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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-

squared test or Wilcoxon rank sum test. Methylation array data, QSG-MSP data, and immunohistochemistry data were analyzed using the Wilcoxon rank sum test. The Spearman's rank correlation coefficient was used to assess correlations. Statistical analyses were conducted using JMP software version 10 (SAS Institute Japan, Japan). *P* values of < 0.05 were considered significant.

3. Results

3.1. Clinical features

There were no significant differences in gender between AIP and PDA cases ($P = 0.4589$), or AIP and NP cases ($P = 0.2129$) (Table 1). There were no significant differences in age between AIP (mean 72 years, range 69.5–77.5) and PDA (mean 70.5 years, range 64.0–73.3) cases ($P = 0.2069$), or AIP (mean 72 years, range 69.5–77.5) and NP (mean 69 years, range 66.0–76.0) cases ($P = 0.4128$) (Table 1).

The serum concentrations of IgG4 were measured in nine AIP (mean 250 mg/dL, range 122–1415) and 11 PDA (mean 23 mg/dL, range 20–33) cases (Table 1). Seven AIP cases had high serum IgG4 levels (cut-off, 135 mg/dL). All PDA cases had low serum levels. The serum concentrations of IgG4 were not measured in NP cases.

3.2. Histological findings

All AIP cases had lymphoplasmacytic inflammation, storiform fibrosis, and abundant IgG4-positive cells (≥10 cells/high-power field) (Fig. 1A and B). No PDA or NP cases exhibited the features of IgG4-RD.

3.3. Beta-values of methylation array and the methylation rate of QSG-MSP

We conducted a methylation array to identify hypomethylated genes in AIP. *SKI* returned high beta-values in NP, representing hypermethylation, and low beta-values in AIP, representing hypomethylation, using HM450K. Notably, *SKI* had the largest difference between beta-values in AIP and NP. Therefore, we compared the methylation levels of *SKI* with clinicopathological data from AIP cases.

The *SKI* beta-value was significantly lower in AIP (mean 0.24, range 0.17–0.27) compared with PDA (mean 0.50, range 0.28–0.67) and NP (mean 0.81, range 0.66–0.84) cases ($P = 0.0014$ and $P = 0.0058$) (Fig. 2A). The *SKI* beta-value for PDA cases (mean 0.50, range,

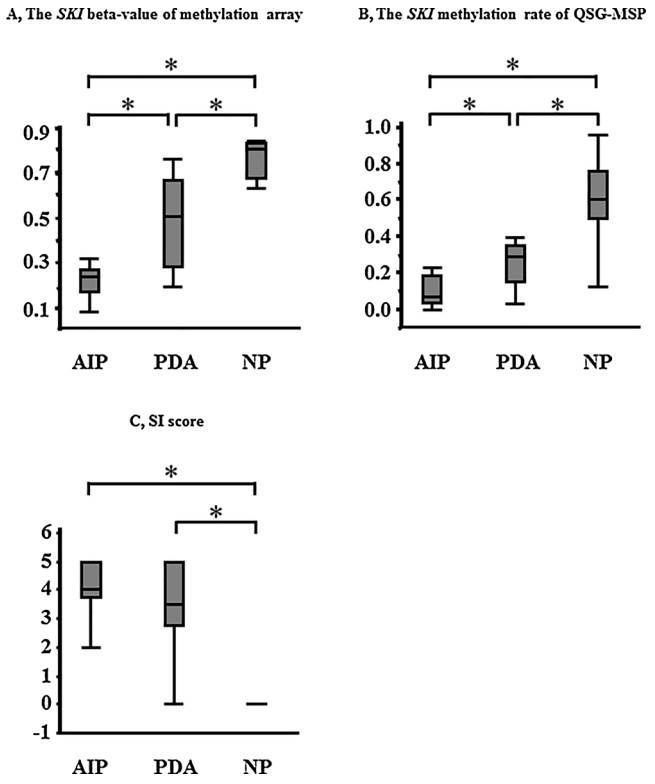

Fig. 2. Box plot of the *SKI* beta-values from methylation array, *SKI* methylation rates of QSG-MSP, and SI scores in patients with autoimmune pancreatitis (AIP) vs. obstructive pancreatitis area in pancreatic ductal adenocarcinoma (PDA) and normal pancreas (NP).

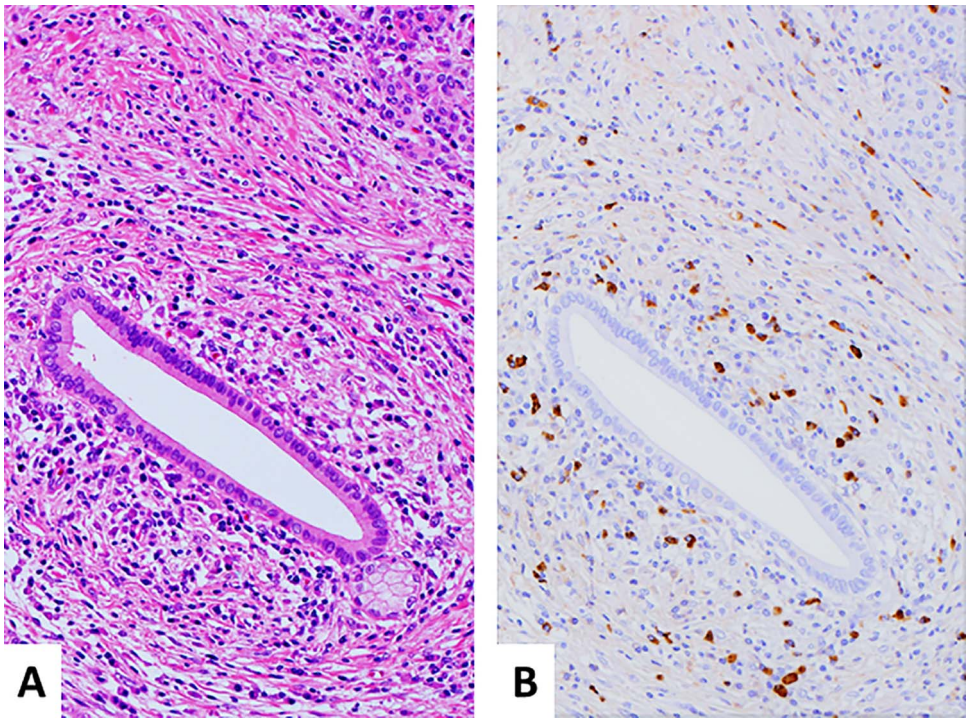

Fig. 1. Pathology of autoimmune pancreatitis (AIP) and SKI immunostaining. (a) Lymphoplasmacytic infiltration with storiform fibrosis in AIP (H&E). (b) IgG4$^+$ plasma cells in AIP tissue (IgG4 immunostaining). Original magnification: × 25.

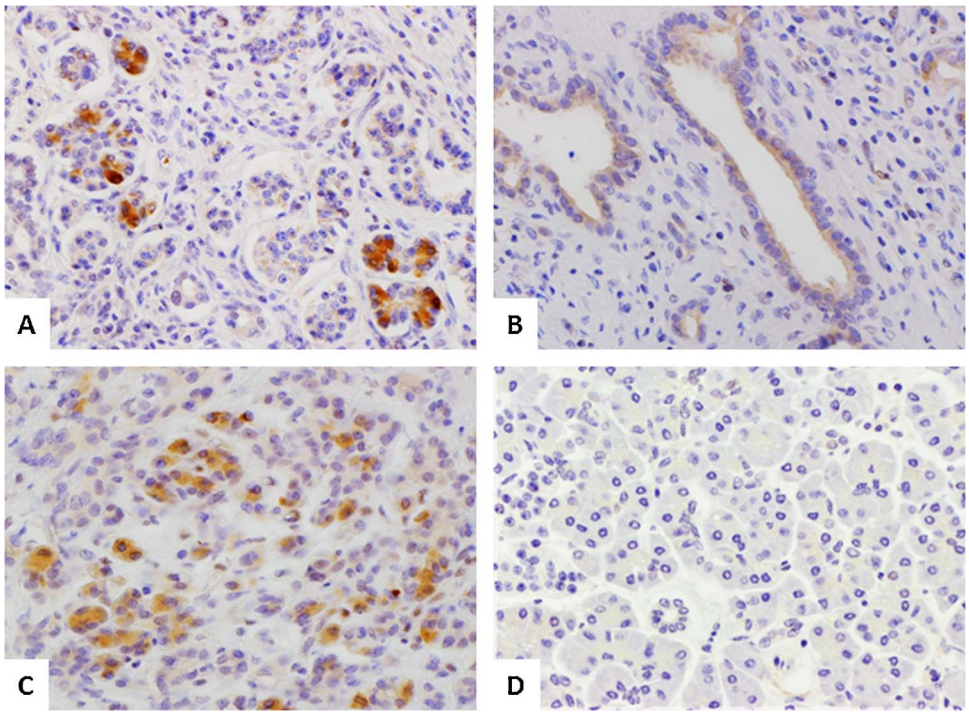

Fig. 3. Immunohistochemical detection of SKI expression in samples from autoimmune pancreatitis (AIP), obstructive pancreatitis area in pancreatic ductal adenocarcinoma (PDA), and normal pancreas (NP). SKI staining in acinar cells (A) and pancreatic duct cells (B) in AIP. SKI staining in acinar cells (C) in PDA and NP (D). Original magnification: × 25.

0.28–0.67) was also significantly lower than that of NP (mean 0.81, range, 0.66–0.84) cases ($P = 0.0169$) (Fig. 2A).

By QSG-MSP, the *SKI* methylation ratio was significantly lower in AIP cases (mean 7.11, range 3.35–18.45) compared with PDA (mean 28.89, range 14.87–35.15) and NP (mean 60.55, range 49.72–76.11) cases ($P = 0.0041$ and $P = 0.0011$) (Fig. 2B). The *SKI* methylation ratio in PDA (mean 28.89, range 14.87–35.15) cases was also significantly lower than that of NP (mean 60.55, range 49.72–76.11) cases ($P = 0.0015$) (Fig. 2B).

3.4. Immunohistochemistry of SKI

Positive SKI signals were identified in the cytoplasm of acinar and pancreatic duct cells in both AIP (Fig. 3A and B) and PDA cases (Fig. 3C). The pattern of SKI-positive cells varied from diffusely positive to scattered positive. The density of positive cells also varied from strong to weak. No positive cells were identified in NP samples (Fig. 3D).

The SI score of immunohistochemical staining was significantly higher in AIP (mean 4.00, range 3.75–5.00) samples than in NP (no signal) samples ($P = 0.0001$) (Fig. 2C). There was no significant difference in SI score between AIP (mean 4.00, range 3.75–5.00) samples and PDA (mean 3.50, range 2.75–5.00) samples ($P = 0.2610$) (Fig. 2C). The SI score was significantly higher in PDA (mean 3.50, range, 2.75–5.00) samples than in NP (no staining) ($P = 0.0001$) (Fig. 2C).

3.5. Correlations between clinicopathological data, QSG-MSP methylation rate, and SI score in AIP

There was no statistically significant correlation between SI score and the *SKI* methylation ratio in AIP cases ($r = -0.6142$, $P = 0.0785$) (Table 2), although they had a tendency towards a moderate negative correlation. There was strong negative correlation between the serum concentrations of IgG4 and the *SKI* methylation ratio ($r = -0.8503$, $P = 0.0075$) (Table 2). There was a moderate positive correlation between the serum concentrations of IgG4 and SI ($r = 0.6739$, $P = 0.0466$) (Table 2). There was no correlation between age and the *SKI* methylation ratio ($r = -0.4430$, $P = 0.2323$), or age and SI ($r = 0.0358$, $P = 0.9218$) (Table 2). There was no correlation between serum IgG4 concentrations and age ($r = 0.0588$, $P = 0.8805$) (Table 2).

Table 2
Correlations between clinicopathological data, beta-values of the methylation array, methylation rates of QSG-MSP, and the SI scores in AIP.

		Age	IgG4	Methylation ratio	SI score
Age	r				
	P				
IgG4	r	0.0588			
	P	0.8805			
Methylation ratio	r	−0.443	−0.8503		
	P	0.2323	0.0075*		
SI score	r	0.0358	0.6739	−0.6142	
	P	0.9218	0.0466*	0.0785	

r, Spearman's rank correlation coefficient; *P*, *p*-value.
Methylation ratio, the methylation rate of QSG-MSP.
* $P < 0.05$.

4. Discussion

We identified a novel methylation abnormality in *SKI* characterized by hypomethylation in AIP patients. There have been few reports regarding methylation array analysis in AIP, and there has been no previous report on the role of *SKI* in AIP.

Differences in the analysis methods used might explain the failure to observe a correlation between the *SKI* beta-values from the methylation array and the QSG-MSP-based *SKI* methylation rate values in AIP. A correlating trend between SI score and the *SKI* methylation rate suggests that immunostaining is an important tool for assessing *SKI* methylation abnormalities, and the evaluation of methylation abnormalities by immunostaining was previously reported for many genes [16].

Our study identifies a clear difference in the methylation of *SKI* between AIP and NP cases and between PDA and NP cases. Inflammatory changes in AIP and PDA may affect the methylation levels of *SKI*. However, as there may be a difference in the methylation

levels of *SKI* between AIP and PDA, additional factors may also be involved. Age might be an important factor that influences methylation abnormalities, but there were no differences in patient age between groups in our study.

SKI encodes a transforming protein of the avian Sloan-Kettering retrovirus that was originally shown to exhibit oncogenic activity [17,18]. In the early stages of tumor development, SKI shows oncogenic activity in repressing the tumor suppressive activities of TGF-beta/Smads in inducing apoptosis and cell growth arrest [18]. In contrast, other findings showed that *SKI* contains anti-oncogenic activity in mammalian cells in the later stages of malignancy involved in invasion and metastasis [18,19]. *SKI* is overexpressed in pancreatic cancer [20], melanoma [15,21], esophageal cancer [22], colorectal cancer [23], and acute myeloid leukemia [24]. In AIP, *SKI* may function as an oncogene, which reflects its activity at early stages of tumor development. Although *SKI* might be an oncogene, its carcinogenic potential might be suppressed by the hypermethylation of its promoter region. Hypomethylation of the *SKI* promoter may result from the characteristic chronic inflammation present in AIP [25] and the hypomethylation of oncogenes was reported to be associated with carcinogenesis [26]. Therefore, *SKI* hypomethylation may be involved in carcinogenesis in AIP.

Only one previous report has described a relationship between *SKI* and pancreatic cancer, but no study has provided a detailed description on the expression pattern of SKI by immunohistochemistry; however, normal pancreas seems to show negative *SKI* expression [20]. In contrast, other reports in breast carcinoma and malignant melanoma cases showed positive SKI immunohistochemical staining in the cytoplasm as well as nucleus of tumor cells [13–15]. In our study, SKI was expressed in acinar cells as determined by immunostaining in OP and AIP, indicating that *SKI* may be associated with pancreatic acinar cell carcinoma. Similarly, SKI expression is also detectable in pancreatic ductal cells, and may therefore be involved in pancreatic duct adenocarcinoma. There are several case reports regarding carcinogenesis in AIP [27]. While reports that *KRAS* abnormalities are present in carcinogenesis with AIP exist [28,29], it remains controversial whether *KRAS* is functionally involved in carcinogenesis associated with AIP. To date, only a single report by Kinugawa et al. [12] has proposed a relationship between AIP-associated carcinogenesis and methylation abnormalities.

The results of the current study suggest there may be an association between the serum concentrations of IgG4 and *SKI* methylation levels. Abnormal methylation of *SKI* may be involved in the onset of AIP, because *SKI* also influences inflammatory and immune responses via TGF-beta 1 [30]. Systemic lupus erythematosus, multiple sclerosis, autoimmune thyroiditis and other autoimmune diseases have been associated with the hypomethylation of genes [31–33]. In autoimmune thyroiditis, hypomethylation of the ICAM1 promoter region increases the expression of ICAM1 and may be involved in the onset of autoimmune thyroiditis [33]. Just as DNA methylation is a source of genetic variation that affects the risk of autoimmune diseases in other autoimmune diseases, *SKI* methylation abnormality in AIP may also be an important factor in AIP risk.

A limitation of our study is that it remains unknown whether *SKI* functions to drive mutations in the carcinogenesis process. To determine the involvement of *SKI* in carcinogenesis, *SKI*-expressing gene transfer in genetically modified mice is required. Furthermore, the accumulation of a larger number of samples from AIP cases is warranted for data that are more robust.

In conclusion, our study suggests that *SKI* hypomethylation might play an important role in AIP, and related carcinogenesis. However, the role of *SKI* has not been clearly elucidated and further studies are needed to understand further the function of *SKI*.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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