PCR using AmpliTaq Gold (Applied Biosystems). The ChIP primers were designed to target a region similar to the target region of

the MSP primers (Table I). The PCR conditions were 95°C for 5 min, 34 cycles at 96°C for 5 s, 59°C for 5 s and 68°C for 7 s, with a final extension reaction at 72°C for 1 min. The amplified products were subjected to 1.0% agarose gel electrophoresis.

*Statistical analysis*

Statistical analyses were conducted using Pearson's $\chi^2$ test. The statistical analysis software, SPSS (version 18.0), was used and $P < 0.05$ was considered significant.

## Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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## Conflict of interest

None declared.

## Abbreviations

ChIP, chromatin immunoprecipitation; 5-AzadC, 5-aza-2′-deoxycytidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MSP, methylation-specific-PCR; NC, negative control; rSNPs, regulatory SNPs; RT, reverse transcription; SEA, sperm protein, enterokinase and agrin; SNPs, single nucleotide polymorphisms; TSA, trichostatin A

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