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doi: 10.1016/j.bbrc.2020.03.120

Methylation-mediated silencing of the LIM homeobox 6 (*LHX6*) gene promotes cell proliferation in human pancreatic cancer

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ABSTRACT

Epigenetic gene silencing by aberrant DNA methylation leads to loss of key cellular pathways in tumorigenesis. DNA methylation-mediated silenced genes in pancreatic cancer were searched for using the methyl-CpG targeted transcriptional activation (MeTA) method, and *LHX6* (LIM homeobox 6), a transcription factor involved in embryogenesis and head development, was selected as a strong candidate gene. *LHX6* was downregulated in most of the pancreatic cancer cell lines (83%, 10/12), mainly through promoter hypermethylation and histone deacetylation. Furthermore, *LHX6* was methylated in primary pancreatic cancer specimens (57%, 16/28) in a tumor-specific manner. Re-expression of *LHX6* inhibited colony formation and proliferation in *LHX6* low-expressing pancreatic cancer cell lines, PK-1 and PK-9. In contrast, knockdown of *LHX6* accelerated cell proliferation in *LHX6* high-expressing pancreatic cancer cell lines, PCI-35 and MIA PaCa-2. In order to analyze LHX6 downstream genes, we performed microarray analyses using LHX6 inducible PK-1 and PK-9 and found that LHX6 induction upregulated several genes that had tumor suppressive functions. Among these, we focused on *TFPI2* (Tissue factor pathway inhibitor-2) and found that *TFPI2* was greatly downregulated in all twelve pancreatic cancer cell lines. Our present results suggest that epigenetic inactivation of *LHX6* plays an important role in pancreatic tumorigenesis by promoting cell proliferation through aberrant transcriptional regulation of several cancer-related genes.

Keywords: LHX6, tumor suppressor, pancreatic cancer, DNA methylation, *TFPI2*

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer-related deaths with an overall 5-year survival rate of less than 8% in Japan [1]. The late onset of symptoms and aggressive tumor biology of this disease mean that only a minority (10-15%) of patients can undergo potentially curative surgery [2]. Therefore, establishment of effective strategies including identification of new biomarkers is urgently needed to improve survival rates through early detection [3]. DNA methylation is especially useful as a promising source of tumor biomarkers because of its stability and tumor specific alteration [4].

We previously developed our invented MeTA (methyl-CpG targeted transcriptional activation) method [5,6] to search for DNA methylation-mediated silenced genes in cancer. In MeTA, cancer-related genes transcriptionally silenced by promoter hypermethylation can be reactivated by the introduction of the plasmid which carries a fusion gene comprised of methyl-CpG binding domain (MBD) and transcription activation domain (TAD) into cancer cells [5]. MBD specifically binds to the promoter regions of hypermethylated genes and TAD reactivates epigenetically silenced genes [7]. Because microarray coupled with MeTA (MeTA-array) searches for hypermethylated genes by a completely different mechanism than DNA demethylating agent-based method, it enables identification of hypermethylated genes that were difficult to find by the previous conventional methods. In a series of our

previous studies, we applied MeTA to three pancreatic ductal carcinoma cell lines as well as a normal pancreatic ductal epithelial cell line HPDE and found three hypermethylated genes (*CSMD2*, *SLC32A1*, and *TRH*) [8]. In order to further search for commonly hypermethylated and silenced genes in pancreatic cancer, we examined twelve pancreatic cancer cell lines by MeTA and selected six genes (*IRX4*, *LHX6*, *NEFL*, *NEFM*, *NEFH*, and *NPTX2*) (MS in submission).

In this study, we focused on *LHX6,* because its hypermethylation has been reported and is a potential tumor suppressor gene in some tumors such as glioma [9], breast [10], lung [11], cervical [12], and head and neck [13] cancers. LHX6 has tandem LIM domains which confer specific protein-protein interactions and a homeodomain which has the ability to bind DNA [14]. LHX6 functions as a transcription factor and plays critical roles in cell fate determination during development, particularly, in the central nervous system (CNS). Although LHX6 has been suggested to affect the Wnt/β-catenin pathway [10,15] in breast and lung cancers or the PI3K/Akt/mTOR signaling pathway [16] in breast cancer, the molecular mechanisms of the transcriptional regulator are not known. In this study, we found *LHX6* to be one of the hypermethylated candidate tumor suppressor genes in pancreatic cancer and tried to elucidate its biological function in pancreatic tumorigenesis.

2. Materials and methods

2.1. Cell culture

A total of 12 human pancreatic cancer cell lines were used: AsPC-1, MIA PaCa-2, PANC-1, and BxPC-3 were purchased from American Type Culture Collection (ATCC), and the remaining 8 cell lines (PK-1, PK-8, PK-9, PK-45P, PK-45H, PK-59, PCI-6, PCI-35) have been described previously [17]. An immortalized human pancreatic ductal epithelial (HPDE) cell line was also used as the control. HPDE was a kind gift from Dr. M-S Tsao (University of Toronto, Canada) [18] and was maintained in Keratinocyte Serum Free Medium supplemented with bovine pituitary extract and epidermal growth factor (Invitrogen).

2.2. Quantitative reverse transcription PCR (qRT-PCR)

Total RNAs were isolated using a FastGene RNA Basic Kit (NIPPON Genetics). cDNA synthesis and qRT-PCR were carried out as described previously [19]. Independent triplicate assays were performed, and nucleotide sequences for primers and probes are shown in Table S1; we designed them to detect all six *LHX6* variants.

2.3. Sodium bisulfite sequencing

Genomic DNAs were extracted using DNeasy Blood and Tissue Kit (Qiagen) and bisulfite-treated with EpiTect Bisulfite Kit (Qiagen). Primers used for bisulfite sequencing were listed in Table S1.

2.4. Methylation-specific PCR (MSP)

The methylation status of *LHX6* was determined by methylation specific PCR (MSP) [20]. The methylated and unmethylated band intensities were quantified by ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) [21] and each methylated-to-unmethylated ratio was calculated; two-fold or more in tumor compared with corresponding normal sample was defined as tumor-specific methylation. PCR products were separated in a 4% agarose gel. PCR products encompassing *LHX6* promoter regions treated with or without *Sss*I methylase (New England Biolabs) were used as templates for fully methylated or fully unmethylated controls, respectively. Primers used for MSP analyses are listed in Table S1.

2.5. 5-aza-2'-deoxycytidine and trichostatin A treatments

Twelve pancreatic cancer cell lines and HPDE cells were treated with 5-aza-2'-deoxycytidine (5-Aza-dC, Sigma‐Aldrich) and trichostatin A (TSA, Wako) according to methods described previously [8].

2.6. Tissue specimens

Primary pancreatic cancers and their corresponding normal tissue counterparts were obtained at surgery from a total of 28 patients with pancreatic cancer at Tohoku University Hospital (Sendai, Miyagi, Japan). These specimens were collected between January 2005 and September 2009, and their clinicopathologic characteristics are summarized in Table S2. The tissue specimens were snap frozen in liquid nitrogen after resection and stored at -80ºC. Written informed consent was obtained from all patients. This study was approved by the Ethics Committee of Tohoku University School of Medicine (2015-1-473 and 2015-1-474).

2.7. Strain and plasmids

E. coli strain DH5αF' was used for plasmid preparation. To construct the *LHX6* expression vector, two DNA fragments, *LHX6* cDNA from the ProQuest two-hybrid human fetal brain cDNA library (Invitrogen) and 3xFLAG tag from p3xFLAG-CMV-10 (Sigma-Aldrich), were cloned into pcDNA6/myc-His (Invitrogen) vector. To allow tetracycline-regulated expression of *LHX6*, the 3xFLAG-*LHX6* fragment was cloned into the pcDNA4/TO/myc-His (Invitrogen) vector to generate pcDNA4/TO/3xFLAG-*LHX6*. Nucleotide sequences of the PCR primers are summarized in Table S1.

2.8. Colony formation assay

PK-1 and PK-9 cells were seeded in 6-well tissue culture dishes and transfected with 4 μg of pcDNA6 empty vector or pcDNA6-3xFLAG-*LHX6* using Lipofectamine 2000 reagent (Invitrogen). Transfected cells were cultured in the medium containing 5 μg/ml of blasticidin and colony formation assay was carried out as described previously [22].

2.9. Establishment of stable cell lines

PK-1 and PK-9 cells were used to allow tetracycline-regulated *LHX6* expression using the T-REx

system (Invitrogen) as described previously [23]. PK-1_TR4 and PK-9_TR4 parental cells were first selected as suitable cells by immunoblotting experiment using the monoclonal TetR antibody (Clone 9G9; 1:1000, Clontech Laboratories). Second, pcDNA4/TO/myc-His vector or pcDNA4/TO/3xFLAG-*LHX6* plasmid was transfected into PK-1_TR4 or PK-9_TR4 parental cells to give six cell lines; PK-1_Vec#2, PK-1_LHX6#10, PK-1_LHX6#13, PK-9_Vec#5, PK-9_LHX6#4, and PK-9_LHX6#9. PK-1-derived cells were maintained in RPMI-1640 medium containing 5 μg/ml blasticidin and 125 μg/ml zeocin, and PK-9-derived cells were maintained in RPMI-1640 medium containing 5 μg/ml blasticidin and 250 μg/ml zeocin.

2.10. Immunoblotting

Immunoblotting experiments were performed as described previously [24]. An antibody that specifically recognizes FLAG (F 1804; 1:1000, Sigma-Aldrich) or β-actin (A-5441; 1:3000, Sigma-Aldrich) was used.

2.11. Cell proliferation assay

Tetracycline-regulated cell lines were seeded in 24-well tissue culture dishes at a density of $3x10^3$ cells/well one day before the assay started: day (-1). After the first assay, cells were treated with or without 1 μg/ml tetracycline for 2, 4, and 6 days. Cell viability was assessed by alamarBlue assay (Invitrogen) as described previously [19]. Experiments were performed in quadruplicate and repeated three times.

2.12. Knockdown of LHX6

The sense sequence (5'-CTACGACACCATGATTGAGAACCTC-3') corresponding to nucleotide positions 639 to 663 relative to the start codon was chosen as a short interfering RNA (siRNA) targeting *LHX6*, and a double-stranded siRNA was purchased from Integrated DNA Technologies. Cells were seeded on 6-well tissue culture dishes and transfected with *GL2* siRNA (a negative control targeting firefly luciferase) [22] or *LHX6* siRNA at a final concentration of 1.25 nM, respectively. Knockdown was performed according to the method described previously [25].

2.13. Microarray analysis

Microarray analyses were performed according to methods described previously [8]. Total RNAs from PK-1_LHX6#13 and PK-9_LHX6#4 cells treated with or without 1 μg/ml tetracycline for 4 days were purified, and Cy-3 labeled cRNAs were prepared for hybridization to Agilent Human Gene Expression 4x44K v2 Microarray Kit. The cut-off value for two-fold upregulation or downregulation was employed for selection of the genes. Results of microarray analysis are available in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under the accession number: GSE135736.

2.14. Statistical analysis

A two-tailed Student's *t*-test was used for statistical analyses of comparative data. Pearson test was used for association between methylation status and clinicopathological parameters. The Kaplan-Meier method was used to analyze patient survival and the significance was tested by Wilcoxon test. *P* values of less than 0.05 were considered as significant. All analyses were performed by using JMP (SAS Institute).

3. Results

3.1. LHX6 is downregulated in pancreatic cancer cell lines mainly through promoter hypermethylation and histone deacetylation

In order to examine whether there is a correlation between DNA methylation and mRNA expression of *LHX6* gene in pancreatic adenocarcinomas, we first searched the MethHC (http://MethHC.mbc.nctu.edu.tw) database, which comprises a systematic integration of a large collection of DNA methylation data and mRNA/miRNA expression profiles in human cancers [26]. Interestingly, DNA methylation at the first exon, gene body, and 3' untranslated region was negatively correlated with mRNA expression of *LHX6* in pancreatic adenocarcinomas (Fig. S1). Among these regions, DNA methylation at the first exon of *LHX6* showed higher negative correlation with mRNA expression. Based on this information, we analyzed mRNA expression levels of *LHX6* by qRT-PCR in 12 pancreatic ductal carcinoma cell lines along with HPDE as the normal pancreatic ductal cell control (Fig. 1A). Except for PCI-35 and MIA PaCa-2, *LHX6* was significantly downregulated in most cancer cell lines compared to HPDE (10/12: 83.3%). To investigate the cause of this cancer-specific *LHX6* downregulation, we next performed genomic bisulfite sequencing of the *LHX6* promoter region (nucleotides from -19 to +328) containing 43 CpG sites (Fig. 1B). Among 10 pancreatic cancer cell lines showing low expression levels of *LHX6*, 7 cell lines (PK-1, PK-9, PK-45P, PK-45H, PK-59, AsPC-1, and BxPC-3) were hypermethylated in the region between nucleotides +238 and +328; probably this region contains important elements. These results were confirmed by methylation-specific PCR (MSP) analysis (Fig. 1C). We further examined *LHX6*

mRNA expression profiles after treatment with 5-aza-2'-deoxycytidine (5-Aza-dC) and/or trichostatin A (TSA) in 12 pancreatic cancer cell lines to clarify the influences of DNA methylation and histone modifications (see Fig. 1D). PCI-35 and MIA PaCa-2 with high expression levels of *LHX6*, as well as AsPC-1 with low expression, failed to respond to both 5-Aza-dC and TSA. Six of seven pancreatic cancer cell lines with the hypermethylated *LHX6* promoter (PK-1, PK-9, PK-45P, PK-45H, PK-59, and BxPC-3) were upregulated by 5-Aza-dC; hypermethylation is suggested to play an important role in transcriptional silencing in the majority of pancreatic cancer cells. On the other hand, two pancreatic cancer cell lines, PCI-6 and PANC-1, without hypermethylation were upregulated by TSA; histone deacetylation statuses are involved in transcriptional silencing in these cells. Similarly, PK-8 which was not hypermethylated was synergistically upregulated by treatment with both 5-Aza-dC and TSA. AsPC-1 was hypermethylated, but neither 5-Aza-dC or TSA changed their expression levels of *LHX6*.

3.2. LHX6 was aberrantly methylated in primary pancreatic carcinomas

In order to examine the methylation status of *LHX6* promoter region in primary pancreatic carcinomas, we performed MSP analyses using 28 paired normal and primary resected pancreatic carcinoma specimens. Aberrant tumor specific hypermethylation in *LHX6* promoter was observed in 16/28

(57.1 %) primary pancreatic carcinomas (Fig. 2). Relationships between *LHX6* promoter methylation and clinicopathological parameters such as differentiation, staging, infiltration and invasion were analyzed, but no significant relationships were observed (Table S2). In addition, *LHX6* promoter methylation status did not show any significant relationships with survival time (Fig. S2).

3.3. Tumor suppressive functions of LHX6 in pancreatic cancer cells

In order to investigate the biological significance of *LHX6*, we first performed colony formation assays in two pancreatic cancer cell lines with hypermethylation-mediated downregulation of *LHX6*, PK-1 and PK-9. As shown in Figs. 3A and B, significantly reduced number of colonies were observed only in forced expression of *LHX6*. We next constructed tetracycline regulated *LHX6* inducible cell lines using PK-1 and PK-9, representatives of the *LHX6* low-expressing cells, and performed alamarBlue assays with or without tetracycline induction. Beforehand, we assessed influence of tetracycline addition on the cell growth. Empty vector-transfected PK-1 and PK-9 cell lines (PK-1_Vec#2 and PK-9_Vec#5) were constructed and found that tetracycline treatment itself does not have any effects on cell growth (Fig. S3). Representative results using PK-1_LHX6#13 and PK-9_LHX6#4 are shown in Figs. 3C-E; induction of FLAG-*LHX6* caused significant growth suppression between days 4 to 6 after tetracycline addition.

Results using two other constructed cell lines, PK-1_LHX6#10 and PK-9_LHX6#9, are also shown; they confirmed the growth suppression in these cells (Fig. S4).

We additionally employed siRNA-mediated knockdown of *LHX6* using high-expressing pancreatic cancer cell lines (PCI-35 and MIA PaCa-2) to see the influence on cell growth. Representative results of PCI-35 are shown in Figs. 3F and G. The specific reduction of *LHX6* expressions by siRNA transfection is shown in Fig. 3G, and corresponding acceleration of cell proliferation is shown in Fig. 3F. Results using another high-expressing pancreatic cancer cell line, MIA PaCa-2, are also shown; they confirmed the growth acceleration in this cell line (Fig. S5). These results suggest that the expression of *LHX6* suppresses the cancer cell growth to some extent.

3.4. Identification of TFPI2 as a LHX6 downstream gene

In order to search for downstream genes of *LHX6* in pancreatic cancer cells, we performed gene expression microarray analyses using PK-1_LHX6#13 and PK-9_LHX6#4 cells with or without tetracycline. A total of 432 genes were identified as ones commonly upregulated or downregulated by two fold or more after tetracycline-mediated induction. Among these candidate *LHX6* downstream genes, we selected several commonly upregulated or downregulated genes shown in Table S3 because these genes

were known to function as tumor suppressor genes or oncogenes. We then performed preliminary semiquantitative RT-PCR analyses of each gene and found that the results of microarray analyses were well confirmed in *TFPI2* (Tissue factor pathway inhibitor 2). *TFPI2* is a tumor suppressor gene that is well-known to be hypermethylated in various types of tumors including colon [27] and pancreatic [28] cancers. Inducible expression of *LHX6* upregulated *TFPI2* by 2.1 and 38.3 in PK-1_LHX6#13 and PK-9_LHX6#4, respectively (Fig. 4A). These results suggest that *TFPI2* is regulated by *LHX6*. In accordance with these results, the extreme downregulation of *TFPI2* was seen in 8 of 10 pancreatic cancer cell lines with suppressed *LHX6* expression (Figs. 1A and 4B).

4. Discussion

In this study, the MeTA method identified *LHX6* as the strong candidate of commonly hypermethylated and silenced gene in pancreatic cancer. *LHX6* was hypermethylated in 7 of 12 (58.3%) pancreatic cancer cell lines and 16 of 28 (57.1%) primary pancreatic cancer specimens, respectively, in a tumor-specific manner. In addition, as shown in Figs. 1A and D, *LHX6* was significantly downregulated in 10 of 12 (83.3%) pancreatic cancer cell lines not only by DNA methylation but also by other types of mechanisms, including histone deacetylation. Two *LHX6* mutations in pancreatic cancer, c.767C>A

(p.T256N) and c.746A>C (p.K249T), have been reported in the COSMIC (Catalogue of Somatic Mutations in Cancer) database (https://cancer.sanger.ac.uk/cosmic). Both mutations are located at the DNA binding region and may inactivate transcriptional activity of *LHX6*. All these data suggest that *LHX6* inactivation may play a key role in pancreatic tumorigenesis and that DNA methylation may be a primary cause of inactivation. Consistent with these results, aberrant methylation of *LHX6* has been shown in other types of cancers such as in the lung [11], cervix [12], and head and neck [13] as well as microcystin-LR (L: leucine, R: arginine, MC-LR) induced hepatocellular carcinoma [29]. Therefore, the role of *LHX6* inactivation may be responsible not only in pancreatic cancer but also in several other cancer types.

Using colony formation and cell proliferation assays, our present results demonstrate that *LHX6* regulates cell growth in pancreatic cancer cells. Furthermore, *TFPI2* was identified as a *LHX6*-downstream gene. *LHX6* regulates *TFPI2* expression, and TFPI2 may be involved in the cell proliferation in pancreatic cancer. There are some inconsistencies between *LHX6* and *TFPI2* mRNA levels in pancreatic cancer cell lines. To understand the relationships between *LHX6* and *TFPI2*, we need to consider the following points. First, we must determine the relationship between *TFPI2* and each *LHX6* variant. There are at least six *LHX6* variants, and we still do not know which one is dominantly expressed in pancreas. Although we tried to analyze LHX6 protein levels in pancreatic cancer cells with HPDE, no suitable antibody is available, and we cannot distinguish variant LHX6 proteins. Second, *TFPI2* itself is downregulated by

promoter hypermethylation; therefore, we must analyze *TFPI2* methylation statuses in pancreatic cancers. Further analyses of these points could clarify the relationship between *LHX6* and *TFPI2*.

Poor survival in pancreatic cancer results from late diagnosis when therapeutic intervention is less effective. Recent analyses of circulating tumor DNA (ctDNA) suggest that somatic mutations in cancer may provide a new valuable tool for early diagnosis because they are based on driver gene mutations that are expected to be found only in cancers [30]. However, liquid biopsy needs to increase specificity and sensitivity for clinical application. In order to accomplish this, it is effective to combine somatic mutations with other biomarkers such as alterations of DNA methylation statuses that are highly responsible for the initiation and progression of pancreatic cancers; DNA methylation of *LHX6* promoter may be potentially a valuable biomarker for detection of pancreatic cancer. It would be enormously interesting to know whether these alterations of DNA methylation statuses can be used in the future liquid biopsy of patients with pancreatic cancer, and other yet-to-be studied cancers.

Declaration of competing interest

The authors declare no financial competing interests.

Acknowledgments

We are grateful to Dr. B.L.S. Pierce (University of Maryland University College, USA) for editorial work in the preparation of this manuscript and to Biomedical Research Core (Tohoku University School of Medicine) for technical support. This work was supported by Grants-in-Aid (Z.G. 19K07771, S.F. 18K07061) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

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Figure legends

Fig. 1. Reduced expression levels of *LHX6* through DNA methylation and histone deacetylation in pancreatic cancer cell lines. (A) The expression levels of *LHX6* were analysed by qRT-PCR. (B) DNA methylation statuses of *LHX6* promoter regions were analysed by genomic bisulfite sequencing. (C) DNA methylation statuses were also confirmed by MSP analysis. (D) Twelve pancreatic cancer cell lines, as well as HPDE, were treated with PBS, 5-Aza-dC, TSA or 5-Aza-dC+TSA, and the expression levels of *LHX6* were analysed by qRT-PCR.

Fig. 2. MSP analyses of *LHX6* in 28 pairs of primary pancreatic tumors (T) and normal pancreatic ductal epithelia (N). M and U indicate methylated- and unmethylated-specific PCR products, respectively. All primary tumors include amplification with the U primer set, a result of the presence of normal contaminating tissue. Note that *LHX6* was highly methylated in primary pancreatic carcinomas in a tumor-specific manner (red characters).

Fig. 3. Effects of forced expression and specific knockdown of *LHX6* on pancreatic cancer cell growth. (A) Colony formation assays were performed to confirm the effect of LHX6 on pancreatic cancer cell

growth. (B) Quantitative analysis of relative colony numbers is shown. Compared with empty vector transfectants, a significant reduction of colony numbers was observed for LHX6 transfectants. These data are shown as the mean ± S.D. of three independent experiments. (C) Inducible expression of FLAG-*LHX6* was confirmed by Western blotting analysis. (D) Inducible expression of FLAG-*LHX6* suppressed the growth of PK-1 and PK-9 cells. Tet addition strongly inhibited cell proliferation of PK-1_LHX6#13 (black dashed line) and PK-9_LHX6#4 (red dashed line) cells. (E) The diagram shows the light microscopic appearance (40x magnification). (F) Cell proliferation was accelerated by knockdown of *LHX6* in PCI-35 pancreatic cancer cells. (G) Knockdown of *LHX6* in PCI-35 cells was confirmed by qRT-PCR analysis. ** and *** denote $P < 0.01$ and $P < 0.001$, respectively.

Fig. 4. LHX6 regulates *TFPI2* expression in pancreatic cancer cell lines. (A) *TFPI2* is upregulated after *LHX6* inducible expression in PK-1 and PK-9 cells. (B) *TFPI2* mRNA expression profiles by qRT-PCR analysis. Note that *TFPI2* was greatly downregulated in all 12 pancreatic cancer cell lines. ** and *** denote $P < 0.01$ and $P < 0.001$, respectively.