Gli Regulates MUC5AC Transcription in Human Gastrointestinal Cells

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Abstract

MUC5AC is a well-known gastric differentiation marker, which has been frequently used for the classification of stomach cancer. Immunohistochemistry revealed that expression of MUC5AC decreases accompanied with increased malignant property of gastric mucosa, which further suggests the importance of MUC5AC gene regulation. Alignment of the 5′-flanking regions of MUC5AC gene of 13 mammal species denoted high homology within 200 bp upstream of the coding region. Luciferase activities of the deletion constructs containing upstream 451 bp or shorter fragments demonstrated that 15 bp region between −111 and −125 bp plays a critical role on MUC5AC promoter activity in gastrointestinal cells. We found a putative Gli-binding site in this 15 bp sequence, and named this region a highly conserved region containing a Gli-binding site (HCR-Gli). Overexpression of Gli homologs (Gli1, Gli2, and Gli3) clearly enhanced MUC5AC promoter activity. Exogenous modulation of Gli1 and Gli2 also affected the endogenous MUC5AC gene expression in gastrointestinal cells. Chromatin immunoprecipitation assays demonstrated that Gli1 directly binds to HCR-Gli: Gli regulates MUC5AC transcription via direct protein-DNA interaction. Conversely, in the 30 human cancer cell lines and various normal tissues, expression patterns of MUC5AC and Gli did not coincide wholly: MUC5AC showed cell line-specific or tissue-specific expression whereas Gli mostly revealed ubiquitous expression. Luciferase promoter assays suggested that the far distal MUC5AC promoter region containing upstream 4010 bp seems to have several enhancer elements for gene transcription. In addition, treatments with DNA demethylation reagent and/or histone deacetylase inhibitor induced MUC5AC expression in several cell lines that were deficient in MUC5AC expression. These results indicated that Gli is necessary but not sufficient for MUC5AC expression: namely, the multiple regulatory mechanisms should work in the distal promoter region of MUC5AC gene.

Introduction

Although the mortality and incidence of gastric cancer has gradually fallen in the last several decades, it is still the fourth most common malignancy and the second leading cause of cancer-related death worldwide [1]. Gastric cancer mainly occurs from gastric mucosa with intestinal metaplasia, which is mostly caused by chronic infection of Helicobacter pylori [2,3]. Intestinal metaplasia in stomach is typically classified into two types: mixed gastric-and-intestinal type (incomplete type) and solely intestinal type (complete type) [4]. In the process of metaplastic change of gastric mucosa, induction of intestinal differentiation and loss of gastric differentiation occur simultaneously or asynchronously [5,6].

For induction of intestinal differentiation, we and other groups have been reported that Cdx must be an indispensable key molecule by regulating transcription of many intestinal marker
genes [7,8,9,10,11,12,13]. On the contrary, the molecular mechanism of loss of gastric genes in the process of metaplastic change in stomach remains poorly understood. It is probably due to inadequate identification of gastric marker genes, and also due to unsolved expression regulation of these gastric genes. Based on these backgrounds, we focused on MUC5AC, which is a well-established gastric marker gene [6,14] and is often used for the clinical assessment of gastric cancer [15]. There have been many previous reports investigating the expression of MUC5AC and prognosis of gastric cancer, but the association between expression of MUC5AC and malignant potential of gastric cancer is still controversial [16,17,18,19]. Nevertheless, MUC5AC is one of the most evident gastric marker clearly decreased in the process of intestinal metaplasia [14,20]. We believe elucidating the mechanism of MUC5AC gene expression in gastrointestinal cells must be useful to understand the loss of gastric differentiation during the development of pre-malignant atrophic gastritis.

Our aim of this study is to find a critical mechanism of MUC5AC expression regulation in human gastrointestinal cells. To date, HIF-1α [21], Smad4 [21,22], Sp1 [22], GATA-4/-6, and HNF-1/4 [-4] are reported to activate murine MUC5AC. Sp1 and Gli are reported to enhance MUC5AC expression in human lung-epithelial and pancreatic cancer cells, respectively [24,25]. Contrastively, ATBF1 is negatively regulate MUC5AC expression in human gastric cancer cells [26]. In spite of these results, molecular mechanisms of MUC5AC expression in human gastrointestinal cells have not been fully elucidated. In the present study, we identified 15 bp sequence in human MUC5AC promoter region which plays an important role in MUC5AC expression in gastrointestinal cancer cells, and also found that the region contains a putative Gli-binding site which does not coincide with previous report [25]. Gli is one of transcriptional factors which has a DNA binding zinc finger domain [27]. In this study, we examined molecular roles of Gli on MUC5AC promoter in gastrointestinal cells. Our results demonstrated that MUC5AC expression depends on cooperative regulatory mechanism of Gli, some epigenetic modulation, and other factors in gastrointestinal cells.

Materials and Methods

Cell Culture

Twenty gastric cancer cell lines, ten colorectal cancer cell lines, and two non-gastrointestinal cancer cell lines were maintained in high-glucose DMEM with 10% fetal calf serum (Gibco/Invitrogen, Carlsbad, CA) at 37°C in a humidified 5% CO2 atmosphere. Names of used cell lines and histological types of gastric cancer cell lines were described in our previous reports [6]. Human T98G and A172 cell lines were purchased from the RIKEN Bio Resource Center (Tsukuba, Japan). For the treatment with DNA demethylation reagent or histone deacetylase (HDAC) inhibitor, 5-Aza-2′-deoxycytidine (5-Aza-dC, Sigma-Aldrich) at 2 μg/ml and/or trichostatin A (TSA, Sigma-Aldrich) at 25–1000 ng/ml were added to the culture medium.

Tumor Samples

For the advanced gastric cancer specimens, we randomly selected 89 gastric adenocarcinoma samples surgically resected at the Fujita Health University Hospital. For the early stage gastric cancer endoscopically resected, we selected 78 specimens banked at the University of Tokyo Hospital. This study was approved by the ethic committees of the University of Tokyo, and also by the institutional ethical review board for human investigation at Fujita Health University. According to the Declaration of Helsinki, written informed consents were obtained from all the study participants for use of resected sample in research.

Immunohistochemistry

Deparaffinization and endogenous peroxidase inactivation of clinical tissues were performed as described previously [28]. For MUC5AC, hydrated heating in 1 mM EDTA buffer (pH 8.0) at 120°C was then performed in a pressure cooker (Delicio 6L; T-FAL, Rumily, France) for 10 min for antigen retrieval. The primary immunostaining with anti-MUC5AC antibody (NCL-MUC5AC, Novocastra, Newcastle-upon-Tyne, UK) at a 1:200 dilution was applied for 12 hours at room temperature. After washing in PBS three times, the secondary immunostaining with Histofine Simple Stain MAX-PO(G) (Nichirei, Tokyo, Japan) was applied for 30 min at room temperature. Based on the evaluation by the two independent pathologists, ratios of MUC5AC-positive cells were classified into four classes: 1) <10%, 2) ≥10% and <50%, 3) ≥50% and <80%, and 4) ≥80%.

For Gli1 immunostaining, hydrated heating in 10 mM citrate buffer (pH 6.0) was done in a pressure cooker for 10 min for antigen retrieval. The sections were then incubated for 1 h at room temperature with antisum rabbit antibody (H-300, sc-20867, Santa Cruz Biotechnology) at a 1:500 dilution. For the amplification of signals, anti-rabbit antibody (Dako, Hamburg, Germany) was applied to the slides for 30 min at room temperature. This was followed by incubation with FITC-conjugated phenol (fluoresceyl-tyramide; Dako) for 15 min at room temperature and incubation with anti-FITC antibody conjugated to HRP (Dako) for 15 min at room temperature was then done.

Plasmid constructions

The primer sequences used in plasmid constructions are listed in Table 1. To construct the vectors for luciferase reporter assays, the 2000 bp and 1433 bp of MUC5AC promoter region were amplified from TIG-112 genome using the primers MUC5AC-F-01/MUC5ACup-R-01 and MUC5ACup-F-02/MUC5ACup-R-01. The amplified products were cloned into pETblue-T-vector (Novagen, Darmstadt, Germany), and then digested with Sall and BamHI. The excised 2 kb and 1.4 kb DNA fragments were inserted into the XhoI/BglII site of pGL4.12 (Promega, Madison, WI, USA) to generate pGL4.12-hMUC5ACup2000bp and pGL4.12-hMUC5ACup1433bp. NcoI-HindIII fragment from pGL4.12-hMUC5ACup2000bp was inserted into the EcoRV/HindIII site of pGL4.12 to generate pGL4.12-hMUC5ACup451bp. The 280 bp, 190 bp, 158 bp, 150 bp, 138 bp, 125 bp and 110 bp of MUC5AC promoter region were amplified from pGL4.12-hMUC5ACup451bp as a template using primers MUC5ACup-280-XhoI, MUC5ACup-190-XhoI, MUC5ACup-158-XhoI, MUC5ACup-150-XhoI, MUC5ACup-138-XhoI, MUC5ACup-125-XhoI, MUC5ACup-110-XhoI and pGL4.12-HindIII, digested with HindIII and XhoI, and inserted into the XhoI/XhoI site of pGL4.12 to generate pGL4.12-hMUC5ACup280bp, pGL4.12-hMUC5ACup190bp, pGL4.12-hMUC5ACup158bp, pGL4.12-hMUC5ACup150bp, pGL4.12-hMUC5ACup138bp, pGL4.12-hMUC5ACup125bp and pGL4.12-hMUC5ACup110bp. The 4010 bp to 1432 bp upstream of MUC5AC promoter region was amplified from genome of TIG-112 genome using the primers MUC5ACup-SacI-F and MUC5A-Cup-PciI-R, digested with SacI and PciI, and inserted into the SacI/PciI site of pGL4.12-hMUC5ACup2000bp to generate pGL4.12-hMUC5ACup1010bp. The 3000 bp to 1432 bp upstream of MUC5AC promoter region was amplified from TIG-112 genome using the primers MUC5ACup-3000-Nhel-F and MUC5ACup-PciI-R, digested with Nhel and PciI, and inserted into the Nhel/PciI site of pGL4.12-hMUC5ACup2000bp.
into the NheI/PciI site of pGL4.12-hMUC5ACup2000bp to generate pGL4.12-hMUC5ACup3000bp. To delete the 15 bp in the MUC5AC promoter region (125 bp to 111 bp upstream), PCR-based mutagenesis was performed using primers MUC5AC-Up15delete-F and MUC5AC-Up15delete-R and cloned into pGL4.12 to generate pGL4.12-hMUC5ACup451bp-delta15bp. The plasmid was confirmed by sequence analysis. The 294 bp Sfi1-HindIII region of pGL4.12-hMUC5ACup4010bp was replaced by the 269 bp Sfi1-HindIII fragment of pGL4.12-hMUC5ACup451bp-delta15bp to generate pGL4.12-hMUC5ACup4010bp-delta15bp.

Gli1 and Gli3 cDNAs were purchased from Open Biosystems (clone ID 3531657 and 40125719, respectively, Huntsville, AL,) and Gli2 cDNA (pENTR223.1-hGli2) was purchased from DNAFORM K.K. (clone ID 100069128, Yokohama, Japan). EcoRI-Xhol fragment containing Gli1 cDNA was inserted into the EcoRI/Xhol site of pGL4.12-hMUC5ACup2000bp to generate pGL4.12-hMUC5ACup2000bp-15bp. EcoRI-EcoRV fragment containing Gli3 cDNA was inserted into the EcoRI/XhoI site of pMXs-IP to generate pMXs-Gli1-IP. EcoRI-XhoI fragment containing Gli1 cDNA was inserted into the NheI/NotI site of pcDNA3.1(+) to generate pcDNA3.1(+)(-Gli1 and pcDNA3.1(+)(-Gli3. BsrGI-AvrII fragment from pENTR223.1-hGli2 was inserted into the Acc65I/XbaI site of pcDNA3.1(+) to generate pcDNA3.1(+)(-Gli2).

Luciferase reporter assay

Cells were cultured on 96-well plates and transiently transfected with mixtures of Renilla luciferase control vector (pGL4.74 (Promega) (15 ng) by using Lipofectamine and Lipofectamine PLUS (Invitrogen). To examine the effect of Gli on the promoter activity, pcDNA3.1(+)(-Gli1, -Gli2, and -Gli3) plasmids were transfected with pGL-based plasmids at the same time. Luciferase assays were performed at 24 or 48 h post-transfected using the Dual luciferase reporter assay system (Promega). Luciferase activities were measured by an AutoLumat Plus LB953 (Berthold, Bad Wildbad, Germany). Luciferase activity was normalized to a Renilla control and the results are shown as mean ± SD from at least three independent experiments.

Production and infection of retrovirus vectors

pMXs-Gli1-IRES-Puro, pMXs-Gli2-IRES-Puro and pMXs-Gli3-IRES-Puro and control vector (pMXs-IRES-Puro) were transfected with pCAG-VSVG into the PLAT-GP packaging cell line for retrovirus vector production (Cell Biolabs, Inc. San Diego, CA) using Lipofectamine and Lipofectamine PLUS (Invitrogen). To examine the effect of Gli on the promoter activity, pcDNA3.1(+)(-Gli1, -Gli2, and -Gli3) plasmids were transfected with pGL-based plasmids at the same time. Luciferase activities were measured by an AutoLumat Plus LB953 (Berthold, Bad Wildbad, Germany). Luciferase activity was normalized to a Renilla control and the results are shown as mean ± SD from at least three independent experiments.

Transfection

Cells were transiently transfected with pcDNA3.1(+)(-Gli1, pcDNA3.1(+)(-Gli2, pcDNA3.1(+)(-Gli3 and pcDNA3.1(+) plasmids for 24–48 h using Lipofectamine and Lipofectamine PLUS, and were used for RT-PCR and luciferase reporter assay.

RT-PCR

Total cellular RNA was prepared using the Isogen RNA isolation reagent (Wako Pure Chemical Industries, Osaka, Japan) as previously reported [29]. RT-PCR was performed via a Superscript One-Step reaction using the Platinum Taq (Invitrogen). The primer sequences used for RT-PCR are shown in Table 2. RNA was reverse-transcribed for 30 min at 50°C, and after an initial denaturation at 94°C for 3 min, cDNA amplification procedures were performed as follows: for MUC5AC, 35 cycles of 94°C for 30 sec, 64°C for 1 min, and 72°C for 1 min; for Gli1 and Gli2, 35 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min; for Gli1 and Gli2, 35 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 1 min; for Gli3, 40 cycles of 94°C for 30 sec. A commercial RNA panel, Human Total RNA Master Panel II, was purchased from Clontech Laboratories (Palo Alto, CA, USA).

siRNA

SH-10-TG cells were transfected with siGENOME SMARTpool siRNA against human Gli1 and/or Gli2 (M-003896-00-0005 and M-004648-002-0005, respectively; Dharmacon, Lafayette, CO, USA) using Lipofectamine RNAimax (Invitrogen) according to the manufacturer’s instructions. RNA was extracted from the cells 48 h after siRNA transfection and used for RT-PCR.

Chromatin immunoprecipitation (ChiP) assay

ChiP assays were performed using a ChiP assay kit (Upstate Biotechnology Inc., Lake Placid, NY) according to the manufacturer’s instructions. For crosslinking, cells (2×106) were incubated at 37°C for 15 min in PBS containing 1% formaldehyde. Cells were collected after two washes with ice-cold PBS and subjected to centrifugation for 5 min at 700 g. Cells pellets were resuspended 400 μl sodium dodecyl sulfate (SDS) lys buffer (ChiP assay kit, Upstate) containing protease inhibitors (0.1 M phenylmethylsulfonylfluoride and 4 μl protease inhibitor cocktail (Sigma)), and incubated for 10 min on ice. DNA was sonicated (setting 5, Handy Sonic, model UR-20P; Tomy Seiko, Co., Ltd., Tokyo, Japan) and incubated for 10 min. Supernatants were diluted 10-fold with Chip dilution buffer (Upstate) and subjected to centrifugation at 13,000 rpm for 10 min. Supernatants were diluted 10-fold with Chip dilution buffer (ChiP assay kit, Upstate). 1% of the supernatant was retained as the

| Table 1. Primers used for plasmid constructions. |
|-----------------------------------------------|
| **Name** | **Sequence** |
| MUC5ACup-F-01 | 5'-attcatcaccattacactcactcactc-3' |
| MUC5ACup-F-02 | 5'-tgcctacgtgtaaggctctttctcgagc-3' |
| MUC5ACup-F-03 | 5'-tggtagccgaggggagtgcccgtc-3' |
| MUC5ACup-280-Xhol | 5'-taggctctagttgaggggagttccgctc-3' |
| MUC5ACup-190-Xhol | 5'-cgccttgcaagcgccctgcagccca-3' |
| MUC5ACup-158-Xhol | 5'-agctctcgttgctttcttggtggg-3' |
| MUC5ACup-110-Xhol | 5'-gcctctctgagggtgaaggagccag-3' |
| MUC5ACup-150-Xhol | 5'-actgctctgtaggtggaggggaaaccac-3' |
| MUC5ACup-138-Xhol | 5'-gggtgctcagacacaccgccctgagg-3' |
| MUC5ACup-125-Xhol | 5'-caactcctgacggctgcccacaaaacag-3' |
| pGL4.12-HindIII | 5'-ccgatttttttttta-3' |
| MUC5ACupSac1-F | 5'-tgcctacgacgtcagaaaaagaagc-3' |
| MUC5ACupPci-R | 5'-agagccaccaactacgcttgagc-3' |
| MUC5ACup3000-Nhe-F | 5'-actgctctgtagctatacatacactactatt-3' |
| MUC5ACup15deletesense | 5'-gaggggagacggacccgctgctggagcggctgcct-3' |
| MUC5ACup15deleter | 5'-gcgccacccgctgctgcagacggctgcct-3' |

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input, and the rest was then subjected to immunoprecipitations. Immunoprecipitations were performed overnight at 4°C with 3 μg of anti-Gli1 antibody (H-300, sc-20687; Santa Cruz Biotechnology, Santa Cruz, CA) and nonimmunized rabbit IgG whole molecule (sc-2027; Santa Cruz Biotechnology). After reverse crosslinking, the obtained DNA was purified using PCR product purification kit (Qiagen, Valencia, CA, USA). Immunoprecipitated DNA was analyzed by PCR using primers 5′-gtctgcttcctcccctgaatag-3′ and 5′-ggaatgcgttctgttttggt-3′ to amplify a fragment of the MUC5AC promoter region. Primer pairs for the villin 1 promoter region were used as a negative control [5].

Results

Decrease of MUC5AC expression accompanied with progression of gastric canceration

To evaluate the expression of MUC5AC in gastric cancer, immunostaining was performed using the clinical specimen representing three-grade gastric epithelial cells from the view of tumorigenesis: non-tumorous but precancerous cells of atrophic mucosa around early gastric cancer (Fig. 1A), malignant cells of early gastric cancer endoscopically resected (Fig 1B), and malignant cells of advanced gastric cancer surgically resected (Fig 1C). Our results revealed that the stronger the malignant property of gastric epithelial cells is, the weaker the expression of MUC5AC is. In other word, expression of MUC5AC decreases accompanied with increased malignant property of gastric mucosa. Some previous studies including ours reported that expression of MUC5AC often attenuates during the development of premalignant intestinal metaplasia [6,14]. Our present data further showed that decrease of MUC5AC expression advances in the process of gastric canceration. From these results, we are convinced that it is important to elucidate the regulatory mechanism of MUC5AC gene expression for understanding tumorigenesis of gastric cancer.

A highly conserved region containing a Gli-binding sequence (HCR-Gli) is present in the promoter of MUC5AC gene

To examine the transcriptional regulation of MUC5AC gene, the upstream 5′-flanking regions of MUC5AC gene of 13 mammal species retrieved from GenBank (dated 8 May, 2013) were aligned using ClustalW (ver 2.1) sequence alignment program [30]. The major transcription initiation site of human MUC5AC gene has been mapped to 48 bp upstream of the ATG translation start site [31]. Alignment of the human sequence with other mammal sequences denoted high homology among orthologs within ~200 bp upstream of the ATG start codon (Fig. 2).

To test the function of the conserved promoter regions identified above, a series of luciferase reporter constructs containing 451 bp or shorter fragments of the human MUC5AC promoter region were generated. Promoter activities of these deletion constructs were measured by luciferase reporter assay in the three MUC5AC-expressing cell lines derived from human gastrointestinal cancer: SW480, SH-10-TG, and KE-39 [6]. As shown in Fig. 3A, the promoter activity of the reporter construct containing upstream 110 bp was much lower than those containing upstream 451, 280, 190, and 158 bp.

To examine the essential region for the luciferase activity between ~158 to ~110 bp more precisely, reporter constructs containing 150, 138, and 125 bp upstream of ATG start codon were generated and examined their activities (Fig. 3B). Although the reporter constructs containing upstream 125 bp showed high luciferase activities, the construct containing upstream 110 bp denoted very low activities equal to a negative control.

Next we made a deletion mutant construct containing upstream 451 bp but lacking the region between ~125 and ~111 bp, and examined the promoter activity (Fig. 3C). Deletion of the 15 bp led to a marked decrease in the luciferase activity, suggesting that the 15 bp sequence between ~111 to ~125 bp is critical for the MUC5AC promoter activity. Based on the thorough literature search [32,33,34,35,36,37,38], we found a putative Gli-binding site in this short 15 bp sequence (5′-GCCCTGCCACCCCAAC-3′ shown in Fig. 3D). We could not find any other transcription factors which bind to this 15 bp sequence by searching transcription factor database. Consequently, we named this 15 bp sequence as a highly conserved region containing a Gli-binding site (HCR-Gli).

Exogenous modulation of Gli affects the endogenous MUC5AC gene expression in gastrointestinal cells

We examined whether Gli1 can upregulate MUC5AC transcription activity in gastrointestinal cell lines. We constructed a retrovirus vectors carrying the Gli1 gene, and transduced it into four gastric (SH-10-TG, MKN1, NCI-N87, AZ-521), one colorectal (WiDr), and three other cancer cell lines (MDA-

Table 2. Primer pairs, annealing temperatures (Tm), and product sizes (Length) for the 5 genes analyzed by RT-PCR.

| Genes   | Forward (F) and reverse (R) primer sequences                      | Tm(°C) | Length |
|---------|------------------------------------------------------------------|--------|--------|
| MUC5AC  | F 5′-acgctgtcgagcaagaagcc-3′                                      | 64     | 396    |
|         | R 5′-acgggtgagctgctgcaat-3′                                      |        |        |
| Gli1    | F 5′-gagggatggtctgtgctgcaat-3′                                   | 60     | 491    |
|         | R 5′-gctctcgcctccctgctgctg-3′                                    |        |        |
| Gli2    | F 5′-gcaacagccgtctcccaatc-3′                                     | 60     | 430    |
|         | R 5′-atctcaacgctctcgtcatt-3′                                     |        |        |
| Gli3    | F 5′-gaagcaacagccgtcattc-3′                                      | 60     | 401    |
|         | R 5′-ggaatgcttgctgctgctgctg-3′                                   |        |        |
| GAPDH   | F 5′-acctcaccccttgtgaatc-3′                                      | 60     | 423    |
|         | R 5′-tccacacccctgtgaatc-3′                                       |        |        |

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MB435, T98G, A172). In the resulting stable transductants, the expression levels of MUC5AC, Gli1, and GAPDH were analyzed by RT-PCR (Fig. 4A). Although there were two exceptions of AZ-521 and WiDr, MUC5AC was found to be universally upregulated by Gli1 overexpression.

We next compared the three Gli homologs (Gli1, Gli2, and Gli3) identified in human. To examine the effect of these transcription factors on MUC5AC expression, AGS and SW480 cell lines transiently overexpressing Gli1, Gli2 and Gli3 were analyzed by RT-PCR (Fig. 4B). Gli1 strongly upregulated MUC5AC expression, which was consistent with the result of stable transductants in Fig. 4A. Gli2 and Gli3 also increased the MUC5AC expression, but their effects were weaker than Gli1.

To address whether Gli1, Gli2, and Gli3 activate the MUC5AC promoter through the 15 bp of HCR-Gli, we performed the luciferase reporter assay using the construct containing upstream 451 bp of the human MUC5AC gene and the deletion mutant lacking the HCR-Gli (Fig. 4C). In the Gli-transfected SW480, SH-10-TC, and KE-39 cells, all the three Gli homologs enhanced activation of the reporter construct containing upstream 451 bp of MUC5AC, but not of the deletion mutant lacking the HCR-Gli. These results indicate that Gli1, Gli2, and Gli3 can efficiently activate MUC5AC transcription through the HCR-Gli. From these results, we speculate that Gli1, Gli2 and Gli3 may play redundant roles but the effect of Gli1 is stronger than that of others upon the upregulation of MUC5AC expression.

We next examined the effect of Gli knockdown by siRNA transfection on MUC5AC expression. After SH-10-TC cells were transfected with Gli1 and/or Gli2 siRNA, the expression levels of MUC5AC, Gli1, Gli2, and GAPDH were analyzed by RT-PCR. As shown in Fig. 4D, double knockdown of Gli1 and Gli2 most efficiently decreased MUC5AC expression, whereas the effects of either Gli1 or Gli2 single knockdown were considerably weaker. These results suggest that Gli1 and Gli2 have redundant functions for MUC5AC expression, which is consistent with our above-mentioned speculation.

Gli interacts to HCR-Gli in the MUC5AC promoter region

To determine whether Gli directly binds to the MUC5AC promoter, chromatin immunoprecipitation (ChIP) assays were performed using anti-Gli1 antibody. As a negative control, the human villin1 promoter sequence was used because villin1 is a typical intestinal marker gene regulated by Cdx [8], and also because exogenous modulation of Gli1 expression level did not change endogenous villin1 expression (our unpublished observation). As shown in Fig. 4E, the HCR-Gli-containing fragment of MUC5AC promoter was co-immunoprecipitated with Gli1 in SW480 and KE-39 cells, whereas the upstream sequence of villin1 promoter was not. Our results demonstrate that Gli1 activates MUC5AC gene transcription through direct interaction between Gli1 and the MUC5AC promoter region.

Taking all the results from luciferase assays with upstream deletion constructs, overexpression and knockdown of Gli, and

Figure 1. Expression of MUC5AC in non-tumorous epithelial cells of atrophic mucosa around early gastric cancer endoscopically resected (A), malignant cells of early gastric cancer endoscopically resected (B), and malignant cells of advanced gastric cancer surgically resected (C). Based on the percentages of cells with immunoreactivity of MUC5AC, the 78 non-malignant or malignant specimens (A, B) and 89 malignant specimens (C) were classified into four categories: 1) < 10%, 2) ≥ 10% and < 50%, 3) ≥ 50% and < 80%, and 4) ≥ 80%.

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**Figure 2. Comparison of the 5’-upstream sequences of MUC5AC gene of 13 mammal species.** Alignment was carried out using the ClustalW (ver 2.1). The translational start site (TSS, arrow) and the translational starting codon (ATG, bold) are indicated. Asterisks (*) represent exact matches in all the sequences.

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Figure 3. Luciferase reporter analyses of a series of MUC5AC promoter constructs in gastrointestinal cell lines and identified highly conserved sequence similar to known Gli-binding sequences. (A–C) Data represent the mean of luciferase activities of SW480, SH-10-TC, and KE-39 cells measured at 24 h after transfection. The error bars showed the standard deviation of the results from three independent experiments. (D) Alignment of the highly conserved sequence in MUC5AC promoter region with previous reported sequences of Gli-binding site. doi:10.1371/journal.pone.0106106.g003
Figure 4. Effects of exogenous modulation of Gli on MUC5AC gene expression. (A) RT-PCR detecting MUC5AC mRNA in 5 gastrointestinal and 3 non-gastrointestinal cell lines infected with retroviral vector encoding Gli1 gene. (B) RT-PCR detecting MUC5AC mRNA in AGS and SW480 cells transfected with pcDNA3.1(+) (vec), pcDNA3.1(+) -Gli1, pcDNA3.1(+) -Gli2 or pcDNA3.1(+) -Gli3. (C) Luciferase reporter analysis of MUC5AC promoter constructs in SW480, SH-10-TC, and KE-39 cells transfected with pcDNA3.1(+) (vector), pcDNA3.1(+) -Gli1, pcDNA3.1(+) -Gli2 or pcDNA3.1(+) -Gli3. (D) MUC5AC expression analyzed by RT-PCR using SH-10-TC cells transfected with control siRNA, Gli1 siRNA alone, Gli2 siRNA alone, or Gli1 and Gli2 siRNAs.
Gli1 lines in which Gli1 and/or recognizing the promoter region of MUC5AC expression, at least partly based on the epigenetic modulation.

In total, expression patterns of MUC5AC and Gli clearly support our conclusion that Gli is necessary for MUC5AC expression in human gastrointestinal cells. In addition, it is also obvious that expression of Gli is not enough for MUC5AC expression, suggesting that other mechanism should work cooperatively on the transcription of MUC5AC gene.

Multiple regulatory mechanisms work in the promoter region of MUC5AC gene

To analyze the far distal promoter region of MUC5AC gene, we generated reporter constructs containing 4010, 3000, 2000, and 1433 bp upstream of ATG start codon and examined their promoter activities (Fig. 6A). Deletion from 4010 bp to 1433 bp region revealed gradual decrease in transcriptional activity, suggesting the presence of enhancer elements in this distal promoter region. However, even in the reporter construct including long 4010 bp, deletion of the 15 bp HCR-Gli region resulted in the drastic decrease of luciferase activity (Fig. 6A).

Together, our results demonstrated that there are at least two regulatory elements in the MUC5AC promoter region: the 15 bp sequence between −111 bp to −125 bp which critical for the promoter activity, and the wider regulatory region in −4010 bp to −1433 bp which can enhance the promoter activity.

To further examine the possible epigenetic regulation on MUC5AC expression, four gastrointestinal cell lines and two glioblastoma cell lines were treated with demethylating agent (5Aza-dC) and/or HDAC inhibitor (TSA). Transcription of MUC5AC was slightly upregulated in all the four MUC5AC-deficient cell lines (Fig. 6B), suggesting that epigenetic regulation such as methylation and/or histone acetylation additionally works as the regulatory mechanism of MUC5AC expression. From the results of four MUC5AC-deficient cell lines, methylation seems to play some universal role on suppression of MUC5AC expression. Synergistic effects of 5-Aza-dC and TSA in A172 and T98G cells also suggested that modification of histone acetylation may have some influence on MUC5AC transcription.

Discussion

MUC5AC, a secreted mucin highly detected in the superficial gastric epithelium, is a well-known gastric marker often used for classification of stomach cancer [20,39]. We have previously reported that expression of MUC5AC in stomach decreases in association with development of intestinal metaplasia [6]. In the present study, we further showed that MUC5AC expression is apparently related to the tumor stage: advanced gastric cancers present reduced levels of MUC5AC compared with early gastric cancer. Despite the anticipated importance of MUC5AC regulation, the molecular mechanisms underlying MUC5AC expression in gastrointestinal cells remain poorly understood. In this study, we searched 5’-upstream of MUC5AC gene and identified the HCR-Gli at −125/−111 bp in its promoter region. Our overexpression/knockdown analyses and luciferase reporter assays revealed that Gli induced MUC5AC expression through the HCR-Gli in gastrointestinal cells. In addition, ChIP analysis showed that Gli1 directly binds to HCR-Gli. The results of RT-PCR using 30 gastrointestinal tumor cell lines and an RNA panel from systemic normal tissues suggested that Gli is necessary but not sufficient for MUC5AC expression, which was consistent with the results from immunohistochemistry of clinical specimens. Furthermore, we found that the far distal upstream region from −1433 to −4010 bp enhances the Gli-dependent MUC5AC expression, at least partly based on the epigenetic modulation.

Recently, Inaguma et al. reported that Gli1 activates the MUC5AC promoter through two putative Gli-binding sites (GBS1 and GBS2) in pancreatic cancer cells [25]. However, the significance of MUC5AC expression is different in gastrointestinal cells and pancreatic cells. Namely, abundant expression of MUC5AC is observed in normal epithelial cells of stomach but its expression is decreased accompanied with development of intestinal metaplasia [14,20]. On the other hand, MUC5AC is generally undetectable in normal pancreas tissue and is sometimes ectopically induced in pancreatic tumor cells. In fact, there are two differences between Inaguma’s report and our results. First, Inaguma et al. showed that both GBS1 and GBS2 respond to upregulation of MUC5AC expression by Gli. The GBS1 overlaps to our identified HCR-Gli, but our results indicated that the GBS2 (corresponding to −159/−168 bp in Fig. 2) has no effect on MUC5AC expression (Fig. 3A). Second, Inaguma et al. reported that Gli1 expression correlates with MUC5AC induction by immunohistochemistry. However, our results revealed that expression of Gli does not always accompany with MUC5AC expression in gastrointestinal cells (Figure 5). These inconsistencies might be due to a difference of originated tissues (pancreatic or gastrointestinal).
No induction of MUC5AC expression in AZ521 cells (Fig. 4A), which completely lack the SWI/SNF complex-based chromatin remodeling activity [28], may suggest the interaction between Gli and the SWI/SNF complex. Although there have been no previous reports concerning the interaction with Gli, SWI/SNF chromatin remodeling complex has been reported to bind with various transcription factors such as AP-1 [40], CREB [41], MyoD [42], Cdx [8], GATA1 [43], and so on.
The 5’-upstream distal region from −1433 to −4010 bp in MUC5AC promoter showed obvious enhancing effect on MUC5AC expression (Fig. 6A). Interestingly, we noticed that the region from −1145 to −3187 bp contains 86 copies of tandem sequences 5’-TCA(C/T)TCA(C/T)-3’ (Fig. 6C) [44]. The tandem repeats are conserved among mammals especially among primate and cetartiodactyla. Human, olive baboon, rhesus monkey, killerwhale, and dolphin have more than 60 repeats of the tandem repeats in their 4010 bp upstream of MUC5AC promoter region. In contrast, horse, dog, cat, panda, and hamster have only less than 10 repeats of the tandem repeats. We have no information for this tandem repeat sequences at present, but it might has some roles on regulation of MUC5AC expression.

Recently, Yamada et al. showed that the CpG methylation status of around −3.7 kb in the MUC5AC promoter is associated with MUC5AC expression in some cancer cell lines, suggesting that the expression of MUC5AC is epigenetically regulated in the distal promoter region [45]. In fact, we found increase in MUC5AC expression by 5-Aza-dC and/or TSA treatment, indicating some epigenetic mechanism involved in MUC5AC gene regulation (Fig. 6C).

To understand gastric tumorigenesis mainly occurred in the atrophic mucosa with chronic gastritis and intestinal metaplasia, we believe the disrupted balance between intestinal and gastric differentiation must be important [8,28]. For intestinal differentiation, many previous reports indicated that Cdx must be an indispensable key molecule based on the transcriptional regulation of many intestinal differentiation marker genes [8,9,10,11,12]. On the contrary, the critical key molecule for gastric differentiation has not been identified yet. Despite the thorough screening, we have not found the essential regulator for our identified gastric marker Cathepsin E (data not shown) [6], and we have elucidated that typical gastric marker MUC5AC gene is partly regulated by Gli, a universal transcription factor which alone cannot realize the tissue-specific expression of MUC5AC gene. Unlike intestinal differentiation, we now speculate that gastric differentiation may depend on cooperative regulatory mechanism of some universal transcription factors and epigenetic modulations.

Figure 6. Multiple regulatory mechanisms of MUC5AC gene expression. (A) Luciferase reporter analysis of a series of constructs covering the far upstream sequence in the MUC5AC promoter. Data represent the mean of luciferase activities in SW480, SH-10-TC, and KE-39 cells measured at 24 h after transfection. The error bars showed the standard deviation of the results from three independent experiments. (B) Expression of MUC5AC analyzed by RT-PCR, using the 4 gastric cancer-derived and 2 glioblastoma-derived cell lines treated with 5-Aza-2’-deoxycytidine (5-Aza-dC) and/or trichostatin A (TSA). (C) Schematic representation of the presumed multiple regulations of human MUC5AC gene.
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In summary, the present study has shown that Gli regulates MUC5AC gene expression via direct protein-DNA interaction through the highly conserved 15 bp sequence between −125 and −111 bp in the promoter region of MUC5AC. Furthermore, immunohistochemical analysis and RT-PCR using systemic normal tissues reveals that Gli is necessary but not sufficient for MUC5AC expression. We conclude that MUC5AC expression is regulated by combination of multiple regulatory mechanisms such as universal transcription factors and epigenetic modulations.

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Conceived and designed the experiments: NY NK-Y KK. Performed the experiments: NK-Y NY Y. Takahashi CN KS MI MF Y. Tsutsumi MI. Analyzed the data: NK-Y NY Y. Takahashi CN KS KK. Contributed to the writing of the manuscript: NK-Y NY MF KK.

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