Promoter hypermethylation and histone hypoacetylation contribute to Pancreatic-duodenal homeobox 1 (*PDX1*) silencing in gastric cancer

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Abstract

Background and Aims: The expression of pancreatic-duodenal homeobox 1 (*PDX1*) in gastric cancer is aberrantly reduced. The aim of this study was to elucidate the regulation of DNA methylation and histone acetylation at the promoter for *PDX1* silencing in gastric cancer.

Methods: *PDX1* expression in response to demethylation and acetylation was detected in human gastric cancer cell lines by RT-PCR and Western blot. Four CpG islands within the 5'-flanking region of *PDX1* gene were analyzed with their transcription activities being detected by dual luciferase assay. Promoter hypermethylation was identified in gastric cancer cell lines and cancer tissues by methylation-specific PCR (MSP) or bisulfite DNA sequencing PCR analysis (BSP). Histone acetylation was determined by chromatin immunoprecipitation (ChIP) assay.

Results: Demethylation by 5-aza-2'-deoxycytidine (5'-aza-dC) and/or acetylation by Trichostatin A (TSA) restored *PDX1* expression in gastric cancer cells. Hypermethylation was found in four CpG islands in 6 of 7 cancer cell lines. However, only the distal CpG island located in the promoter fragment of *PDX1*, F383 (c.-2063 ~ -1681nt upstream of the ATG start codon) displayed significant transcriptional activity that could be suppressed by SssI methylase and increased by 5'-aza-dC and TSA. More than 70% of the single CpG sites in F383 were methylated with hypermethylation of F383 fragment more common in gastric cancerous tissues compared to the paired normal tissues (p<0.05). ChIP assay showed F383 was also associated with low hypoacetylation level of the histones.

Conclusion: Promoter hypermethylation and histone hypoacetylation contribute to *PDX1* silencing in gastric cancer.

Keywords: *PDX1*, gastric cancer, DNA methylation, histone acetylation

Abbreviation: *PDX1*, pancreatic-duodenal homeobox 1; 5'-aza-dC, 5'-aza-2'-deoxycytidine; TSA, trichostatin A; MSP, methylation-specific PCR; BSP, bisulfite DNA sequencing PCR analysis; ChIP, chromatin immunoprecipitation; DNMT, DNA methyltransferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; q-RT-PCR, quantitative real-time reverse transcription PCR; *USF1*, upstream stimulatory factor 1; *Ptf1α*, pancreas transcription factor 1α; *HNF3β*, hepatocyte nuclear factor 3β; IUGR, intrauterine growth retardation; HSS, nuclease hypersensitive sites.

Introduction

In mammals, epigenetic regulation including DNA hypermethylation and histone modification represents the major epigenetic mechanisms implicated in regulation of gene transcription [1]. DNA methylation within the promoter of tumor suppressor genes is a common phenomenon in cancer cells. It results in the transcriptional silencing of these genes and promotes tumor development [1-4]. Promoter hypermethylation has been reported to induce aberrant reduction of some homeobox genes such as Aristaless-like homeobox-4[5], *CDX1*[6-8] and *CDX2*[9] in cancers including squamous esophageal cancer, hematologic malignancies and colorectal cancers.

Histone acetylation/deacetylation alters the status of open chromatin domains and thus affects gene transcription. This process is modulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Loss of histone H3 and H4 acetylation attributes to the imbalanced recruitment of HDAC and results in transcription repression of tumor suppressors in cancers [10].

Pancreatic duodenal homeobox-1 (*PDX1*) is a homeobox gene that belongs to the ParaHox subfamily [11, 12]. *PDX1* plays a critical role in embryogenesis and tissue differentiation[13-18]. During the development of mice embryos, deletion or mutation of *pdx1* gene allele resulted in pancreatic malformation[17,18]. In *pdx1*-null mice embryos, the malformations of the stomach-duodenum junction caused the lack of gastric empty and subsequent stomach distension [16]. The cell-type specific expression of *PDX1* is controlled by 5'-flanking sequences that contain several

conserved promoter regions [19-21]. Area I (-2852 to -2547) and II (-2247 to -2071) direct endocrine cell expression [19,20] while area III (-1973 to -1694) directs the transient expression in β-cells [22, 23]. Our previous work has implicated *PDX1* as a putative tumor suppressor with reduced expression in gastric cancer [24,25].

In this project, we investigated the epigenetic regulatory mechanism of *PDX1* gene in gastric cancer. We showed that both promoter hypermethylation and histone deacetylation accounted for *PDX1* silencing. Unlike the responsive sequences around the translation starting codon identified in β-cells [26], a functional fragment, F383, located at area III (-2063 to -1681) mediated the epigenetic regulation of *PDX1* in gastric cancer cells.

Methods and Materials

Cell Lines and Tissue Samples

Human gastric cancer cell lines AGS, KATOIII, SNU1 were from the American Type Culture Collection (Manassas, VA). SGC7901 was from the Academy of Military Medical Science (Beijing, China), TMK1, MKN45 and BCG823 were maintained in our lab and described in the previous study [24, 27, 28]. All cell lines were grown as described previously[24]. 30 pairs of human gastric cancerous tissues with the adjacent normal tissues were collected from the specimen archives of the Department of Gastroenterology, the Affiliated Hospital of Sun Yat-sen University. CpGnome universal methylated DNA (Invitrogen, Carlsbad, CA) was used as the positive control for methylation.

Reverse Transcription Polymerase Chain Reaction

As per the manufacturer's protocol, Mini-RNease RNA extraction kit (Qiagen, Hilden, Germany) and Thermoscript RT system reagent (Gibco BRL, Gaithersburg MD) were used for RNA extraction and reverse transcription. PCR was performed using 2μl cDNA template, 0.2U Hot-start Taq DNA Polymerase ,forward and reverse primers and dNTPs Mix in a final volume of 20μl. The sequences of *PDX1* primers were shown in our previous publication [24]. And PCR was carried out for 35 cycle with 94℃ denaturation for 30 seconds, 56℃ annealing for 30 seconds and 72℃ elongation for 45 seconds.

Western Blot

Whole cell lysates were prepared and assayed for protein expression by western blot as reported previously[24]. Immunoblots were developed and visualized by the enhanced chemiluminescence (ECL) system (Amersham, Piscataway, NJ) according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the internal loading control.

5'-aza-dC and TSA Treatment

Cancer cells were seeded in 6-well plates at a density of 2 x 10^5 cells per well 12 hours before the drug treatment. The cells were treated with the following drugs alone or in combination: 5'-aza-dC (5, 10 or 20μM, Sigma, St Louis, MO) and TSA (500 nM, Sigma). Cells were harvested after 24 hours. Double diluted water (DDW) was used as the dissolvent control.

Construction of Putative Promoter Fragments

To indentify functional CpG islands and evaluate the association of DNA methylation and promoter transcription, the CpG islands of the *PDX1* promoter had to be identified. We searched *PDX1* genomic sequence at -2000nt to +600nt using Promoter Database (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home) and found four enriched CpG islands by MethPrimer (http://www.urogene.org/methprimer/index1.html). The four islands were respectively

located at -1975 to -1704 (island 1), -1226 to -1066 (island 2), -818 to -694 (island 3), and -112 to +521 (island 4) (Fig.2a). Then four fragments containing these putative CpG islands were amplified and inserted into the firefly luciferase reporter vector, pGL3basic (Promega, Madison, WI). A XhoI site was added into the 5' terminus of the forward primers and Hind III site was added to the reverse primer. With the upstream nucleotide adjacent to the ATG translation start codon, henceforth defined as c.1, the four reporter constructs were named F383 (-2063 to -1681), F314 (-1320 to -1007), F283 (-864 to -583) and F724 (-170 to +545), respectively. The cloned fragments were all verified by restriction enzyme digestion and sequencing. All primers for construction of the *PDX1* promoter were designed according to the genomic sequence of the *PDX1* gene (accession no. NC_000013.9) published in GenBank with their sequences being listed in Supplementary Table 1.

Transient Transfection, In Vitro Methylation, and Luciferase Reporter Assay

Reporter constructs F383, F314, F283 and F724 (0.8 µg/well) were transfected into cells pre-seeded into 24-well tissue culture plate by Lipofectamine 2000 (Invitrogen). Empty pGL3basic vector was used as control while PRL-CMV (0.008 µg /well, Promega) was used to normalize the reporter gene. 24 hours after transfection, cells were harvested and firefly luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega) with a model TD-20/20 Luminometer (EG&G Berthold, Australia). The value of firefly luciferase activity was normalized to that of renilla luciferase. Promoter activity was presented as the fold

change of relative luciferase unit (RLU) compared to the basic vector control. RLU equals the value of the firefly luciferase unit, divided by the value of the renilla. Each experiment was performed in triplicate, and at least 3 sets of independent transfection experiments were performed. SssI methylase, which is capable of non-specifically methylating all CpG dinucleotides, was used for the *in vitro* methylation of putative promoter fragments. In each case, 3 μg of plasmid DNA (triplicates in 24-well plate), pGL3basic and all reporter constructs were incubated in a 20μl reaction system containing 4 units of SssI methylase, 2μl of 20 x S-adenosylmethionine and 2μl of 10 x NEBuffer for 4 hours at 37℃ followed by 20 minutes at 65℃ to stop the reaction.

Genomic DNA Isolation, Bisulfite DNA Sequencing PCR Analysis (BSP) and Methylation Specific PCR Analysis (MSP)

Genomic DNAs were isolated from cells or resected specimens using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA samples were treated with sodium bisulfite to convert cytosine to uracil. Briefly, 1.0 μg of genomic DNA from each sample was denatured with 2 mol/L NaOH at 37℃ for 10 minutes, followed by incubation with 3 mol/L sodium bisulfate (pH 5.0, Sigma) at 50℃ for 16 hours, Two microliters of 20 μl bisulfite-treated DNA was amplified at 95℃ for 20 minutes and then 40 cycles at 94℃ for 30 seconds, 52℃ for 30 seconds, and 72℃ for 45 seconds and a final extension at 72℃ for 10 minutes using the bisulfate treated DNA sequencing PCR (BSP) primers (Supplementary Table 2). For cell lines, PCR products were purified and then inserted into pGEM-T

vector (Promega); 10-12 white clones were selected for each sample and then sequenced to determine the aberrant methylation of each CpG island. Moreover, 2 normal gastric tissues were used as normal controls.

Methylation status of human gastric cancerous tissues and paired normal tissues was evaluated by MSP analysis. After isolation and modification with sodium bisulfite, genomic DNA samples were amplified with methylation or unmethylation specific primers with their sequences listed in supplementary table 2. The total 25μl PCR reaction contained 10pmol/L primers, 25μmol/L deoxynucleoside triphosphates, 2μl of bisulfate-treated DNA, 0.5U of hot-start Taq polymerase, and the respective buffers. The condition for PCR amplification was as follows: 20-minute hot start at 95℃ followed by 40 cycles at 94℃ for 30 seconds,52℃ for 30 seconds,72℃ for 45 seconds and a final extension at 72℃ for 10 minutes. PCR products were visualized on a 2% agarose gels stained with ethidium bromide. Moreover, a normal gastric tissue was used as normal control.

Chromatin Immunoprecipitaion (ChIP) and Quantitative Real-time Reverse Transcription PCR (q-RT PCR)

Evaluation of histone acetylation levels was performed using a ChIP assay with a commercially available kit (Upstate Biotechnologies, Charlottesville, VA) according to the manufacturer's protocol. Approximately 2 x10^6 cells were treated with 1% formaldehyde to cross-link the protein to DNA for 15 min at 37℃. After washing, the cell pellets were resuspended in lysis buffer and sonicated to yield an average DNA

size of about 500bp. The sonicated cells were subsequently deposited by centrifugation and diluted with ChIP dilution buffer. 20μl of the diluted lysates was left as the input control. Samples were incubated with protein A-Sepharose and sonicated salmon sperm DNA for 1 hour at 4℃, then incubated with 5μg specific antibodies against acetyl-histone H3 (lys 9 and 14) and acetyl-histone H4 (lys 5, 8, 12 and 16) (Upstate Biotechnology), or normal rabbit IgG (Santa Cruz Biotechnology) overnight at 4℃ followed by incubation with Protein A-Sepharose for 1 hour. After washing, the−sepharose/antibody/histone complexes were eluted with elution buffer (1% SDS and 0.1 M NaHCO₃). The cross-linking was then reversed by 5M NaCl at 65℃ for 4 hour. The DNAs were extracted with phenol/chloroform, precipitated with ethanol and applied for quantitative analysis by q-RT PCR.

q-RT PCR reactions were performed for 60 cycles with 94℃ for 15 seconds,58℃ for 15 seconds, and 72℃ for 40 seconds by using Applied Biosystems Sequence Detection System 7900 (ABI Prism 7900HT, Applied Biosystems, California, USA) in 20μl mixture including 10μl power SYBR GREEN PCR Master Mix (Applied Biosystems, Foster City, CA). Inspection of melt curves and *CT* values generated were used for quantification of the copy numbers of four fragments and the results were expressed as the fold change compared to that obtained with IgG. The primer sequences are listed in Supplementary Table 3. The amount of immunoprecipitated DNA was normalized to the input DNA. Each treatment was set up in triplicate, and two independent ChIP experiments were performed.

Statistical Analysis

The results of luciferase activities are expressed as mean ± SEM. Student t test and Fisher's Exact Test were used to determine the statistical significance between different groups by the statistical software, SPSS13.0**.** A p value of less than 0.05 was considered significant.

Results

Restoration of *PDX1* expression in cancer cells by demethylation and acetylation

We have reported that *PDX1* expression in gastric cancer cells is down-regulated [24]. To examine if epigenetic regulation was involved in *PDX1* silencing, we first treated 7 gastric cancer cell lines with 5'-aza-dC. As shown in Figure 1A, the endogenous mRNA expression of *PDX1* in gastric cancer cells was weak and partly up-regulated by 5'-aza-dC in these cell lines. Similarly, *PDX1* expression could be up-regulated by the HDAC inhibitor, TSA (Figure 1B). Furthermore, 5'-aza-dC and TSA could increase *PDX1* expression in a dose dependent manner (Figure 1C). To evaluate the interaction of 5'-aza-dC and TSA on *PDX1* expression, we randomly chose the two cell lines TMK1 and KATOIII. As shown in Figure 1D and E, a slightly synergistic effect was observed when cells were treated with combination of 5'-aza-dC and TSA. These results suggested that *PDX1* expression in gastric cancer cells was modulated by epigenetic regulation that included both DNA methylation and histone deacetylation.

Hypermethylation of *PDX1* promoter in gastric cancer cell lines

To define the putative responsive sequence for epigenetic regulation, we first searched a 2600bp genomic sequence including the ATG translation starting codon (-2000nt to +600nt) of the *PDX1* gene using Methprimer software (*http://www.ucsf.edu/urogene /methprimer*). Four putative CpG islands were found located at -1975nt to -1704nt, -1226nt to -1066nt, -818nt to -694nt and -112nt to +521nt, respectively (Figure 2A).

We then examined the methylation status of these CpG islands in 7 gastric cancer cell lines by MSP assay. Complete methylation in all four CpG islands was found in SGC7901, AGS, TMK1 and SNU1 cells while partial methylation was found in all CpG islands except F383 in BCG823 and MKN45 cells. However, we showed no DNA methylation of all four islands examined in KATOIII cells as well as in normal gastric tissue (Figure 2B). To confirm the accuracy of the MSP methodology, we treated TMK1 cells with 5'-aza-dC. We found that the complete methylation in all four CpG islands was successfully reversed into partial methylation (Figure 2C). These results suggested that hypermethylation of *PDX1* promoter existed in most of gastric cancer cell lines.

Identification of a functional CpG island within the 5'-flanking region of *PDX1* gene

To determine if the sequences contained by CpG islands were critical for *PDX1* transcription, we cloned these fragments into pGL3basic vector to generate 4 luciferase reporter constructs, F383, F314, F283 and F724. After transient transfection into cells, a dual luciferase assay showed that F383, the most distal fragment displayed substantial promoter activity in all three cell lines tested (Figure 3A). This fragment was included in promoter Area III described previously (19, 20). The RLU of F383 in AGS, TMK1 and KATOIII cells were 14.37 ± 3.38, 14.51 ± 4.12 and 11.32 ± 0.17, respectively. On the contrary, the transcription activities of F314, F283 and F724 were not significantly different from that of pGL3basic vector (Figure 3A).

Subsequently, we evaluated the effect of *in vitro* methylation and demethylation on transcription activity of F383. As shown in figure 3B, *in vitro* methylation by treatment with SssI methylase reduced the promoter activities of F383 by 0.52 ± 0.05 times in AGS cells ($p<0.01$), 0.68 ± 0.001 times in TMK1 cells ($p<0.05$) and 0.25 ± 0.03 times in KATOIII cells ($p<0.001$). Consistent with this, *in vitro* demethylation by pretreatment with 5'-aza-dC increased the promoter activities of F383 to 1.47 ± 0.10 fold in AGS cells ($p<0.001$), 1.02 ± 0.08 fold in TMK1 cells ($p>0.05$), and 1.01 ± 0.06 fold in KATOIII cells ($p>0.05$). The promoter activity of F383 was slightly increased in TMK1 cells might attribute to non-specificity of 5'-aza-dC treatment. However, the promoter activity of F383 didn't changed with 5'-aza-dC because none of the four CpG islands studied are methylated in the KATOIII cells. In general, these results indicate that the distal CpG island located at -1975nt to -1704nt, contained by F383, was not only critical for *PDX1* transcription but also the responsive sequence for DNA methylation.

Hypermethylaiton of CpG dinucleotides within F383 fragment

Next, we analyzed the methylation status of CpG dinucleotides within the F383 fragment using BSP analysis. As shown in figure 4A (wildtype sequence), 17 CpG sites were presented in this fragment. All of them were candidate sites for methylation since none of them was altered into T after bisulfite modification (Figure 1A, modified sequence). Again no methylation was found in all 17 CpG sites in KATOIII cells, and all other cell lines displayed high levels of methylation in the 17 CpG sites.

As low as 1.96% ± 1.24% and 0.98% ± 0.97% of methylation were found in the two normal tissues (p<0.0001 compared to cancer cell lines, Figure 4B and C). The detailed methylation ratios for the 17 CpG dinucleotides in 7 gastric cancer cell lines were listed in supplement table 4. Only a few showed methylation at the third (8.3%) and the 13th CpG site (16.7%) in the two normal gastric tissues, however, the significant higher methylation ratios for the 17 CpG dinucleotides in 7 gastric cancer cell lines were examined (Supplement table 4).

Aberrant hypermethylation of F383 in gastric cancerous tissues

To validate our *in vitro* findings, we analyzed the methylation status of F383 in 30 pairs of gastric cancerous tissues and their matched adjacent normal tissues by MSP assay(Figure 5). In adjacent normal tissues, F383 was unmethylated in 16 (53.3%) samples and partially methylated in another 14 (46.7%) samples. In gastric cancer, F383 was completely methylated in 3 (10%) samples and partially methylated in another 27 (90%) samples (vs adjacent normal control, p<0.001). Further analysis indicated that the 3 gastric cancer tissues with complete methylation were all intestinal type and at advanced stages.

Role of histone acetylation in *PDX1* transcription

To characterize the role of acetylation in *PDX1* silencing, we detected the association between *PDX1* promoter and acetylated histones using ChIP assay followed by q-RT-PCR. We showed that F314, F283 and F724 fragments, which had no obvious

promoter activity (Figure 3A), could all bind to acetylated histone H3 and H4,most notably in TMK1 cells (Figure 6A). However, the association between these fragments and acetylated histones in KATOIII cells were at low levels (Figure 6A). This finding indicated the presence of hypoacetylation in KATOIII cells but not in TMK1 cells in these promoter areas. Regarding F383, its association with acetylated histone H3 or H4 were absent or only at low levels in both cell lines (Figure 6A), suggesting F383 is a common fragment for deacetylated regulation in both cell lines. To further confirm this finding, we examined the association between acetylated histones and F383 in cells treated with TSA. We found that the level of histone H3 and histone H4 associated with F383 was significantly increased by TSA. The fold change of acetylated H3 and H4 were 249.60 ± 17.27 and 1.84 ± 0.09 in TMK1 cells and 3.92 ± 0.11 and 4.06 ± 0.11 in KATOIII cells ($p<0.05$, Figure 6B). Finally, we treated the reporter constructs of F383 with TSA in $vitro$. We showed that TSA upregulated the transcription activity of F383 to as high as 8.70 ± 0.04 folds in KATOIII cells ($p<0.001$, Figure 6C). These findings suggest that this fragment is an important responsive sequence for histone deacetylation.

Discussion

PDX1 is a critical regulator for the development and differentiation of some endocrine tissues. Several transcription factors such as *USF1* (upstream stimulatory factor 1) [29], *HNF3β* (hepatocyte nuclear factor3β) [19] and *Ptf1α* (Pancreas transcription factor 1α) [21,22] were capable of stimulating *PDX1* transcription in β-cells [19-23, 29]. Here we reported that promoter methylation and histone acetylation accounted for *PDX1* gene silencing in gastric cancer. A functional fragment, F383, located within the *PDX1* promoter area III (-2063 to -1681) mediated the epigenetic regulation of *PDX1*. *PDX1* expression could be effectively restored by reversing promoter methylation and histone deacetylation. This supports our previous finding that *PDX1* was a novel tumor suppressor for gastric cancer [24].

Typical promoter methylation is restricted to cytosines in CpG dinucleotides located within CpG islands. Through bioinformatic analysis, we identified four putative CpG islands. Island 1 (-1975 ~ -1704), 2 (-1226 ~ -1066) and 4 (-112 ~ +521) were all located within or near the three nuclease hypersensitive sites (HSSs) [19,20]. In previous studies the HSS sites were located between -2560 and -1880 (HSS1), -1330 and -800 (HSS2), and -260 and +180 (HSS3), respectively [19,20]. Thus, these fragments should be the acting fragments for *PDX1* transcription. Similarly, MSP also revealed complete methylation can be seen in tumor cells, albeit only a small percentage, however it not at all seen in normal cells. Also completely unmethylated F383 could be seen in normal cells but not at all seen in tumor cells. Partial methylation was much higher in tumor as compared to normal cells. This speaks to

the process by which aberrant methylation occured in cancer development continuum, supporting it was a well candidate for epigenetic regulation. This finding was partially supported by a recent publication in which a proximal CpG island (-360 to +200) was methylated and responsible for the *pdx1* permanent silencing in β-cells of rat with IUGR [26].

To further assess the biological function of these CpG islands, we evaluated the transcriptional activities of reporter constructs. Only the most distal island (F383) displayed significant promoter activity. F314, F283 and F724 were associated with histone hypoacetylation in KATOIII cells but exerted no transcriptional activities. This suggested that the epigenetic modifications in CpG island 2, 3 and 4 were not essential for *PDX1* transcription. There were three possible interpretations for this phenomenon. Firstly, island 2, 3 and 4 were located beyond the active promoter area of *PDX1* while F383 was located within the well-defined promoter fragment, area III, described previously [19,20]. Secondly, **i**sland 2, 3 and 4 were located in the center of HSS2 and HSS3 while HSS sites were most likely occupied by non-histone proteins (transcription factors). This point was supported by our finding that TSA treatment could only increase the transcription**al** activity of F383 but not the other three fragments. Lastly, the transcription factors might antagonize the suppressive effect of epigenetic regulation. Although the transcription factors for islands 2 and 3 were unknown, a positive regulator *USF1* could bind to its responsive element located at island 4 to stimulate *PDX1* expression in β-cells [29]. A recent study reported recruitment of the histone deacetylase 1 (HDAC1) and deacetylation of histones H3 and H4 were associated with loss of USF-1 binding at the proximal promoter of *Pdx1* and subsequently resulted to *pdx1* silencing in the rat fetal with IUGR [26].

Nevertheless, The CpG island at F383 fragment must not be the only methylated functional sequence of *PDX1* gene. No methylation was found in this island in KATOIII cells, and promoter activity of F383 didn't significantly regulated by SssI

methylase or 5'-aza-dC. However, *PDX1* expression in this cell line could be upregulated as well by 5'-aza-dC and/or TSA (Figure 1). We proposed that the effect may be mediated by the methylation of scatter single CpG sites we have not defined here. Similarly, our previous study reported that single CpG site but not CpG island attributes to *XAF1* transcription silencing in gastrointestinal cancer cells[28]. Further, F383 might be not the only promoter sequence regulating *PDX1* expression in KATOIII cells. Recent study reported that conserved area IV at -6200nt to -5670nt from 5'-flanking sequence also regulated *pdx1* transcription [21]. Area IV was an upstream control region in the rat *pdx-1* gene located between -6200nt and -5670nt, which is also present in the human gene. So area IV might be a putative promoter, containing a hypermethylated CpG island and regulate *PDX1* expression in KATOIII cells.

In conclusion, epigenetic regulation including promoter methylation and histone deacetylation contributed to *PDX1* silencing in gastric cancer cells with the predominant responsive sequences (-2063 to -1681) localising to the *PDX1* promoter area III.

Acknowledgement

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Figures Legends

Figure 1. Restoration of *PDX1* expression in cancer cells by demethylation and acetylation. A, gastric cancer cell lines were treated with double diluted water (DDW),the dissolvent control (C) or 5μM 5'-aza-dC (A) for 24 hours. **B,** gastric cancer cell lines were treated with DDW (C) or 500nM TSA (T) for 24 hours. *PDX1* mRNA expression was detected by RT-PCR with *GAPDH* being used as the internal control. **C,** Cancer cells were treated with the indicated concentration of 5'-aza-dC or TSA for 24 hrs, *PDX1* and *GAPDH* expressions were detected by RT-PCR. **D & E,** TMK1 and KATOIII cells were treated with 5'-aza-dC and TSA alone or in combination for 24 hours, *PDX1* mRNA and protein expressions were detected by RT-PCR (**D**) and western blot (**E**). *GAPDH* was used as an internal control. All of these pictures were representatives of three to four independent experiments with similar findings.

Figure 1
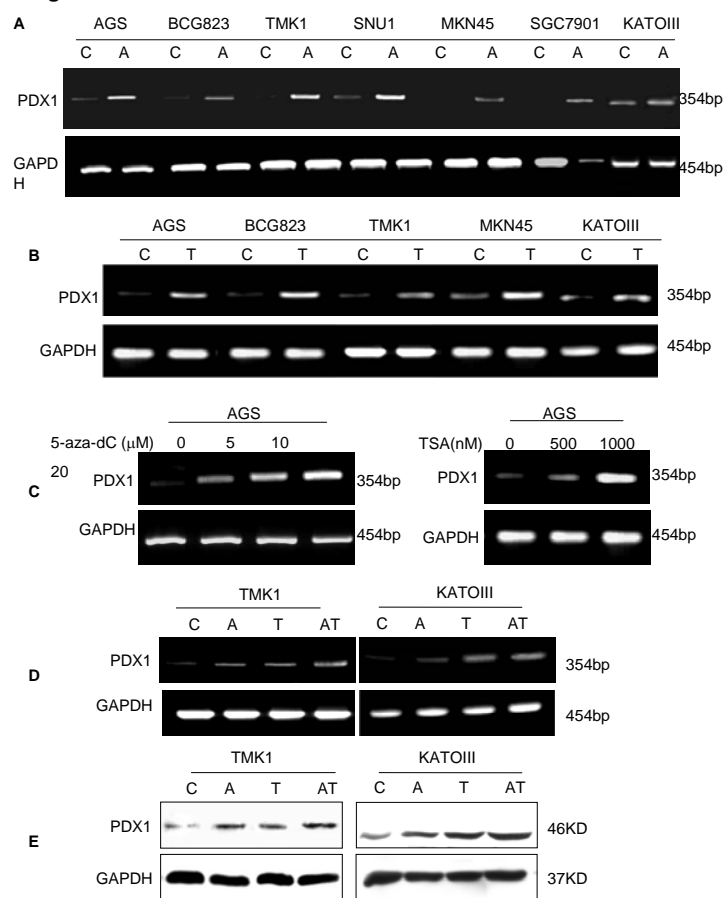
Figure 2. Hypermethylation of CpG islands of the *PDX1* gene in cancer cell lines.

A, The schematic diagram of putative CpG islands within the *PDX1* promoter identified by bioinformatics analysis. The axis is the percentage of the dinucleotide, guanine and cytosine. The genomic DNA sequences (-2000nt to +600nt) of *PDX1* was analyzed by Methprimer software. Locations of 4 putative CpG islands were displayed in areas with gray shadow. **B,** MSP result of four putative CpG islands in 7 gastric cancer cell lines and one normal gastric tissue. M, methylated; U, unmethylated. **C,** Alteration of methylation status in TMK1 cells induced by 5'-aza-dC.
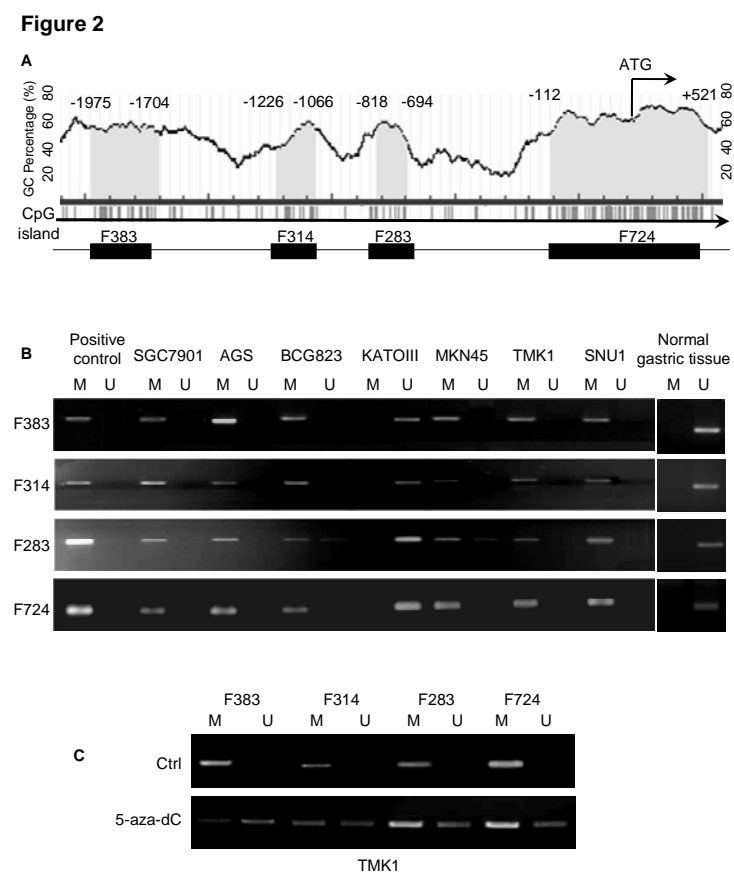
Figure 2

Figure 3. Identification of a functional CpG island within the 5'-flanking region of the *PDX1* gene. A, The four reporter constructs were transfected transiently into AGS, TMK1 and KATOIII cells and dual luciferase assays were performed to assess their transcriptional activities. Firefly luciferase activity were normalized to renilla activity. Promoter activity was presented as the fold change of relative luciferase unit (RLU) compared to the pGL3basic vector. RLU equals the value of the firefly luciferase unit, divided by the value of the renilla. This result was expressed as the mean of triplicate treatments ± standard deviation. **B,** Reporter construct F383 was transfected into cells in absence or presence of SssI methylase (4U) and 5'-aza-dC. (5μM). Transcriptional activities were assessed by dual-luciferase assays (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs control). The RLUs of SssI methylase and 5'-aza-dC were normalized to that of the non-treatment control (Ctrl). All of these experiments were repeated for three to four times with identical results.

Figure 3
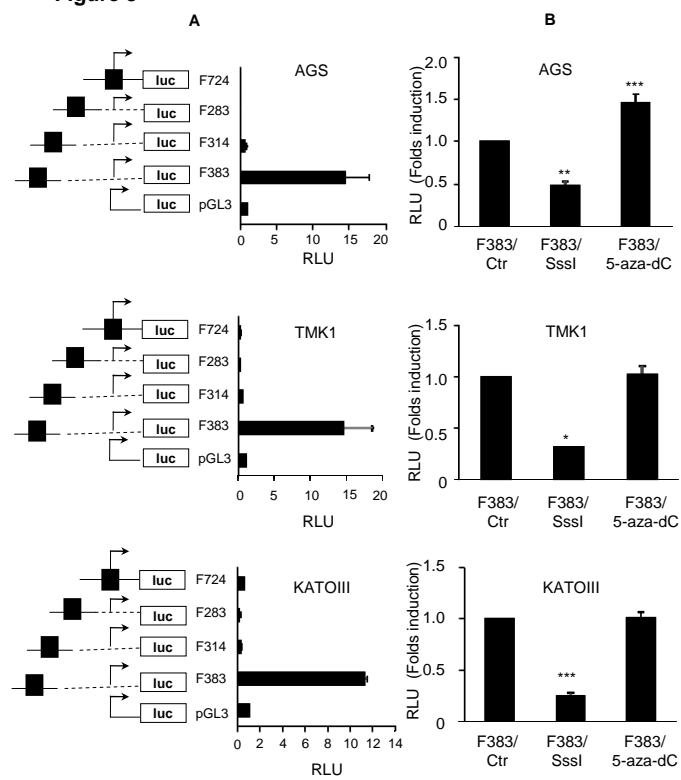
Figure 4. Methylation status of CpG sites within the functional promoter fragment. Fragment F383 was amplified using bisulfite-modified genomic DNA as template and inserted into pGEM-T4 vector. Ten to 12 white clones for each cell line were selected for sequencing. **A,** Wildtype and modified sequence of F383 in BCG823 cells. The BSP primer sequences were underlined and the methylated CpG dinucleotides that were unable to be altered by bisulfite modification were labeled in bold and italicise. **B,** Methylation status of the 17 CpG dinucleotides within F383 fragment in cancer cell lines. **C,** Percentage of methylated CpG sites within F383 in cancer cell lines. 1N & 2N, normal gastric tissues. *p < 0.0001 vs either 1N or 2N.
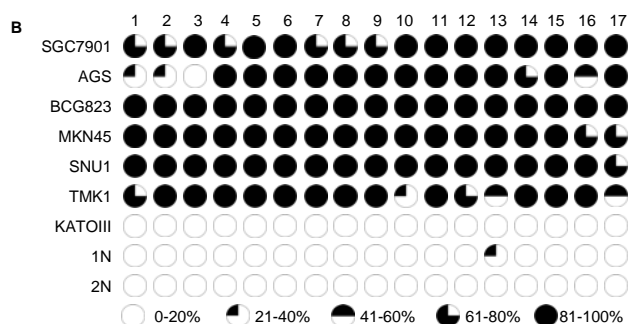

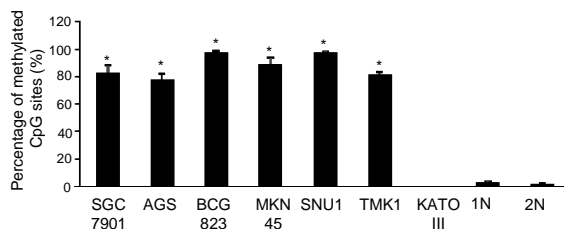
Figure 4

Figure 5. Hypermethylation of the *PDX1* promoter in gastric cancerous tissues. A,

The methylation status of F383 in 30 pairs of gastric cancer (T) and their adjacent

normal (N) tissues was analyzed by MSP. M, methylated; U, unmethylated. PC,

positive control. These figures were the representatives of three independent

experiments using the same DNA sample with repeatable results. **B,** Percentage of

methylated F383 fragment in gastric cancerous tissues and their adjacent normal

tissues, $p < 0.001$ between cancerous and normal tissues.

Figure 5

Figure 6. Role of histone acetylation in *PDX1* transcription. A, Cross-linked and sonicated lysates of TMK1 and KATOIII cells were immuno-precipitated with anti-acetyl-histone H3 and H4 antibodies. Different promoter fragments of *PDX1* were amplified quantitatively by q-RT-PCR. Rabbit IgG was used as the antibody control. The result was expressed as the fold induction compared to that of IgG (* $p < 0.05$, **$p < 0.01$ vs IgG control). **B,** TMK1 and KATOIII cells were treated with TSA (500nM) or DDW, the dissolvent control (Ctrl). *PDX1*-associated histone acetylation was detected by ChIP and q-RT-PCR (* $p < 0.05$, **$p < 0.01$, compared to ctrl). **C,** F383 was transfected into KATOIII cells in presence of TSA (500 nM) or the dissolvent control (Ctrl). Transcriptional activities were assessed by dual-luciferase assay (*** $p < 0.001$, vs control).

Figure 6

A

B

C