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Promoter hypomethylation contributes to the expression of MUC3A in cancer cells

Sho Kitamoto, Norishige Yamada, Seiya Yokoyama, Izumi Houjou, Michiyo Higashi, Suguru Yonezawa *

Department of Human Pathology, Field of Oncology, Kagoshima University, Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan

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ABSTRACT

MUC3A is a membrane-bound glycoprotein that is aberrantly expressed in carcinomas and is a risk factor for a poor prognosis. However, the exact mechanism of MUC3A expression has yet to be clarified. Here, we provide the first evidence that *MUC3A* gene expression is controlled by the CpG methylation status of the proximal promoter region. We show that the DNA methylation pattern is intimately correlated with *MUC3A* expression in breast, lung, pancreas and colon cancer cell lines. The DNA methylation status of 30 CpG sites from −660 to +273 was mapped using MassARRAY analysis. MUC3A-negative cancer cell lines and those with low MUC3A expression (e.g., MCF-7) were highly methylated in the proximal promoter region, corresponding to 9 CpG sites (−345 to −75 bp), whereas MUC3A-positive cell lines (e.g., LS174T) had low methylation levels. Moreover, 5-aza-2′-deoxycytidine and trichostatin A treatment of MUC3A-negative cells or those with low MUC3A expression caused elevation of *MUC3A* mRNA. Our results suggest that DNA hypomethylation in the 5′-flanking region of the *MUC3A* gene plays an important role in *MUC3A* expression in carcinomas of various organs. An understanding of epigenetic changes in *MUC3A* may contribute to the diagnosis of carcinogenic risk and to prediction of outcome in patients with cancer.

1. Introduction

Mucins are heavily *O*-glycosylated proteins that are found in the mucus layer and differentially expressed at the surface of many epithelial cells. They are responsible for the physical properties of mucus gels and are involved in epithelial cell protection and maintenance of the local molecular microenvironment [1–4]. There is also increasing evidence that aberrant expression of mucins contributes to the pathogenesis of cancer [5–7]. We have demonstrated that expression of MUC1 and MUC4 is a poor prognostic factor, whereas MUC2 expression is associated with a favorable outcome in various human neoplasms including pancreatic ductal adenocarcinomas. We have stressed that mucins are indicators of the potential for malignancy [7–9], and we have shown that MUC5AC expression is an effective tool for early detection of pancreatic neoplasms [10,11].

MUC3A was identified and mapped to a mucin cluster on chromosome 7q22, and categorized as a membrane-associated mucin [12–14]. An association between MUC3A expression and poor prognosis has been shown in pancreatic, breast, gastric and renal cancers [15–18]. Gum et al. have reported that the 5′-flanking region of *MUC3A* has promoter activity and that transcription is initiated from multiple sites in a region spanning about 180 nucleotides [19]. However, the exact regulatory mechanism of *MUC3A* is not fully understood. We have described mechanisms of epigenetic regulation for *MUC1*, *MUC2*, *MUC4*, *MUC5AC* expression [20–23], and thus we hypothesized that *MUC3A* may also be regulated epigenetically.

Methylation of CpG sites in genomic DNA plays an important role in gene regulation, and especially in gene silencing [24], and generally the promoter region of a transcribed gene is hypomethylated [25,26]. In the present study, we mapped the CpG methylation status of *MUC3A* from −620 to +209 using MassARRAY analysis in human cancer cell lines. Based on the results of this analysis, we also designed methylation-specific PCR (MSP) primers to identify the CpG sites related to gene expression. Moreover, to examine whether MUC3A mRNA expression is regulated by the DNA methylation status and histone H3 modification, we also treated MUC3A-negative cells or cells with low MUC3A expression (MUC3A-negative/low cells) with a DNA methylation inhibitor, 5-aza-2′-deoxycytidine (5-AzadC) and a histone deacetylase inhibitor, trichostatin A (TSA). In this paper, we provide the first evidence that *MUC3A* gene expression is tightly linked to DNA methylation in the proximal promoter region.

2. Materials and methods

2.1. Cells and treatment

Human breast cancer cell lines MCF-7 and MDA-MB-453; the human lung cancer cell line NCI-H292; human pancreatic carci-

* Corresponding author. Fax: +81 99 265 7235.
E-mail address: syoneza@m2.kufm.kagoshima-u.ac.jp (S. Yonezawa).

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noma cell lines HPAFII and AsPC-1; and the human colon adenocarcinoma cell lines LS174T and Caco2 were obtained from American Type Culture Collection (Manassas, VA, USA). MCF-7, HPAFII, Caco2 and LS174T cells were cultured in Eagle's minimum essential medium (Sigma, St. Louis, MO, USA); NCI-H292 and AsPC-1 cells were cultured in RPMI-1640 medium (Sigma); and MDA-MB-453 cells were cultured in Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA, USA). All media were supplemented with 10% fetal bovine serum (Invitrogen) including 100 U ml^{-1} penicillin and 100 $\mu g ml^{-1}$ streptomycin (Sigma). MUC3A-negative cells or cells with low MUC3A expression were split 24 h before treatment with epigenetic-modifying agents. NCI-H292 cells were incubated with 100 μM 5-AzadC (Sigma) and/or 100 nM TSA (Sigma) for 5 days; MCF-7 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.

2.2. Quantitative reverse transcription PCR (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, USA). Total RNA (20 μL) was reverse transcribed with a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA), as described previously [22]. The primers and probes were designed and synthesized by Applied Biosystems. The product number of the Target Assay Mix used for MUC3A was Hs03649367_mH. 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.

2.3. MUC3A gene promoter sequencing

Genomic DNA was extracted from the seven cell lines using a DNeasy tissue system (Qiagen) according to the manufacturer's instructions. DNA was PCR amplified using seven pairs of sense and antisense primers (Table 1) for the full-length *MUC3A* promoter.

2.4. Quantitative methylation analysis

Quantitative methylation analysis of the *MUC3A* promoter was performed using a MassARRAY compact system (Hitachi, Tokyo, Japan) [27]. DNA from the cell lines was extracted using a DNeasy tissue system (Qiagen). A 1-mg sample of DNA was converted with sodium bisulfite using an EZ DNA methylation kit (Zymo Research, Orange, CA, USA) and the modified DNA was amplified by PCR. The target regions were amplified using the primer pairs shown in Table 1. Each forward primer was tagged with a 10mer (5′-AGGAAGAGAG-3′) to balance the PCR, and each reverse primer had a T7-promoter tag (5′-CAGTAATACGACTCACTATAGGGAGAAGGCT-3′) for *in vitro* transcription. PCR amplification was performed with the following parameters: hot start at 94 °C for 15 min; followed by denaturing at 94 °C for 20 s, annealing at 56 °C for 30 s, and extension at 72 °C for 1 min for 45 cycles; and final incubation at 72 °C for 3 min. Unincorporated dNTPs were dephosphorylated by adding 2 μl of premix including 0.3 U shrimp alkaline phosphate (SAP; Sequenom, San Diego, CA, USA). The reaction mixture was incubated at 37 °C for 40 min and SAP was then heat inactivated for 5 min at 85 °C. After SAP treatment, 2 μl of the PCR products were used as a template for *in vitro* transcription, and RNase A cleavage was used for the reverse reaction, following the manufacturer's instructions (Sequenom). The samples were conditioned and spotted on a 384-pad Spectro-CHIP (Sequenom) using a MassARRAY nanodispenser (Samsung, Irvine, CA, USA), followed by spectral acquisition on a MassARRAY analyzer compact MALDI-TOF–MS (Sequenom). The resultant methylation calls were analyzed with EpiTyper software v1.0 (Sequenom) to generate quantitative results for each CpG site or an aggregate of multiple CpG sites.

2.5. DNA extraction and DNA MSP analysis

DNA from the 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

Table 1
Synthetic oligonucleotides used in this study.

Name	Primer sequence	Position
MUC3A promoter-sequencing primers		
MUC3A-S1F	AGATGGGGTTTCACTATGTTGG	−1078 to −1057
MUC3A-S1R	CTGAGTTTCAATTCCTCCTGCT	−429 to −408
MUC3A-S2F	GCAGACTGAGGGACAGAAGG	−483 to −464
MUC3A-S2R	CTCTGGAAGAAACCTCTCCTCA	+230 to +251
10mer-tagged or T7-tagged primers		
MUC3A-1F	aggaagagagTTGTTGTTTAGGTTGGAGTGTAGTG	−860 to −836
MUC3A-1R	cagtaatacgactcactatagggagaaggctACAACCTCTCCTAAATTTCAATTCC	−422 to −398
MUC3A-2F	aggaagagagAGAGATGGGGTTTTGTTATTTTGTT	−387 to −363
MUC3A-2R	cagtaatacgactcactatagggagaaggctACAACCTCTCCTAAATTTCAATTCC	−422 to −398
MUC3A-3F	aggaagagagGGAATTGAAATTTAGGAGAGGTTGT	−422 to −398
MUC3A-3R	cagtaatacgactcactatagggagaaggctAACAAAACAAACTTCAAAACCAAAA	−48 to −24
MUC3A-4F	aggaagagagGGTTGTGGGGTTGAGTTAATAGTAG	−293 to −269
MUC3A-4R	cagtaatacgactcactatagggagaaggctCCTTTCCTAAAACATCACATCAAAA	+98 to +122
MUC3A-5F	aggaagagagGGTTTTTTTTGGATGTTTAAGGTTT	+22 to +46
MUC3A-5R	cagtaatacgactcactatagggagaaggctAAAATAACAAAAACCCAACTCCCT	+291 to + 314
MSP primers		
MUC3A-UF*	GTTAAGGGTATTTTTTATGGGTGTT	−362 to −338
MUC3A-UR*	CAAAAACATAACCCTAAAACATACACA	−81 to -55
MUC3A-MF**	GTTAAGGGTATTTTTTACGGGTGTC	−362 to −338
MUC3A-MR**	AAACGTAACCCTAAAACGTACACG	−81 to −58

Each synthetic oligonucleotide is listed with the position number with respect to the translational start site. In MSP analysis.
* Indicates the U primer for unmethylated alleles.
** Indicates the M primer for methylated alleles.