Cancer specific promoter CpG Islands hypermethylation of HOP homeobox (HOPX) gene and its potential tumor suppressive role in pancreatic carcinogenesis

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Abstract

Background: We have recently identified HOP homobox (HOPX) as a tumor suppressor gene candidate, characterized by tumor-specific promoter DNA hypermethylation in human cancers, and it can remarkably inhibit tumors’ aggressive phenotypes. In this current study, we for the first time examined methylation level of HOPX and tested the functional relevance in pancreatic cancer (PC).

Methods: Clinical features of HOPX promoter hypermethylation was investigated in 89 PC tissues, and immunohistochemistry was added. We also examined its functional relevance in phenotype assays such as soft agar, proliferation, invasion, and cell cycle analysis.

Results: PC tissues had HOPX gene hypermethylation as compared to the corresponding normal pancreas tissues, and its uniqueness was robust to discriminate tumor from normal tissues (AUC = 0.85, P < 0.0001). Unexpectedly, HOPX was increased in expression in tumor tissues, and immunohistochemistry revealed its predominant expression in the Langerhans islet cells, where HOPX was reduced in expression for PC cells with promoter hypermethylation. HOPX transfectants exhibited G1 arrest with subG1 accumulation, and inhibited tumor forming and invasive ability.

Conclusion: Defective expression of HOPX which is consistent with promoter DNA hypermethylation may explain aggressive phenotype of pancreatic cancer, and intense expression of HOPX in the Langerhans cells may in turn uniquely contribute to pancreatic carcinogenesis.

Keywords: HOP homeobox, Pancreatic cancer, Methylation

Background

Global hypomethylation is often accompanied by dense hypermethylation of the specific promoters in human cancers [1-3]. Promoter hypermethylation results in gene silencing, and such genes have proved to have potent tumor suppressive function and is rather rare [1,4]. We previously developed pharmacologic reversal of epigenetic silencing and uncovered a myriad of transcriptionally repressed genes in human cancers [5,6]. Using this technique, we have identified several unknown tumor suppressor gene candidates, which included HOP homeobox (HOPX) [7,8].

HOPX gene (GeneBank accession number NT022853), also known as HOP, NECC1, LAGY or OB1, was initially identified as a gene essential for cardiac growth and development [9]. Three spliced transcript variants, HOPX-α, -β, and -γ, encode the same protein, which contains a putative homeodomain motif that acts as an adapter protein to mediate transcription [10]. HOPX expression is ubiquitous in wide arrays of normal tissue, but not in malignant tissues including choriocarcinoma, lung, uterine endometrial, and gastrointestinal...
Methods

Cell lines and tissue samples
The pancreatic cancer cell lines, PK-8, KLM-1, and NOR-P1 were kindly provided from the Cell Resource Centre for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Six other cell lines, PK-S9, PK-45 H, PK-45P, MIA Paca2, PANC-1, or the esophageal squamous cell carcinoma (ESCC) cell line TE15 [16] and gastric cancer cell line KatoIII were purchased from RIKEN BioResource Centre (Ibaraki, Japan). All cell lines except MIA Paca2 were maintained in RPMI 1640 Medium (GIBCO), containing 10% fetal bovine serum. The pancreatic cancer cell lines, PK-8, KLM-1, and NOR-P1 were kindly provided from the Cell Resource Centre for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Six other cell lines, PK-S9, PK-45 H, PK-45P, MIA Paca2, PANC-1, or the esophageal squamous cell carcinoma (ESCC) cell line TE15 [16] and gastric cancer cell line KatoIII were purchased from RIKEN BioResource Centre (Ibaraki, Japan). All cell lines except MIA Paca2 were maintained in RPMI 1640 Medium (GIBCO), containing 10% fetal bovine serum.

Clinical tissue samples were categorized according to TNM classification, 7th edition of the Union Internationale Contre Le Cancer (UICC) and the 6th edition of the Japan Pancreas Society (JPS). The patients’ characteristics were depicted in Additional file 1 Table S1. All tissue samples were collected at the Kitasato University Hospital, and informed consent was obtained. The present study was approved by the Ethics Committee of the Kitasato University.

Bisulfite treatment of DNA and sequencing analysis
Genomic DNA from homogenized bulky tissues and cell lines was extracted using QIAamp DNA Mini Kit (QIAGEN). Bisulfite treatment was done by bisulfite treatment (Table 1). All cell lines except MIA Paca2 were maintained in RPMI 1640 Medium (GIBCO), containing 10% fetal bovine serum.

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Quantitative-methylation-specific PCR (Q-MSP)
TaqMan methylation specific PCR (Q-MSP) was carried out using iQ Supermix (Bio-Rad) in triplicate on the iCycler iQ™ Real-Time PCR Detection system (Bio-Rad). PCR conditions and the primer sequences are provided in Table 1. Serial dilutions of bisulfite modified DNA from KatoIII were used as positive control and TE15 as negative control, respectively. The methylation value was defined by a ratio of HOPX-β divided by β-actin and then multiplied by 100, according to the comparative cycle threshold (CT) method [17].

RNA purification and reverse transcriptase-polymerase chain reaction
Total RNA from homogenized bulky tissues and cell lines was extracted using RNeasy Mini Kit (QIAGEN), and reverse-transcribed with a SuperScript III Reverse Transcriptase kit (Invitrogen). Quantitative real time RT-PCR (Q-RT-PCR) for HOPX-β or HOPX-core was performed using iQ™ SYBR Green Supermix (Bio-Rad) in triplicate on the iCycler iQ™ Real-Time PCR Detection system (Bio-Rad), either (Table 1). Relative quantitative analysis adjusted for β-actin was performed according to the CT method [17]. Table 1 depicts sequences of primers/probes and PCR condition.

Immunoprecipitation and Western blotting
Whole cells lysates were obtained using RIPA buffer (Pierce, Rockford, IL) supplemented with 10 μL/ml Halt™ Protease Inhibitor Cocktail Kit (Pierce) and Halt™ Phosphatase Inhibitor Cocktail Kit (Pierce). Immunoprecipitation (IP) was performed using DynaBead Protein G (Dynal Biotech, Oslo, Norway), 1 μg of anti-HOPX mouse IgG1K monoclonal antibody (3D6, Sigma), and 400 μg of each cell lysates. The anti-HOPX mouse IgG1K monoclonal antibody (3D6, dilution of 1:1000, Sigma), anti-HOPX rabbit IgG polyclonal antibody (FL-73, dilution of 1:200, Santa Cruz Biotechnol- ogy, Santa Cruz, CA, USA), anti-V5 mouse IgG2a monoclonal antibody (dilution of 1:5000, In vitrogen), and anti-β-actin mouse IgG2a monoclonal antibody (dilution of 1:1000, Sigma) were used for Western blotting (WB). The anti-HOPX rabbit IgG polyclonal antibody (FL-73, dilution of 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-V5 mouse IgG2a monoclonal antibody (dilution of 1:5000, Invitrogen), and anti-β-actin mouse IgG2a monoclonal antibody (dilution of 1:1000, Sigma) were used for Western blotting (WB) or IP/WB.

5-Aza-dC and TSA treatment
Cells (1×10⁶ cells/T-75 flask) were treated with 1 or 5 μM of the demethylating agent 5-aza-2′-deoxycytidine (5-Aza-dC) (Sigma-Aldrich) dissolved in 50% acetic acid or mock-treatment with PBS including the same amount of acetic acid every 24 hrs for 4 days. When combined with the histone deacetylase inhibitor trichostatin A (TSA) (Sigma-Aldrich), 300 nM TSA was added to the medium for the final 24 hrs.

Immunohistochemistry
Formalin fixed, paraffin-embedded histological sections (3 μm thick) were immunostained using the HOPX...
| Method               | Gene          | Forward primer (5'→3')          | Fluorescent (5'→3')            | Reverse primer (5'→3')          |
|----------------------|---------------|---------------------------------|--------------------------------|---------------------------------|
| bisulfite sequencing  | HOPX-β        | TAGTTGTGTGGAGAGGGCCGG           | FAM-CGGAGATAGAAGGGCGGCGGAGGGTCG-TAMRA | AAACATCCCAACACACAAATCGGGAAGAA   |
| Q-MSP†                | HOPX-β        | TTGGGAGAGGTTTTTTAAGCG          | FAM-ACCACCCCAACACACAAATGCGGGAAGAA | AAACATCCCAACACACAAATCGGGAAGAA   |
| Q-MSP                | β-actin       | TGGTATGGAGAGGGGTATGAAATT       | FAM-ACCACCCCAACACACAAATGCGGGAAGAA | AAACATCCCAACACACAAATCGGGAAGAA   |
| RT-PCR§               | HOPX-α and γ  | CAAGACGCTGGCTGCGCTT            | GCGGAGAGGAGAAGACAGAGAT         | GCGGAGAGGAGAAGACAGAGAT          |
| RT-PCR/Q-RT-PCR      | HOPX-β        | GCTCCCTTTTCCGGGAGGAA           | GCGGAGAGGAGAAGACAGAGAT         | GCGGAGAGGAGAAGACAGAGAT          |
| RT-PCR/Q-RT-PCR      | HOPX-core     | CAGAGGACCAGGACTGAATCC          | GCGGAGAGGAGAAGACAGAGAT         | GCGGAGAGGAGAAGACAGAGAT          |
| RT-PCR/Q-RT-PCR      | β-actin       | GCTCGTGCGAGACACGGCTC          | CAACATGTCTGCGACACTCTCTCT       | CAACATGTCTGCGACACTCTCTCT       |
| PCR for cloning#     | HOPX          | CACCATGTCCGGCGGAGACCCGGAGGCGG | GCTGTGAGGGCTGAGCACCTGCTCTCT    | GCTGTGAGGGCTGAGCACCTGCTCTCT    |

Abbreviations: Tm, annealing temperature.

1: Bisulfite sequencing PCR was done at 95°C for 3 min followed by 40 cycles at 95°C for 1 min, 72°C for 2 min, and final extension at 72°C for 10 min, in a 50 μl reaction volume containing 1 μl treated DNA, 5 μl 10× PCR buffer, 0.2 μm o/l MgCl₂, 0.2 μmol/l each primer and 0.2 μl Platinum™ Taq DNA Polymerase.

2: Q-MSP was done at 95°C for 3 min followed by 45 cycles at 95°C for 20 sec, 60°C for 20 sec, and 72°C for 30 sec, in a 25 μl reaction volume containing 100 ng treated DNA, 300 nmol/l fluorescent probe, and 25 μl iQ™ supermix.

§: RT-PCR was done at 95°C for 3 min followed by 30 cycles at 95°C for 1 min, and final extension at 72°C for 10 min, in a 50 μl reaction volume containing 1 μl cDNA, 5 μl 10× PCR buffer, 0.2 μmol/l dNTP mixture, 1.5 mmol/l MgCl₂, 0.2 μmol/l each primer and 0.2 μl Platinum™ Taq DNA Polymerase.

#: PCR was done at 94°C for 2 min followed by 35 cycles at 94°C for 15 sec, 64.8°C for 15 sec, 68°C for 30 sec, and final extension at 68°C for 7 min, in a 50 μl reaction volume containing 1 μl cDNA, 5 μl 10× Pf×50™PCR Mix, 0.3 μmol/l dNTP mixture, 0.3 μmol/l each primer and 1 μl Pf×50™ DNA Polymerase.
Figure 1 (See legend on next page.)
antibody (3D6, dilution of 1:200). And immune complexes were detected using the 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate, as a chromogen for 30 seconds or 2 minutes.

**Plasmid and transfection**
A full length cDNA of HOPX was previously isolated and subcloned into pCDNA3.1D/V5-His-TOPO vector (pCDNA3.1-HOPX) [8]. The vector with self-ligation was used as a mock control. Plasmid vectors were transfected into 2 pancreatic cancer cell lines (MIA Paca2 and PANC-1) using Lipofectamine 2000 reagent (Invitrogen). Stable clones with HOPX or mock were established by G418 (GIBCO) selection (MIA Paca2 and PANC-1) using Lipofectamine 2000 reagent. Plasmid vectors formed and each experiment was done in triplicate.

**Cell cycle assay**
Cells (1×10^6 cells/ml) were fixed in 75% ethanol, 5×10^5 cells stained with propidium iodide (Guava cell cycle ligation was used as a mock control. Plasmid vectors were transfected into 2 pancreatic cancer cell lines (MIA Paca2 and PANC-1) using Lipofectamine 2000 reagent (Invitrogen). Stable clones with HOPX or mock were established by G418 (GIBCO) selection (MIA Paca2, 800 μg/ml; PANC-1, 1200 μg/ml).

**Proliferation assay**
Cell proliferation and viability (2×10^3 cells/well) were measured using the Premix WST-1 Cell Proliferation Assay System (Takara Bio, CO., Tokyo, Japan) in 96-well plates. Experiments were performed in triplicate.

**Invasion assay**
Cells were seeded at density of 1×10^6 cells/well in the 24-well BD BioCoat Matrigel Invasion Chamber (BD Biosciences Discovery Labware, Bedford, MA) filled with 500 μl DMEM (GIBCO). As a chemoattractant, 10% FBS in 750 μl DMEM (GIBCO) was used for the assay. After incubation for 22 hrs, the membrane of the upper chamber was fixed and stained by Diff-Quick reagent (Sysmex, Kobe, Japan). Invaded cells were counted in for randomly selected sites per membrane.

**Anchorage-independent colony formation assay**
Anchorage-independent cell growth was analyzed by plating 0.36% top agarose (Bacto™ Ager, Becton Dickson and Company, Franklin Lakes, NL) containing 1×10^5 cells on a surface of 0.72% bottom agarose in 6-well plates. Two independent experiments were performed and each experiment was done in triplicate.

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Figure 2 (See legend on next page.)
PC cell lines. We therefore added experiments of IP/WB (Figure 1C), which could detect very weak but consistent expression of HOPX protein in KLM-1, PK-8, and PK-45 H at 10 kDa [7,9,14]. As HOPX transfectants and positive control TE15 showed considerable expression (Figure 1C), we concluded that HOPX expression showed very little, if any, in PC cells.

Characteristics of promoter B methylation in PC cell lines
We initially examined the HOPX-β promoter methylation in all the 8 PC cell lines by bisulfite treatment followed by direct sequencing, and promoter B of HOPX gene was proved to be completely methylated in cytosine residues of CpG islands in 7 PC cells except PANC-1 and MIA Paca2 (Figure 2A, B).

In order to demonstrate whether HOPX silencing of PC cells is due to epigenetic abnormalities, demethylating agents such as 5-Aza-dC and/or trichostatin A were added to PC cells, and reactivation of HOPX transcripts was evaluated by RT-PCR and Q-RT-PCR (Figure 2C). Differently from other GI cancers, reactivation was recognized in small portions of PC cells to the small extent (Figure 2C). Reactivation by the most optimal demethylating conditions was only found in PK-45 H and PK-59, suggesting that reduction of HOPX gene could be partially explained by epigenetic alterations of HOPX promoter B. HOPX absent expression in MIA Paca2 was also confirmed even after epigenetic reversion. We could have therefore postulated homozygous deletion to explain this absent expression. However, DNA could be amplified for the promoter regions of HOPX-β, and actual cloned sequence largely showed unmethylated in MIA Paca2 (Figure 2B). Other cancer cell lines also showed very little expression of HOPX-β, so constitutive transcription signal to activate HOPX-β expression was defective in PC cell lines. However, this result does not represent meaningless significance of HOPX methylation, and we continued methylation analysis in primary PC tissues.

Expression of HOPX transcripts and protein in PC tissues and the corresponding normal pancreas tissues
We first examined the expression status of HOPX transcripts for both the primary tumors and the corresponding pancreas tissues in the 5 consecutive advanced PC patients by both semi-quantitative RT-PCR and Q-RT-PCR. As a result, HOPX-β transcripts were rather robustly over-expressed in the primary PC tissues as compared to the corresponding normal tissues (Figure 3A). WB also showed HOPX protein over-expression in tumor tissues as compared to in the corresponding normal tissues (Figure 3B).

In order to confirm predominant localization of HOPX protein in primary PC, we then performed immunohistochemistry (Figure 3C, D). Surprisingly, HOPX was strongly immunostained almost exclusively for pancreatic islet cells by short term exposure (30 seconds) of DAB. Neither cancer cells nor normal pancreatic components such as acinar and ductal cells showed staining of HOPX (Figure 3D). On the other hand, islet cells in normal pancreas also showed considerable immunostaining of HOPX (Figure 3D). These findings suggested that predominant expression of HOPX transcripts and protein in primary PC represents those of islet cells.

Instead of intense immunostaining of pancreas islet cells, pancreatic duct and a portion of acinar cells were also immunostained by intermediate exposure (2 minutes) of DAB (Figure 4A). The cellular localization of HOPX existed mainly in cytoplasm. Under such conditions, we investigated the IHC staining of HOPX for 11 cases with high methylation value and 9 cases with low methylation value, respectively. In high methylation value tissues, absent expression of HOPX was confirmed despite frequent inclusion of heterogeneity. However, 9 samples with low methylation value exhibited relatively strong HOPX expression, while only one sample showed negative expression (Figure 4A). These results indicated that expression of HOPX protein was associated with promoter hypermethylation.

Using the same primers and probes in gastric cancer study [8], we examined both 89 primary PC tissues and 84 corresponding non tumor tissues by Q-MSP analysis (Figure 4B). The most optimal cut-off value was calculated for 1.5 from a receiver-operator characteristic (ROC) analysis in order to maximize both sensitivity and specificity of PC detection (Figure 4C), where sensitivity was 83.2%, and specificity was 76.2%.

The overall methylation value detected in primary PC tissue (14.50 ± 16.53) was significantly higher than that in the non tumoral tissues (3.64 ± 12.02) (P < 0.0001) (Figure 4B). In addition, the methylation values within primary PC tissues were significantly higher than those within non tumor
Figure 3 (See legend on next page.)
tissues in individual patients, whereas methylation values did not significantly differ in each stage (Figure 4D).

We further investigated whether the HOPX-β methylation value was able to predict patient's outcomes. Log-rank plot analysis [18] showed that any cut-off value could not represent prognostic stratification in PC (Figure 4E). We preliminarily analyzed the correlation between HOPX-β hypermethylation and the clinicopathological parameters, but none of any clinicopathological variables was associated with methylation status of HOPX-β (Additional file 2 Table S2).

**HOPX stable transfectants caused suppression of aggressive PC cell phenotypes**

Two cell lines of pancreatic adenocarcinoma such as PANC-1 and Mia Paca2 cells were transfected with pcDNATM3.1-HOPX with V5-tagged and established stable HOPX-expressing cell lines. In the HOPX stable cell lines, exogenous mRNA expression level in cells with the most abundant expression was comparable to physiological expression level in human PC tissues. HOPX protein was confirmed by 3D6 antibody and anti-V5 antibody. Exogenously expressed tagged HOPX was detected as approximately 15 kDa which is consistent with mRNA levels (Figure 5A).

HOPX transfectants showed both less viability by WST assay (Figure 5B) and remarkable reduction of colonies in soft agar (Figure 5C) as compared with mock cells. Moreover, we found considerable suppression of invasion activity in HOPX-expressing cells by Matrigel invasion assay (Figure 5E). Cell cycle analysis further revealed that HOPX increased fractions of both subG1 fraction and G0/G1, accompanied by decreased fraction of both S and G2/M, indicating that both G1 arrest and apoptotic sensitivity may be at least partially involved in tumor suppressive traits of HOPX-expressing cell (Figure 5D and Additional file 3 Table S3).

**Discussion**

We have recently identified HOPX as genes specifically methylated in human cancers [7,8] after developing algorithm utilizing pharmacological unmasking microarray (PUM) [5,6]. Among the identified candidates of TSGs, HOPX is of particular interest in terms of methylation and functional involvement in tumor aggressiveness. Other groups also recapitulated the similar finding that HOPX promoter DNA is hypermethylated specifically in endometrial cancer [15]. In this present study, we for the first time added pancreatic cancer to the list of organs in which HOPX is involved in carcinogenesis.

HOPX harbors 2 discrete promoter regions, promoter A and promoter B. Promoter B has CpG islands, while promoter A does not have them, and cancer-specific hypermethylation is recognized in the promoter B in primary PC tissues as well as other GI cancers [7,8]. Such independent regulation of the discrete promoter regions was reported in other critical methylation genes such as RASSF1 [19], and possession of the complex promoter regions may indicate their functional importance in biological relevance. On the other hand, epigenetic reactivation of HOPX gene expression was much less than expected in PC cell lines as compared to other GI cancer cell lines. Allowing for actual expression in primary cancer tissues, constitutive HOPX expression signal was derived from carcinoma-stroma interaction in primary PC cells.

Pancreatic cancer is a ductal carcinoma, however it is controversial which normal components (ductal cells, acinar cells, or islet cells) of the pancreatic tissues are precursor cells for PC [20,21]. Pour et al. proved that transplantation of islets into the submandibular gland of Syrian golden hamsters followed by treating with nitrosamine N-nitrosobis-(2-oxopropyl)amin (BOP), a carcinogen for PC led to the development of ductal pancreatic adenocarcinoma in this site, while PC did not occurred after transplanting ductal and acinar cells into this gland [22]. Schmied et al. has also insisted that islet cells contribute to pancreatic carcinogenesis in an animal model and disease exploration [23,24]. In mice with hamster islets implanted in the splenic lobe of the mouse pancreases, pancreatic ductal adenocarcinomas developed in the implanted animals, but not in control mice, after BOP treatment [25]. These findings strongly supported the hypothesis that PC is generated from islet cell origin. In this current study, we for the first time revealed that islet cells expressed abundant HOPX protein in primary PC tissues as well as the normal pancreas. It is intriguing hypothesis that cancer cell with low expression of HOPX is derived from islet cells which constitutively express abundant HOPX, and that promoter DNA hypermethylation is causative for gene silencing.

Clinical findings also supported hypothesis that the islet cell is alternatively involved in PC carcinogenesis.
Figure 4 (See legend on next page.)
[23], in which remarkable alteration of quality of islet cells was observed in primary PC tissues. Ten out of the 14 cancer specimens showed a significant loss of beta cells (P < 0.005) and eight of them also showed a significant increase of alpha cells (P < 0.005), all of them from hyperglycemic patients. Most affected islets were found within zone 1 (intratumoral) and zone 2 (peritumoral), to a lesser extent in zone 3 (acini close to tumor) and none in zone 4 (acini remote from tumor). The incidence of 72% with alteration of islets in their material correlates with the frequency of abnormal glucose levels in human pancreatic cancer patients. In our study, HOPX is remarkably increased in primary PC tissues, and it was predominantly expressed in the islet cells. These findings suggested that alteration of HOPX expression in the islet cells may explain the link of PC to diabetes mellitus, and this mechanistic possibility should be paid attention in the next future, as oncogenic role of islet cells remains elusive during PC carcinogenesis.

HOPX actually suppressed tumor aggressiveness of PC cells (PANC-1 and MIA Paca2). WST assay showed that HOPX suppressed cell viability putatively representing cell proliferation ability. In cell cycle analysis, HOPX increased subG1 and G0/G1 phases, representing apoptotic induction and inhibition of DNA synthesis, suggesting that cell cycle abnormalities may be linked to cell viability. More importantly, HOPX could inhibit tumor-forming ability in soft agar, which is supposed to represent metastatic trait of tumor cells [26]. Interestingly, HOPX has been demonstrated to suppress tumorigenesis in soft agar in ESCC and gastric cancer as well as pancreatic cancer, hence anchorage independent growth suppression is the common feature of HOPX expression in human cancers. Finally, HOPX also affects Matrigel invasion less than other phenotypes in PC. We are also interested in alternate aspects of tumor suppressor gene critical for tumor aggressiveness. We further elucidated that HOPX is a putative tumor suppressor gene critical for tumor aggressiveness in PC. We would like to know more specific and definitive conclusions as to these concerns in the near future.

Collectively, we found that HOPX methylation is a very frequent and cancer specific event in PC development. We further elucidated that HOPX is a putative tumor suppressor gene critical for tumor aggressiveness in PC. We are also interested in alternate aspects of HOPX in terms of a role in islet cells. We must confirm
Figure 5 (See legend on next page.)
more detailed mechanism involved in remarkable phenotype alteration by HOPX abnormalities in PC in future study.

Conclusions
Defective expression of HOPX which is consistent with CpG islands promoter DNA hypermethylation may explain aggressive phenotype of pancreatic cancer, and intense expression of HOPX in the Langerhans islet cells may in turn uniquely contribute to pancreatic carcinogenesis.

Additional files

Additional file 1: Table S1. Characteristics and prognostic analysis in 89 patients with pancreatic cancer.

Additional file 2: Table S2. Correlation analysis between HOPX-β methylation status and clinicopathological variables (n=89).

Additional file 3: Table S3. Distribution of cell cycle phase.

Abbreviations
HOPX: Homeodomain-only protein; PC: Pancreatic cancer; Q-MSP: TaqMan methylation specific polymerase chain reaction; Q-RT-PCR: Quantitative reverse transcriptase-polymerase chain reaction; UIICC: The union internationale contre le cancer; JPS: Japan pancreas society; ROC curve: Receiver-operator characteristic curve; SD: Standard deviation; DSS: Disease specific survival.

Competing interests
There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Authors’ contributions
MW conceived of the study, performed the study, drafted the manuscript and participated in coordination. KY participated in coordination and assisted in editing of manuscript. HK, AO, HK, KN, and AE helped in the collection and analysis of clinical data. MW participated in coordination. All authors read and approved the final manuscript.

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References
1. Jones PA, Baylin SB: The epigenomics of cancer. Cell 2007, 128(4):683–692.
2. Bird AP: CpG-rich islands and the function of DNA methylation. Nature 1986, 321(6067):209–213.
3. Goeltz SE, Vogelstein B, Hamilton SR, Feinberg AP: Hypomethylation of DNA from benign and malignant human colon neoplasms. Science 1985, 228(4696):187–190.
4. Herman JG, Baylin SB: Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med 2003, 349(21):2024–2034.
5. Yamashita K, Upadhyay S, Osada M, Hoque MO, Xiao Y, Morii M, Sato F, Meltzer SJ, Sidransky D: Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma. Cancer Cell 2002, 2(6):485–495.
6. Kim MS, Yamashita K, Baek JH, Park HL, Carvalho AL, Osada M, Hoque MO, Upadhyay S, Morii M, Moon C, et al: N-methyl-D-aspartate receptor type 2B is epigenetically inactivated and exhibits tumor-suppressive activity in human esophageal cancer. Cancer Res 2006, 66(7):3409–3418.
7. Yamashita K, Kim MS, Park HL, Tokumaru Y, Osada M, Inoue H, Morii M, Sidransky D: HOP/0B1/NECC1 promoter DNA is frequently hypermethylated and involved in tumorigenic ability in esophageal squamous cell carcinoma. Mol Cancer Res 2008, 6(1):31–41.
8. Okai A, Yamashita K, Kikuchi S, Sakuramoto S, Katada N, Kukudo K, Kobayashi H, Kim MS, Sidransky D, Watanabe M: Potential utility of HOP homeobox gene promoter methylation as a marker of tumor aggressiveness in gastric cancer. Oncogene 2010, 29(26):3263–3275.
9. Chen F, Kook H, Milewski R, Gilter AD, Lu MM, Li JJ, Naarain R, Schnapp R, Jen K, Biben C, et al: HOP is an unusual homeobox gene that modulates cardiac development. Cell 2002, 110(6):713–723.
10. Kook H, Yung WW, Simpson RJ, Kee HJ, Shin S, Lowry JA, Loughlin FE, Yin Z, Epstein JA, Mackay JP: Analysis of the structure and function of the transcriptional coregulator HOP. Biochemistry 2006, 45(33):10584–10590.
11. Asanoma K, Matsuda T, Kondo H, Kato K, Kishino T, Niikawa N, Wake N, Kato H: NECC1, a candidate choriocarcinoma suppressor gene that encodes a homeodomain consensus motif. Genomics 2003, 81(1):15–25.
12. Asanoma K, Kato H, Inoue T, Matsuda T, Wake N: Analysis of a candidate gene associated with growth suppression of choriocarcinoma and differentiation of trophoblasts. J Reprod Med 2004, 49(8):617–626.
13. Chen Y, Petersen S, Pacyna-G Engelbach M, Piatas A, Petersen I: Identification of a novel homeobox-containing gene, LAGY, which is downregulated in lung cancer. Oncology 2003, 64(4):450–458.
14. Chen Y, Pacyna-G Engelbach M, Deutschmann N, Niesporek S, Petersen I: Homeobox gene HOP has a potential tumor suppressive activity in human lung cancer. Int J Cancer 2007, 121(5):1021–1027.
15. Yanaguchi S, Asanoma K, Takao T, Kato K, Wake N: Homeobox gene HOPX is epigenetically silenced in human uterine endometrial cancer and suppresses estrogen-stimulated proliferation of cancer cells by inhibiting serum response factor. Int J Cancer 2009, 124(1):2577–2588.
16. Nishihira T, Hashimoto Y, Katayama M, Mori S, Kuroki T: Molecular and cellular features of esophageal cancer cells. J Cancer Res Clin Oncol 1993, 119(8):441–449.
17. Luijckx KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001, 25(4):402–408.
18. Waraya M, Yamashita K, Katagiri H, Iishi K, Takahashi Y, Furuta K, Watanabe M: Preoperative serum CA125 and dissected peripancreatic tissue margin as determiners of long-term survival in pancreatic cancer. Ann Surg Oncol 2009, 16(5):1231–1240.
19. Dammann R, Schagdarsurengin U, Liu L, Otto N, Girmon D, Dralle H, Boehm BO, Pfeifer GP, Hoang-Vu C: Frequent RASSF1A promoter hypermethylation and K-ras mutations in pancreatic carcinoma. Oncogene 2003, 22(24):3816–3812.
20. Pour PM, Pandey KK, Batra SK: What is the origin of pancreatic adenocarcinoma? Mol Cancer 2003, 2:13.
21. Hennig R, Ding XZ, Adrian TE: On the role of the islets of Langerhans in pancreatic cancer. Histol Histopathol 2004, 19(3):999–1011.
22. Pour PM, Weide L, Liu G, Kazakoff K, Scheetz M, Toshkov I, Ikematsu Y, Fienhold MA, Sanger W: Experimental evidence for the origin of ductal-type adenocarcinoma from the islets of Langerhans. Am J Pathol 1997, 150(3):2167–2180.
23. Schmied BM, Ulrich AB, Matsuzaki H, Li C, Friess H, Bohrer MW, Andron-Sandberg A, Adrian TE, Pour PM: Alteration of the Langerhans islets in pancreatic cancer patients. Int J Pancreatol 2000, 28(3):187–197.
24. Schmied BM, Ulrich AB, Matsuzaki H, Li C, Pour PM: In vitro pancreatic carcinogenesis. Ann Oncol 1999, 10(4):41–45.
25. El-Ghamari M, Bergmann F, Schmied BM, Weitz J, Ulrich A: Islet cells contribute to pancreatic carcinogenesis in an animal model. Pancreas 2011, 40(2):242–246.
26. Takahashi M, Furihata M, Akimitsu N, Watanabe M, Kaul S, Yumoto N, Okada T: A highly bone marrow metastatic murine breast cancer model established through in vivo selection exhibits enhanced anchorage-independent growth and cell migration mediated by ICAM-1. Clin Exp Metastasis 2008, 25(5):517–529.
27. Katoh H, Yamashita K, Waraya M, Margalit O, Ooki A, Tamaki H, Sakagami H, Kokubo K, Sidransky D, Watanabe M: Epigenetic silencing of HOPX promotes cancer progression in colorectal cancer. Neoplasia 2012, 14(7):559–571.
28. Kook H, Lepore JJ, Gitler AD, Lu MM, Wing-Man Yung W, Mackey J, Zhou R, Ferrari V, Gruber P, Epstein JA: Cardiac hypertrophy and histone deacetylase-dependent transcriptional repression mediated by the atypical homeodomain protein Hop. J Clin Invest 2003, 112(6):863–871.
29. Yin Z, Gonzales L, Kolla V, Rath N, Zhang Y, Lu MW, Kimura S, Ballard PL, Beers MF, Epstein JA, et al: Hop functions downstream of Nkx2.1 and GATA6 to mediate HDAC-dependent negative regulation of pulmonary gene expression. Am J Physiol Lung Cell Mol Physiol 2006, 291(2)L191–L199.
30. Kee HJ, Kim JR, Nam XI, Park HY, Shin S, Kim JC, Shimoto Y, Takahashi M, Jeong MH, Kim N, et al: Enhancer of polycomb1, a novel homeodomain only protein-binding partner, induces skeletal muscle differentiation. J Biol Chem 2007, 282(10):7700–7709.
31. Attwooll C, Oddi S, Cartwright P, Prosperini E, Agger K, Steensgaard P, Wagener C, Sardet C, Moroni MC, Helin K: A novel repressive EZF6 complex containing the polycomb group protein, EPC1, that interacts with EZH2 in a proliferation-specific manner. J Biol Chem 2005, 280(21):11999–12008.
32. Aoto T, Saitoh N, Sakamoto Y, Watanabe S, Nakao M: Polycomb group protein-associated chromatin is reproduced in post-mitotic G1 phase and is required for S phase progression. J Biol Chem 2008, 283(27):18905–18915.

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