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Promoter hypomethylation of *SKI* in autoimmune pancreatitis

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ABSTRACT

The relationship between methylation abnormality and autoimmune pancreatitis (AIP)—a representative IgG4-related disease—has not yet been elucidated. We identified *SKI* might have a significant methylation abnormality in AIP through methylation array analysis using the Illumina Infinium Human Methylation 450K BeadChip array, and investigated the relationship of *SKI* with AIP clinicopathological features. The methylation rate of *SKI* was assessed by quantitative SYBR green methylation-specific PCR, and the degree of SKI expression in tissue specimens was assessed by immunohistochemistry in 10 AIP cases, 14 cases of obstructive pancreatitis area in pancreatic ductal adenocarcinoma (PDA) without a history of AIP, and 9 normal pancreas (NP) cases. The *SKI* methylation ratio was significantly lower in AIP than in PDA and NP. Additionally, the immunohistochemical staining-index (SI) score for SKI was significantly higher in AIP than NP, although there was no significant difference between AIP and PDA. There was a strong negative correlation between SI score and *SKI* methylation ratio, and between the serum concentrations of IgG4 and the *SKI* methylation ratio. There was a moderate positive correlation between the serum concentrations of IgG4 and SI. *SKI* is thought to be an oncogene indicating that *SKI* hypomethylation and carcinogenesis might be linked to AIP. Furthermore, the correlation between serum concentrations of IgG4 and *SKI* methylation levels suggest *SKI* might be involved in the pathogenesis of AIP. However, the role of *SKI* has not been clearly elucidated. Further studies are needed to understand further the function of *SKI*.

1. Introduction

IgG4-related disease (IgG4-RD) is a group of incompletely-understood inflammatory conditions that involve the formation of masses in multiple organs [1] and are characterized by high IgG4 serum values [1]. Autoimmune pancreatitis (AIP) is a representative IgG4-RD [2], and is pathologically characterized by lymphoplasmacytic inflammation and storiform fibrosis [3]. Pathologically, many IgG4-positive cells are observed in AIP lesions [4]. Steroid therapy is effective in IgG4-RDs, including AIP [5].

AIP is an autoimmune mediated chronic pancreatitis and its pathophysiology and clinical symptoms are characteristic of autoimmune diseases [6,7]. Autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus (SLE) occur when tissue damage is caused by an autoimmune response. Recently, it was reported that most susceptibility gene polymorphisms of autoimmune diseases exist in enhancer regions [8]. This suggests the importance of the epigenome controlling the enhancer function [9]. Regarding DNA methylation,

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analysis of epigenome-wide studies in patient specimens of SLE and rheumatoid arthritis (RA) reported the DNA hypomethylation of important genes involved in the onset of disease [8,10]. Because AIP is an autoimmune disease, methylation abnormalities may be involved in its pathogenesis.

Chronic inflammation causes epigenetic changes in genes. Methylation abnormalities sometimes induce carcinogenesis. Chronic pancreatitis is also an inflammatory condition, and 5% of chronic pancreatitis patients develop carcinogenesis [11]. In chronic pancreatitis patients, the cancer risk is 13.3 times higher than in healthy subjects [11].

Methylation abnormalities in AIP may cause AIP or AIP itself may cause methylation abnormalities, and these methylation abnormalities might cause secondary changes such as carcinogenesis. However, methylation abnormalities of AIP have rarely been investigated. Genomic analysis by DNA methylation array is a commonly used method to evaluate methylation abnormalities associated with carcinogenesis and autoimmune diseases.

Using DNA methylation array results from AIP tissues, we identified *SKI* as a candidate gene. We examined the methylation levels of *SKI* using Quantitative SYBR green methylation-specific PCR (QSG-MSP) and immunohistochemistry. This study evaluated the methylation levels of *SKI* with the clinicopathological data of AIP patients.

2. Materials and methods

2.1. Patients and tissue samples

We identified 10 AIP cases, 14 pancreatic ductal adenocarcinoma (PDA) cases without a history of AIP, and 9 normal pancreas (NP) cases that were resected for non-pancreatic carcinoma without a history of AIP, at Shinshu University Hospital or one of its affiliated hospitals from 1996 to 2013. In PDA cases, the obstructive pancreatitis (OP) area was used for various measurements. All AIP cases met the diagnostic criteria for AIP. Specimens were retrieved from archived tissue blocks. This study was approved by the Ethics Committee of Shinshu University, Japan. The demographic data of patients are provided in Table 1. All lesions were reviewed by two pathologists (T.U. and H.O.) for pathologic diagnosis and areas for manual macrodissection were selected by hematoxylin and eosin (H&E)-stained tissue sections. For comparison with AIP and chronic inflammation other than AIP, the OP area was used for analyses. For comparison between AIP and normal pancreas without tumor, the following 9 normal samples were used: one case of injury due to accident and 8 cases of pancreatic tissue excised together with extra-pancreatic lesion. These cases did not have any neoplastic lesions in the pancreas.

2.2. Methylation array

Methylation analysis was performed on 10 AIP cases, 14 PDA cases, and 4 NP cases using the Illumina Infinium Human Methylation 450K (HM450K) BeadChip array (Illumina, San Diego, CA). Images were obtained using the Genome Studio version 2011.1 methylation module (Illumina). The methylation score for each CpG group was represented as a beta-value according to the fluorescence intensity ratio. Beta-values may be any value between 0 (non-methylated) and 1 (completely methylated). The HM450K protocol required 1 μg of bisulfite-converted DNA. For bisulfite treatment, genomic DNA was extracted from 5-μm-thick, formalin-fixed and paraffin-embedded unstained tissue sections of 10 AIP, 14 PDA, and 4 NP samples, using the QIAamp DNA Minikit (Qiagen Inc., Valencia, CA). DNA was modified with sodium bisulfite treatment using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) following the manufacturer's instructions.

Table 1
Clinical features of patients with autoimmune pancreatitis (AIP), pancreatic ductal adenocarcinoma (PDA), and normal pancreas (NP).

	AIP	PDA	NP
Age	72.0 (69.5–77.5)	70.5 (64.0–73.3)	69.0 (66.0–76.0)
Sex			
Male	9	11	6
Female	1	3	3
IgG4 (mg/dL)	250.0 (122.0–1415.0)	23.0 (20.0–33.0)	na

*Normal value for IgG4: < 70 mg/dL (cut-off: 135 mg/dL).
AIP, autoimmune pancreatitis; PDA, pancreatic ductal adenocarcinoma; NP, normal pancreas; na, not available.

2.3. Quantitative SYBR green methylation-specific PCR (QSG-MSP)

QSG-MSP was performed to quantify the levels of CpG DNA methylation of *SKI* using the Applied Biosystems 7500 real time PCR system (Applied Biosystems, Foster City, CA) as previously reported [12]. Primers for QSG-MSP were designed using Methyl Primer Express Software v1.0 (Applied Biosystems). The presence of CpG islands was determined using Methyl Primer Express v1.0 software (Applied Biosystems). Primer sequences were 5′-TAAAGTCGGGGATGGTAGGAC-3′ (forward) and 5′-CGATCGCGATTCTTAAAAAAC-3′ (reverse). Primers for the control *ACTB* (beta-actin) gene were 5′-TGGTGATGGAGGAGG TTTAGTAAGT-3′ (forward) and 5′-AACCAATAAAACCTACTCCTCCCT TAA-3′ (reverse) as previously described [12]. Quantitative PCR was performed in a 25-μL reaction volume with 12.5 μL of 2 × SYBR Green PCR Master Mix (Applied Biosystems), 2.5 pmol of each primer, and 25 ng of bisulfite-treated DNA sample. Thermal cycling was as follows: 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Only samples with amplification at the correct melting temperature were used for further analyses of methylation. The amount of methylated DNA (percentage of methylated reference) was calculated as follows: ratio of quantity of target gene to quantity of target gene of test sample divided by quantity of *ACTB*.

2.4. Immunohistochemistry

Immunohistochemical staining was performed on 4-μm-thick formalin-fixed, paraffin-embedded, tissue sections, using anti-SKI antibody (1:500; Santa Cruz, CA, USA). Briefly, deparaffinized tissue sections were treated with 0.3% H_2O_2 for 30 min to inhibit endogenous peroxidase followed by antigen retrieval using microwave heating in EDTA/Tris buffer (pH 9.0) for 25 min. Overnight incubation at 4 °C with primary antibody against SKI diluted in bovine serum albumin was followed by incubation with the secondary antibody (NOVOLINK-Polymer Detection Systems) for 1 h at room temperature. Staining was developed by reaction with 3,3′-diaminobenzidine substrate-chromogen solution, followed by counterstaining with hematoxylin.

Staining results for the above antibody were evaluated by two pathologists (T.U. and H.O.) using a semi-quantitative method. We defined a staining-index (SI), which was calculated as the sum of two scores: an intensity score (IS) and a percentage score (PS). Previous studies showed positive expression of SKI by immunohistochemical analyses in both the cytoplasm and nucleus of malignant tumors, such as breast carcinoma and malignant melanoma [13–15]. The IS was scored on a four-tier scale based on the degree of cytoplasmic and nuclear staining intensity in most epithelial cells (score 0: no staining; 1: weak; 2: moderate; 3: strong). The PS was scored on a four-tier scale based on the percentage of cells exhibiting positive cytoplasmic and nuclear staining among all target cells (score 0: no staining; 1: < 10%; 2: 10–50%; 3: > 50%). Therefore, total SI scores ranged between 0 and 6.

2.5. Statistical analysis

Statistical analyses of clinical data were performed using the Chi-