TWIST1 hypermethylation is observed in pancreatic cancer

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Abstract. Despite the growing evidence demonstrating that *TWIST1* is a noteworthy tumor biomarker, little is known about the clinical significance of *TWIST1* methylation in human primary pancreatic cancer. In the present study, the association of *TWIST1* methylation with clinicopathological characteristics was examined in human primary pancreatic cancer. Primary pancreatic cancer specimens and corresponding healthy pancreatic non-tumorous tissues from 33 patients with pancreatic cancer were used. Methylation levels of *TWIST1* were compared with clinicopathological characteristics. The *TWIST1* methylation level was higher in pancreatic cancer compared to corresponding non-neoplastic pancreatic tissues. The mean *TWIST1* methylation was 66.7% for pancreatic cancer tissue and 15.0% for corresponding nonneoplastic pancreatic tissue (P=0.0004). These results suggested that *TWIST1* methylation is a useful biomarker for the screening of pancreatic cancers. Studies using independent data sets are required to confirm these findings.

Introduction

Given that pancreatic cancer is the fourth most common cause of cancer-related mortality and has the lowest patient survival rate of any solid cancer type (1,2), early diagnosis and therapy remain a major challenge.

Pancreatic cancer has been shown to be epigenetic, since it is a genetic disease characterized by widespread and profound alterations in DNA methylation. *TWIST1* is a highly conserved transcription characteristic that belongs to the family of basic helixloop-helix proteins and is involved in embryonic development through the regulation of the migration-invasion program [termed the epithelial-mesenchymal transition (EMT)] during neural crest migration, while regulating mesodermal determination, myogenesis and morphogenesis (3-5). Although growing evidence demonstrates that *TWIST1* methylation is a notable tumor biomarker in various tumors (6-9), little is known concerning the clinical significance of *TWIST1* methylation in human primary pancreatic cancer. These correlations were investigated in the present study. Results showed that *TWIST1* is methylated more frequently in pancreatic cancer compared to non-neoplastic pancreatic tissue, providing a new diagnostic marker for pancreatic cancer.

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Materials and methods

Materials. Formalin-fixed, paraffin-embedded primary pancreatic cancer tissues and corresponding non-neoplastic pancreatic tissues from 33 patients who underwent surgical resection between 2004 and 2009 were evaluated. DNA was prepared from cells in microdissected, 5-μm histopathological sections, as described previously (9). Clinicopathological characteristics were available for the patients. The mean age of the patients (17 males) was 64.4 years. Eighteen tumors were located in the pancreatic head, 10 in the pancreatic body and 3 in the pancreatic tail. One tumor was stage I, 4 were stage II, 15 were stage III, 7 were stage IVa and 6 were stage IVb (classified according to the classification of pancreatic carcinoma of the Japan Pancreas Society). The study protocol was approved by the Institutional Review Board of Yamaguchi University Graduate School of Medicine. Informed consent was obtained from each patient.

Sodium bisulfite modification of DNA. Bisulfite treatment was performed as reported previously (9). Two micrograms of genomic DNA in 50 μl of water were denatured with 5.5 μl of 2 M NaOH at 37˚C for 10 min, followed by incubation with 30 μl of 10 mM hydroquinone and 520 μl of 3 M sodium bisulfite (pH 5.0) at 50˚C for 16 h in the dark. DNA was then purified with 50 μl of water and a DNA Cleanup kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions, incubated with 5.5 μl of 3 M NaOH at room temperature for 5 min, precipitated with 1 μl of 20 mg/ml glycogen, 33 μl of 10 M ammonium acetate and 260 μl of 100% ethanol, washed with 70% ethanol and finally re-suspended in distilled water. DNAs used as positive controls for methylated and unmethylated alleles were *Sss*I methyltransferase-treated placental DNA (New England Biolabs, Ipswich, MA, USA) and lymphocyte DNA, respectively.

KRAS mutations. DNA sequencing was used to evaluate mutations in exon 2 of *KRAS*, as described previously (10).

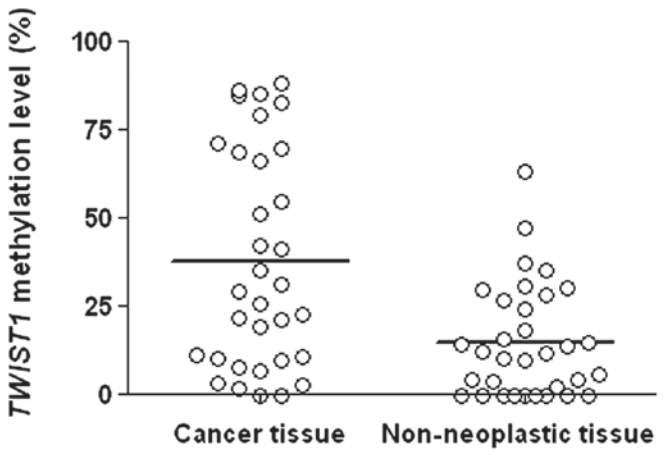

Figure 1. Distribution of *TWIST1* methylation in primary pancreatic cancer and non-neoplastic tissues is shown. Each sample is indicated by an open circle. The horizontal lines are the mean level in each group.

Methylation assay. The *TWIST1* Combined Bisulfite Restriction Analysis (COBRA) primers were F: 5'-TGTGTA GAAGTTGTTGTTATT-3' and R: 5'-CRAAAAAAACTAT CCTAAC-3' (9). PCR amplification was performed for a total of 40 cycles with an annealing temperature of 55˚C. The PCR product was digested with *Bst*UI (New England Biolabs). The digested PCR products were separated by electrophoresis on 4% agarose gels. Digested fragments, which represent methylated DNA, were quantified by densitometry.

Results

The *TWIST1* methylation lever was higher in pancreatic cancer compared to corresponding non-neoplastic pancreatic tissue (Fig. 1). The mean *TWIST1* methylation was 66.7% for pancreatic cancer tissue and 15.0% for corresponding non-neoplastic pancreatic tissue (P=0.0004). After setting the cut-off point at 15.0%, which was the mean level of *TWIST1* methylation in nonneoplastic pancreatic tissue, the correlations between *TWIST1* methylation status and clinicopathological characteristics were studied. No correlations were detected between *TWIST1* methylation status in cancer tissue and clinicopathological characteristics (Table I).

Table I. Correlations between *TWIST1* methylation status and clinicopathological findings in pancreatic cancer patients.

	TWIST1 methylation status		
Characteristics	Methylated	Unmethylated	P-value[a]
Age (years)			
>60	12	7	0.719
≤60	10	4	
Gender			
Male	13	4	0.282
Female	9	7	
Histological type[b]			
Well	7	4	1.000
Mod-poor	15	7	
TNM stage[c]			
I, II, III	14	7	1.000
IVa, IVb	8	4	
Perineural invasion[d]			
+	19	8	0.375
-	3	3	
Venous invasion[d]			
+	16	7	0.696
-	6	4	
Lymphatic invasion[d]			
+	20	9	0.586
-	2	2	
KRAS mutation			
+	14	8	1.000
-	4	3	

[a]Analyzed by the Fisher's test. [b]Well, well-differentiated adenocarcinoma; mod-poor, moderately or poorly differentiated adenocarcinoma. [c]Classified based on the International Union Against Cancer tumor-node-metastasis classification. [d]Classified based on the classification of pancreatic carcinoma of the Japan Pancreas Society.

Discussion

The *TWIST1* methylation level was higher in pancreatic cancer compared to non-neoplastic pancreatic tissue. To the best of our knowledge, this is the first study on the distinct difference in *TWIST1* methylation levels in healthy pancreatic tissues and pancreatic cancer, suggesting a potential for *TWIST1* as a biomarker for the early detection of pancreatic cancers using pancreatic juice DNA-based assays.

Hypermethylation of DNA in promoter CpG islands results in the transcriptional silencing of cancer-related genes (11). In the present study, hypermethylation of *TWIST1* was observed frequently in pancreatic cancer. However, upregulation of *TWIST* mRNA in pancreatic cancer has been reported (12). This discrepancy may be due to the lack of a direct correlation between *TWIST1* methylation and *TWIST1* expression in primary colorectal (9) and breast cancers (13). Characteristics other than *TWIST1* methylation may also affect *TWIST1* expression in pancreatic cancer. *TWIST1* expression is regulated by hypoxia, a common characteristic in solid cancers, in an HIF-1α- and HIF-2α-dependent manner (14,15). Alternately, *TWIST1* promoter methylation might be an early event, preceding compensatory *TWIST1* overexpression (13).

In conclusion, increasing evidence has demonstrated that *TWIST1* exhibits a unique characteristic as a tumor marker. Since *TWIST1* methylation levels are higher in pancreatic cancers compared to corresponding healthy pancreatic tissues, *TWIST1* methylation may be a feasible epigenetic marker for the detection of pancreatic cancer, using a pancreatic juice DNA test. Confirmatory studies using independent data sets are required to support these findings.

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