GSH2 promoter methylation in pancreatic cancer analyzed by quantitative methylation-specific polymerase chain reaction

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Abstract. Tumor suppressor gene silencing via promoter hypermethylation is an important event in pancreatic cancer pathogenesis. Aberrant DNA hypermethylation events are highly tumor specific, and may provide a diagnostic tool for pancreatic cancer patients. The objective of the current study was to identify novel methylation-related genes that may potentially be used to establish novel therapeutic and diagnostic strategies against pancreatic cancer. The methylation status of the GS homeobox 2 (GSH2) gene was analyzed using the sodium bisulfite sequencing method. The GSH2 methylation ratio was examined in primary carcinomas and corresponding normal tissues derived from 47 patients with pancreatic cancer, using quantitative methylation-specific polymerase chain reaction. Methylation ratios were found to be associated with the patient's clinicopathological features. GSH2 gene methylation was detected in 26 (55.3%) of the 47 pancreatic cancer patients, indicating that it occurs frequently in pancreatic cancer. A significant association with methylation was observed for tumor-node-metastasis stage (P=0.031). GSH2 may be a novel methylation-sensitive tumor suppressor gene in pancreatic cancer and may be a tumor-specific biomarker of the disease.

Introduction

Pancreatic cancer has the highest mortality rate among all cancer types, with an overall five-year survival rate of <5% (1-3). In the United States, pancreatic cancer has been estimated to cause ≥36,800 mortalities annually (2). Thus, novel treatment and detection methods are urgently required for pancreatic cancer patients.

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Aberrant methylation of CpG-rich sequences is a common epigenetic alteration in human cancers, including pancreatic cancer (4,5). In numerous cases, a tumor may arise following the methylation of the promoter of a tumor suppressor gene, leading to gene silencing. This is typically an early event in tumorigenesis, thus these events may be used as a diagnostic marker to detect early-stage pancreatic cancer (6). Therefore, an understanding of the altered gene methylation patterns and the underlying molecular mechanisms in pancreatic cancer may have a significant clinical impact.

GS homeobox 2 (GSH2), also know as GSX2, is a homeobox gene involved in the regulation of mammalian organ development downstream of the sonic hedgehog (Shh) signaling pathway (7). GSH2 is hypermethylated in astrocytomas (8), and Shh signaling is involved in the initiation and progression of pancreatic cancer (9-11). However, the methylation status of GSH2 in pancreatic cancer patients remains unclear. In the present study, the methylation status of the GSH2 gene transcriptional regulation region (TRR) was examined in primary carcinoma and paired normal tissues derived from 47 patients with pancreatic cancer, and the association of methylation with the clinicopathological features of the patients was also assessed. These findings suggest that GSH2 methylation status may provide a novel diagnostic tool for pancreatic cancer.

Materials and methods

Cell line and culture. The pancreatic cancer PANC1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA), and PaTu8988 cells were a gift from Dr H.P. Elsasser (Phillips University, Marburg, Germany). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies Inc., Rockville, MD, USA) and incubated at 37˚C in a humidified chamber with 95% air and 5% CO_2.

Sample collection and DNA preparation. The tissue and patient data usage protocol was approved by the Ethics Committee of the General Hospital of Shenyang Military Command (Shenyang, Liaoning, China). Written informed consent was obtained from each patient. A total of 47 primary tumor and corresponding normal tissue specimens were obtained from the Second Military Medical University affiliated to Changhai Hospital (Shanghai, China) between September 2007 and

September 2009. Immediately after surgical resection, the tissue samples were stored in liquid nitrogen. Tumor tissues containing >70% tumor cells were used as primary tumor samples, and the corresponding adjacent normal tissues without any tumor cell infiltration were selected as normal tissue samples. Genomic DNA from the tissues was extracted using the phenol/chloroform method and ethanol precipitation.

Sodium bisulfite modification. Genomic DNA (1 μg) was processed using the EZ DNA Methylation™ kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. The bisulfite-modified DNA was subsequently suspended in deionized water (20 μl) for immediate use or stored at -80˚C.

Bisulfite-specific PCR (BSP) and DNA sequencing. GSH2 promoter methylation detection was conducted using custom primers designed to specifically amplify the bisulfite-converted DNA of the GSH2 TRR (Shanghai Shenggong Biology Engineering Technology Service, Ltd., Shanghai, China). The primers used were as follows: Forward, 5'-GTTTAAAGGGAGGCGATTAGATAG-3' and reverse, 5'-TTCTCTCTCCAACTCCAAAAATTA-3'. For PCR analysis, bisulfite modified DNA (2 μl from each sample) was combined in 25 μl of reaction mixture (Takara Biotechnology Co., Ltd., Dalian, China) containing 1X PCR buffer, 2.0 mM $MgCl_2$, 2.5 mM dNTP, 1 mM primer and 800 U/l EX Taq DNA HS. The reaction mixture was preheated at 95˚C for 5 min and amplified using a touchdown PCR program as follows on a PCR system (TProfessional Thermocycler; Biometra GmbH, Göttingen, Germany): 9 cycles of 95˚C for 30 sec, 59˚C for 30 sec (next cycle touchdown 0.5˚C) and 72˚C for 30 sec; 42 cycles of 95˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec; and a final extension of 4 min at 72˚C. The products subsequently underwent direct sequencing analysis, or were cloned into the pMD-18-T vector (Takara Biotechnology Co., Ltd.) prior to sequencing analysis; from each sample, 10-25 clones were randomly selected for DNA sequencing.

Quantitative methylation-specific PCR (qMSP). The bisulfite-treated DNA was amplified using qMSP, in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using the following primers and probes: GSH2 forward, 5'-GTTTTCGATGCGTAGGATGC-3' and reverse, 5'-ACGTTTTTAACTAAACCTACGCGC-3'; GSH2 probe, 5'-FAM-ATCCCTCTTTAATCCT-MGB-3'; β-ACTB forward, 5'-TGGTGATGGAGGAGGTTTAGTAAGT-3' and reverse, 5'-AACCAATAAAACCTACTCCTCCCTTAA-3'; and β-ACTB probe, 5'-FAM-TTTGTTATTGTGTGTTGG GTG-MGB-3'. The PCR amplification was conducted as follows: Initial denaturation step of 95˚C for 10 min, then 45 cycles of 95˚C for 15 sec, 70˚C for 15 sec and 60˚C for 60 sec. The bisulfite-treated DNA that was obtained from the Panc1 cells and was fully methylated by SssI methylase was used as a positive control. β-actin was used as an internal control to correct for differences in quality and quantity between samples.

Statistical analysis. The associations between GSH2 methylation and the clinicopathological parameters were analyzed using χ^2 or Student's t-tests with SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Methylation of the GSH2 gene TRR in pancreatic tissues and pancreatic cancer cells. According to the National Center for Biotechnology Information genome database, the GSH2 gene TRR features a CpG island between exons 1 and 2. BSP and MSP primers were designed to bind the 3' end of this region (Fig. 1A). BSP PCR-based sequencing analysis was then performed to assess the methylation status of the GSH2 gene TRR in four tissue groups: Two pancreatic cancer cell lines (PANC1 and PaTu8988), two cases of pancreatic cancer, their adjacent normal tissue, and two cases of normal pancreatic tissues (Fig. 1B).

To further confirm that hypermethylation of the GSH2 gene TRR occurs in pancreatic cancer, BSP cloning-based sequencing analysis was conducted to identify the methylation patterns in these same samples in addition to one sample of white blood cells from a healthy volunteer. Marked CpG methylation was identified in the pancreatic cancer tissue and cell lines (Fig. 2A and B), but not in the normal tissue (Fig. 2C-E). These data are consistent with the results of the BSP PCR-based sequencing analysis.

Based on these data, methylation of the GSH2 promoter was evaluated in a panel of 47 pancreatic cancer tissues and adjacent normal tissues using qMSP (Fig. 3). The mean GSH2 expression level in the pancreatic cancer tissues was significantly higher than that of the adjacent normal tissues (cancer tissue: Mean, 0.10; 95% confidence interval, 0.06-0.14; normal tissue: Mean, 0.04; 95% confidence interval, 0.01-0.06; P=0.0089). Based on these data, hypermethylation was defined by a value of >0.04. Hypermethylation of the GSH2 gene was detected in 26 out of the 47 (55.3%) primary pancreatic cancer tissue samples, indicating that GSH2 TRR hypermethylation occurs frequently in pancreatic cancer.

Association of GSH2 gene TRR methylation with clinicopathological parameters in patients with pancreatic cancer. To further assess the diagnostic significance of GSH2 methylation, the methylation results were assessed with regard to the clinicopathological features of the patients. No significant associations were observed between the GSH2 methylation status and patient gender or age, tumor size, tumor location or lymph node metastasis. However, a significant association was observed between GSH2 methylation levels and tumor-node-metastasis stage (P=0.031; Table I). This supports the qMSP findings, suggesting that GSH2 methylation frequently occurs in pancreatic cancers.

Discussion

Pancreatic cancer is one of the most lethal human cancers, and an effective treatment remains elusive (12). Hypermethylation of gene promoters is a common event during carcinogenesis and tumor progression. The present study investigated the methylation of the GSH2 promoter in pancreatic cancer cell lines, pancreatic cancer and corresponding adjacent normal pancreatic tissues, and normal pancreatic tissue from

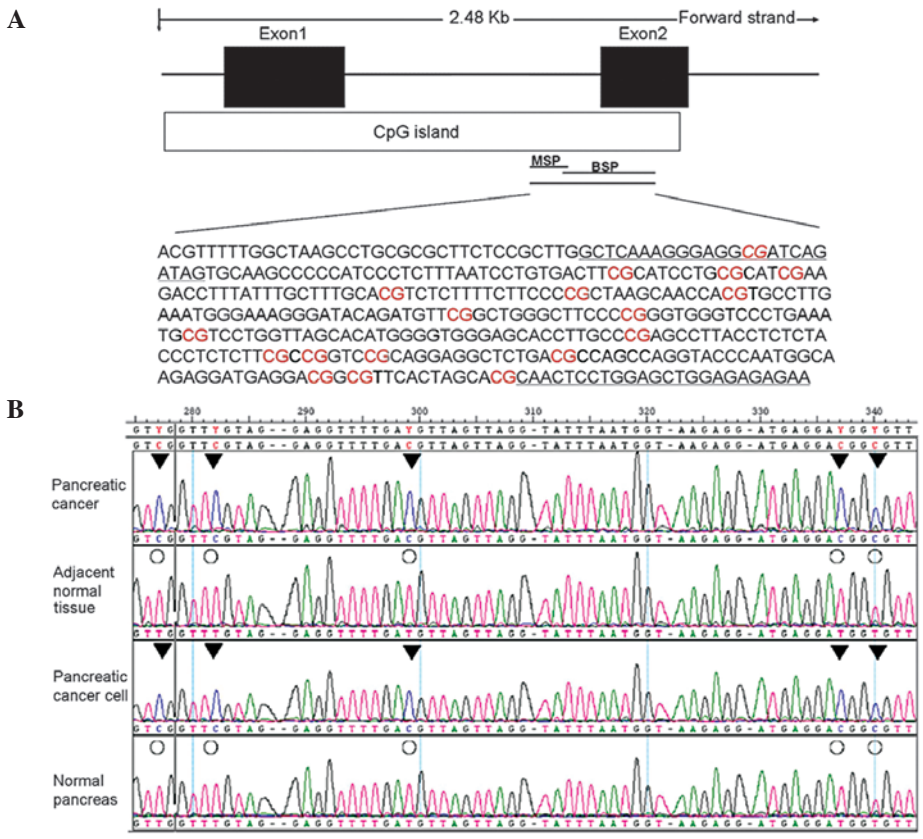

Figure 1. (A) A schematic of the structure of the GS homebox 2 gene and the topology of the BSP and MSP primers, indicating the position of the CpG island (containing 18 CpG sites) and the BSP primer binding sites. (B) Representative BSP cloning-based sequencing analysis results for four different groups of pancreatic tissues and cell lines. BSP, bisulfite-specific polymerase chain reaction; MSP, methylation-specific polymerase chain reaction.

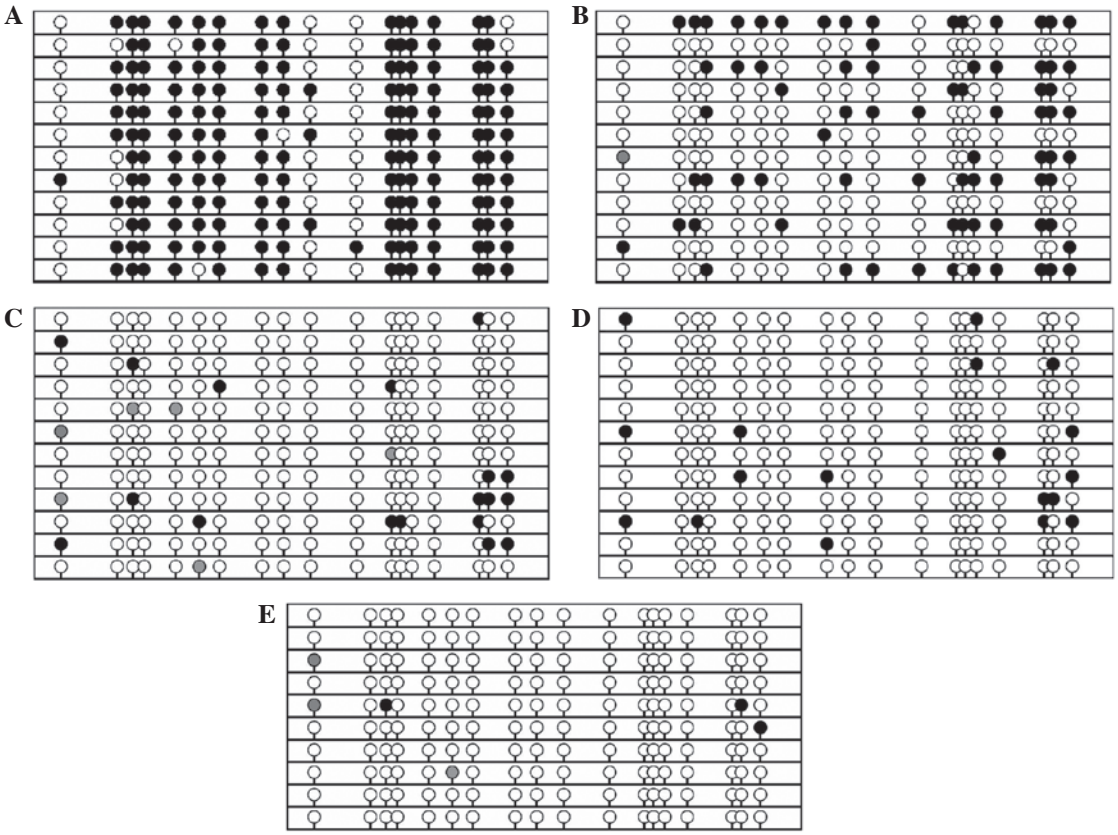

Figure 2. Representative methylation status of 18 CpG island sites using bisulfate-specific polymerase chain reaction cloning-based sequencing analysis. Dark circles indicate a methylated site; white circles indicate an unmethylated site. (A) Pancreatic cancer cell line (Panc1). (B) Pancreatic cancer tissue sample PC34. (C) Healthy human white blood cell. (D) Adjacent normal tissue samples PN34. (E) Normal pancreatic tissue sample N4.

Table I. Clinicopathological features and GSH2 methylation in pancreatic carcinoma (n=47).

Clinicopathological feature	Total cases	GSH2 methylation		P-value
		+	–	
Patients, n	47	26	21	
Gender, n				0.805[a]
Male	30	17	13	
Female	17	9	8	
Age, years	47	61.31±7.47[b]	58.90±7.81[b]	0.288[c]
Maximal tumor size, mm	47	4.58±2.07[b]	4.46±1.77[b]	0.835[c]
Histology, n				0.435[a]
Well-differentiated	34	20	14	
Poorly-differentiated	13	6	7	
Lymph node metastasis, n				0.160[a]
Positive	26	12	14	
Negative	21	14	7	
TNM stage, n				0.031[a]
I/II	22	9	13	
III/IV	25	18	7	

Age range, 45-80 years; maximal tumor size range, 10-100 mm. [a]χ^2 test; [b]mean ± standard deviation; [c]Student's t-test. GSH2, GS homeobox 2; TNM, tumor-node-metastasis.

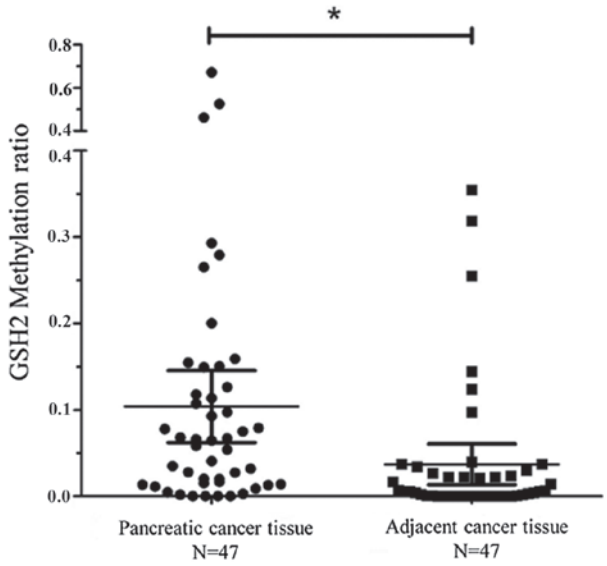

Figure 3. Quantification of DNA methylation in the GSH2 gene promoter. The methylation status of GSH2 in 47 pancreatic cancer tissues and paired adjacent normal tissues was assessed by quantitative methylation-specific polymerase chain reaction. Error bars indicate standard deviation of the mean (*P<0.05). GSH2, GS homeobox 2.

disease-free subjects. The data indicated that GSH2 promoter methylation is highly associated with pancreatic cancer. The GSH2 promoter was found to be hypermethylated in the pancreatic cancer tissues compared with the adjacent normal tissues and normal pancreatic tissue from healthy subjects. Furthermore, aberrant hypermethylation of GSH2 was found to be associated with advanced-stage disease. To the best of our knowledge, the present study is the first to report the correlation of GSH2 promoter hypermethylation with pancreatic cancer and tumor progression.

GSH2 is a homeobox gene that is activated as a downstream target of the Shh pathway; this pathway is essential in organ development and embryonic pancreatic development (7). Misregulation of Shh signaling has been associated with a number of cancer types, including pancreatic carcinoma (13). For example, Shh ligand overexpression induces PanIN-1 and -2-like lesions and may contribute to the formation of desmoplasia in pancreatic cancer (14,15). Conversely, inhibition of Shh signaling suppresses the self-renewal capacity of pancreatic cancer stem cells and reverses chemotherapy resistance (16). Shh may also downregulate GSH2 in ventral telencephalic progenitors (17), suggesting that Shh signaling is able to suppress GSH2 under certain conditions.

The results of the current study indicated that hypermethylation of the GSH2 promoter is a common feature of pancreatic cancer. This may be due to the aberrant activation of Shh signaling commonly observed in pancreatic cancers (18). Furthermore, these data indicate that GSH2 promoter methylation may be used as a novel indicator of Shh activation, as well as a diagnostic tool for pancreatic cancer patients. The precise transcriptional mechanisms that control GSH2 remain unclear, however, the present findings suggest that a better understanding of these mechanisms may inform diagnostic and therapeutic approaches for pancreatic cancer.

In summary, the results of the current study demonstrated that hypermethylation of the GSH2 promoter is associated with the progression of pancreatic cancer, suggesting that this may serve as a diagnostic and prognostic marker for pancreatic

cancer patients. Following this preliminary result, further studies are required to investigate the molecular mechanisms underlying the regulation of changes to GSH2 methylation, and the association between GSH2 hypermethylation and the biological features of pancreatic cancer. In future, the investigation of a large study population should be conducted in order to further support the current results.

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References

1. Landis SH, Murray T, Bolden S and Wingo PA: Cancer statistics, 1998. CA Cancer J Clin 48: 6-29, 1998.
2. Li D, Xie K, Wolff R and Abbruzzese JL: Pancreatic cancer. Lancet 363: 1049-1057, 2004.
3. Warshaw AL and Fernández-del Castillo C: Pancreatic carcinoma. N Engl J Med 326: 455-465, 1992.
4. Chen WD, Han ZJ, Skoletsky J, *et al*: Detection in fecal DNA of colon cancer-specific methylation of the nonexpressed vimentin gene. J Natl Cancer Inst 97: 1124-1132, 2005.
5. Feinberg AP and Tycko B: The history of cancer epigenetics. Nat Rev Cancer 4: 143-153, 2004.
6. Brune K, Hong SM, Li A, *et al*: Genetic and epigenetic alterations of familial pancreatic cancers. Cancer Epidemiol Biomarkers Prev 17: 3536-3542, 2008.
7. Corbin JG, Gaiano N, Machold RP, Langston A and Fishell G: The Gsh2 homeodomain gene controls multiple aspects of telencephalic development. Development 127: 5007-5020, 2000.
8. Wu X, Rauch TA, Zhong X, *et al*: CpG island hypermethylation in human astrocytomas. Cancer Res 70: 2718-2727, 2010.
9. Bailey JM, Mohr AM and Hollingsworth MA: Sonic hedgehog paracrine signaling regulates metastasis and lymphangiogenesis in pancreatic cancer. Oncogene 28: 3513-3525, 2009.
10. Morton JP, Mongeau ME, Klimstra DS, *et al*: Sonic hedgehog acts at multiple stages during pancreatic tumorigenesis. Proc Natl Acad Sci USA 104: 5103-5108, 2007.
11. Pasca di Magliano M, Sekine S, Ermilov A, Ferris J, Dlugosz AA and Hebrok M: Hedgehog/Ras interactions regulate early stages of pancreatic cancer. Genes Dev 20: 3161-3173, 2006.
12. Boj SF, Hwang CI, Baker LA, *et al*: Organoid models of human and mouse ductal pancreatic cancer. Cell 160: 324-338, 2015.
13. Jemal A, Siegel R, Xu J and Ward E: Cancer statistics, 2010. CA Cancer J Clin 60: 277-300, 2010.
14. Bailey JM, Swanson BJ, Hamada T, *et al*: Sonic hedgehog promotes desmoplasia in pancreatic cancer. Clin Cancer Res 14: 5995-6004, 2008.
15. Thayer SP, di Magliano MP, Heiser PW, *et al*: Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature 425: 851-856, 2003.
16. Huang FT, Zhuan-Sun YX, Zhuang YY, *et al*: Inhibition of hedgehog signaling depresses self-renewal of pancreatic cancer stem cells and reverses chemoresistance. Int J Oncol 41: 1707-1714, 2012.
17. Xu Q, Guo L, Moore H, Waclaw RR, Campbell K and Anderson SA: Sonic hedgehog signaling confers ventral telencephalic progenitors with distinct cortical interneuron fates. Neuron 65: 328-340, 2010.
18. Maréchal R, Bachet JB, Calomme A, *et al*: Sonic hedgehog and Gli1 expression predict outcome in resected pancreatic adenocarcinoma. Clin Cancer Res 21: 1215-1224, 2015.