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Association Between Immunoglobulin G4–related Disease and Malignancy within 12 Years after Diagnosis: An Analysis after Longterm Followup

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ABSTRACT. ***Objective.*** Because it is uncertain whether immunoglobulin G4–related disease (IgG4-RD) is associated with malignancy, we evaluated the incidence of cancer development in a large cohort of patients with IgG4-RD.

Methods. The study enrolled 158 patients diagnosed as having IgG4-RD between 1992 and 2012. We calculated the standardized incidence ratio (SIR) and cumulative rate of malignancies in this group and searched for risk factors associated with the occurrence of tumors.

Results. A total of 34 malignancies were observed in the patients with IgG4-RD over a mean followup period of 5.95 ± 4.48 years. The overall SIR of malignancies was 2.01 (95% CI 1.34–2.69). The SIR of patients who exhibited a tumor within 1 year after IgG4-RD diagnosis was 3.53 (95% CI 1.23–5.83), while that of subjects forming a malignancy in subsequent years was 1.48 (95% CI 0.99–1.98). The cumulative rate of malignancy development was significantly higher in patients with IgG4-RD within 12 years after diagnosis than in the Japanese general population. Comparable results were obtained for an autoimmune pancreatitis subgroup. The serum concentrations of several disease activity markers at diagnosis were significantly higher in patients with malignancies than in those without.

Conclusion. We identified a close association between IgG4-RD and malignancy formation within 12 years after diagnosis, particularly during the first year. An active IgG4-RD state is presumed to be a strong risk factor for malignancy development. (First Release October 15 2015; J Rheumatol 2015;42:2135–42; doi:10.3899/jrheum.150436)

Key Indexing Terms:
IMMUNOGLOBULIN G4–RELATED DISEASE
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ACTIVITY MARKER

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Immunoglobulin G4–related disease (IgG4-RD) is a systemic condition characterized by high serum IgG4 concentration and IgG4-bearing plasma cell infiltration in affected organs[1,2,3]. The concept of IgG4-RD was established through extensive evaluation of extrapancreatic lesions complicating autoimmune pancreatitis (AIP)[2]. IgG4-RD characteristically involves multiple organs, and is believed to manifest as Mikulicz disease[4], respiratory disorders[5], sclerosing cholangitis[6], retroperitoneal fibrosis[3], tubulointerstitial nephritis[7], and prostatitis[8]. Although corticosteroid therapy is effective for IgG4-RD, relapses sometimes occur during dose tapering and maintenance phases[9,10,11].

The longterm outcome of this new disease entity has been described to include the complication of malignancy development[12,13,14]. Yamamoto, *et al* reported that patients with IgG4-RD were significantly more prone to malignancies than the general population in a followup study of 105 patients[15]. Shiokawa, *et al* uncovered similar results in 108 patients with AIP[16]. Because the occurrence of malignancies in the first year after diagnosis was significantly higher than in subsequent years, the authors proposed that AIP may feature

aspects of a paraneoplastic syndrome and that the fate of patients with AIP was closely influenced by the occurrence and treatment of malignancies. On the other hand, Hirano, *et al* reported that IgG4-RD was not significantly associated with malignancy after evaluating 113 patients with IgG4-RD[17]. This may have been because Hirano excluded cases of malignancies that were diagnosed concomitantly with IgG4-RD, and Yamamoto and Shiokawa did not. Hart, *et al* also noted that 116 patients with type 1 AIP did not have significant numbers of malignancies when compared with 344 control subjects from a primary care clinic[18]. Accordingly, the issue of whether an association exists between IgG4-RD and malignancy remains controversial.

Close relationships have been identified between chronic inflammation and malignancy, including one between gastric cancer and gastritis from *Helicobacter pylori*[19,20] and another between hepatocellular carcinoma and viral hepatitis[21,22]. Similarly, the chronic inflammatory state of IgG4-RD may be related to malignancies in systemic organs in which IgG4-RD is found. Further, because IgG4-RD is recognized as an autoimmune disease that occurs predominantly in the elderly, a deficiency in immune surveillance may trigger the occurrence of this disease, which in turn induces associated malignancies[23]. Older patient age also appears to be a major contributing factor to the occurrence of malignancies in general[24]. It will be necessary to precisely identify the time of IgG4-RD onset, affected lesions, and various other risk factors in a large number of patients following longterm observation to clarify whether IgG4-RD is related to malignancy. The aim of our present study is to determine the key relationships between IgG4-RD and malignancy, such as whether IgG4-RD is significantly complicated by malignancy.

MATERIALS AND METHODS

Patients. We enrolled 158 patients with IgG4-RD (119 men and 39 women, median age at IgG4-RD onset: 72 years) who had been diagnosed based on the Japanese Comprehensive Diagnostic Criteria for IgG4-RD[25] between 1992 and 2012 at our clinic or affiliated hospitals. The cohort included 109 patients with type 1 AIP (84 men and 25 women, median age at AIP onset: 66 yrs), among whom 103 patients possessed extrapancreatic lesions. AIP was diagnosed according to the International Consensus Diagnostic Criteria 2011 (ICDC 2011), which were based mainly on characteristic imaging findings, high serum IgG4 concentration, the presence of extrapancreatic lesions (other IgG4-RD), and steroid responsiveness and less on pathological findings because pancreatic biopsy samples have been difficult to obtain in sufficient sample sizes for correct diagnosis[26]. One hundred and eleven patients received steroid therapy when involved vital organs exhibited a risk of serious organ dysfunction or failure, such as obstructive jaundice due to pancreatic head swelling or urethral stenosis from retroperitoneal fibrosis, or when severe symptoms, including unbearable abdominal pain, were evident. Steroid treatment was carried out at our institute according to the Japanese consensus guidelines for AIP, which recommend a minimum of 3 years of maintenance therapy[11].

Survey for complication of malignancy. Of the 158 patients, we searched for the complication of malignancy in 142 subjects by examining medical records dated until December 2013. For the 16 patients who discontinued treatment at our institutions during the study period, we sent questionnaires for the clinical survey of malignancy and obtained replies from 8 individuals. We screened for the occurrence of malignancy up until the time of last contact for the remaining 8 patients with whom we had lost contact during the survey period. Malignancies before IgG4-RD diagnosis were not analyzed in our present study.

Analysis for correlation between IgG4-RD and malignancy. The standardized incidence ratio (SIR) of malignancy in our cohort was calculated to evaluate whether IgG4-RD was significantly complicated by malignancy by adopting the cancer incidence rates for the Japanese general population as stratified by sex, 5-year age groups, and calendar year[27]. The SIR was calculated by dividing the actual number of malignancies by the expected number if the cohort exhibited malignancies at the same age-stratified rate as the Japanese general population. Further, we determined the 95% CI using normal approximation based on Poisson's distribution. The occurrence of malignancy in IgG4-RD was considered to be significantly elevated when the lower value in this interval exceeded 1.00. We analyzed 2 patient groups in our present study: one that included patients who were diagnosed as having IgG4-RD and malignancy concurrently and another that excluded such patients. A concurrent diagnosis of IgG4-RD and malignancy meant that a malignancy was diagnosed during the period of intensive examination using computed tomography and magnetic resonance imaging for the detection of IgG4-RD, which usually lasted about 1-3 months after the suspicion of IgG4-RD. However, in most cases, intensive examination using these image tests continued about 3 months after diagnosis because of the evaluation for steroid effects and the check for relapse occurrence, suggesting that malignancies may be easily found in these periods 3 months after IgG4-RD diagnosis. Accordingly, we defined the period of a concurrent diagnosis as within 3 months before or after IgG4-RD diagnosis.

To evaluate the possibility of paraneoplastic syndrome manifesting in IgG4-RD, we calculated and compared the SIR of patients in whom malignancies were found to complicate IgG4-RD within 1 year and 1 year or more after diagnosis.

Identical procedures were performed for the AIP subgroup in our cohort because several previous studies were restricted to patients with AIP only.

The Kaplan-Meier method was used to estimate the cumulative rate of malignancy development. Cancer incidence rate curves were calculated using the data per 100,000 people according to year, age, and sex as reported by the Ministry of Health, Labor, and Welfare of Japan. The log-rank test was adopted to test hypotheses concerning the differences in malignancy development between the IgG4-RD group and the Japanese general population. Information on the Japanese general population was obtained with regard to sex, cancer site, 5-year age groups, and calendar year during the period of 1975-2008[27,28]. Because the general population sample size was very large, the widths of its 95% CI were nearly zero.

Risk factors for the occurrence of malignancy in IgG4-RD. We searched for risk factors of malignancy complications by comparing clinical variables between all patient groups with and without malignancies, including IgG4-RD onset age, sex, serum levels of various activity markers [IgG4, IgG, complement proteins, soluble interleukin 2 receptor (sIL-2R), and circulating immune complex (CIC)], number of lesions, experience of corticosteroid therapy, and occurrence of relapse. We defined relapse as a reappearance of IgG4-RD symptoms, elevation of disease activity markers, and identification of active lesions in diagnostic imaging. Because alcohol intake, smoking, and diabetes mellitus (DM) are also considered major risk factors for the development of malignancies, we evaluated the effects of those factors as well. Alcohol intake and smoking were defined as daily consumption of > 20 g of alcohol and > 10 cigarettes, respectively, at the diagnosis of IgG4-RD. DM was assessed before or around IgG4-RD diagnosis, whereby a fasting glucose level of > 126 mg/dl and/or glycosylated hemoglobin level of > 6.5% was judged as indicative of DM[29]. The same procedures were done for the AIP subgroup.

Statistics. Differences between groups were analyzed using the Mann-Whitney test for continuous data and the chi-squared test or Fisher's exact test for categorical data. Statistical analyses were performed using Stat Flex version 6 software (Artech Co. Ltd.). All tests for Kaplan-Meier

analysis were calculated with the IBM SPSS Statistics Desktop for Japan (version 19.0; IBM Japan Inc.). A p value of < 0.05 was considered statistically significant.

Ethics. Our present study was approved by the ethics committee of our institute (Approval Code 2602).

RESULTS

Malignancies complicating IgG4-RD. Among the 158 patients with IgG4-RD who were followed for a mean period of 5.95 ± 4.48 years, we identified 36 malignancies in 34 patients, which included 5 cases each of lung, colon, and prostate cancer and 4 cases each of gastric and pancreatic cancer (Table 1). Among the 109 patients with AIP, we detected 30 malignancies in 28 patients, which included 5 cases of prostate cancer and 4 cases each of lung and pancreatic cancer (Table 1). When malignancies other than those occurring concurrently with IgG4-RD diagnosis were analyzed in our cohort, a total of 29 malignancies were found in 27 patients. When the period for developing malignancies after IgG4-RD diagnosis was set at before and after 5 years, 26 patients demonstrated the occurrence of malignancies before 5 years versus 8 patients afterward. Consequently, the occurrence of malignancies tended to be most frequent within 5 years after IgG4-RD diagnosis (Figure 1).

In our present study, the malignancies found in 11 patients were successfully treated by surgery, chemotherapy, or radiotherapy, after which 8 patients experienced no relapse during or after subsequent corticosteroid therapy. The remaining 3 patients did not receive corticosteroid therapy. We encountered no cases of genuine paraneoplastic syndrome that were apparently improved following surgery and/or chemotherapy alone.

Table 1. Number of malignancies in patients with IgG4-RD and AIP.

Type of Malignancy	Total No. Malignancies in Patients with IgG4-RD	Total No. Malignancies in Patients with AIP
Total	36	30
Lung	5	4
Colon	5	3
Prostate	5	5
Stomach	4	3
Pancreas	4	4
Kidney	2	1
Lymphoma	2	2
Biliary tract	1	1
Liver	1	1
Esophagus	1	0
Breast	1	1
Ovary	1	1
Thyroid	1	1
Skin	1	1
Tongue	1	1
Myelodysplastic syndrome	1	1

IgG4-RD: immunoglobulin G4–related disease; AIP: autoimmune pancreatitis.

We observed that 12 patients experienced malignancy in the same organ affected by IgG4-RD: pancreatic and lung cancer in 4 patients each, prostate in 2, and bile duct and kidney cancer in 1 each.

Overall analysis of IgG4-RD's association with the occurrence of malignancy. The expected incidence of cancer in our IgG4-RD cohort according to rates for the Japanese general population during an overall followup of 940 person-years was 16.9. Based on this, we first calculated the overall SIR of malignancies after IgG4-RD diagnosis, which was 2.01 (95% CI 1.34–2.69) and indicative that IgG4-RD was significantly complicated by malignancy (Table 2). Next, we selected the patients whose malignancies were not diagnosed concurrently with IgG4-RD and witnessed an SIR of 1.60 (95% CI 1.07–2.13), which also represented a significant result.

Comparison of SIR of malignancies found within 1 year after IgG4-RD diagnosis with that of tumors detected in subsequent years. The SIR of malignancies identified within 1 year after IgG4-RD diagnosis was 3.53 (95% CI 1.23–5.83), whereas that for subsequent years was 1.48 (95% CI 0.99–1.98), indicating a significant occurrence of malignancies in the first year after IgG4-RD diagnosis that became less frequent afterward (Table 2).

Analysis of type 1 AIP in IgG4-RD. The expected incidence of cancer in our AIP cohort according to rates for the Japanese general population during an overall followup of 740 person-years was 13.4. Based on this, the calculated SIR restricted to type 1 AIP was 2.08 (95% CI 1.32–2.85), indicating that type 1 AIP was significantly associated with malignancy as well. Moreover, the SIR of malignancies within 1 year after type 1 AIP diagnosis was 3.91 (95% CI 1.02–6.80), whereas that for subsequent years was 1.57 (95% CI 0.90–2.23), suggesting that the occurrence of malignancies in type 1 AIP was also significantly more frequent within the first year and less frequent afterward (Table 2). Next, we selected the patients whose malignancies were not diagnosed concurrently with AIP and witnessed an SIR of 1.71 (95% CI 1.01–2.41), also a significant result.

Kaplan-Meier analysis of cumulative malignancy rate between the IgG4-RD group and the Japanese general population. In Kaplan-Meier testing, the lower limit of the 95% CI for cumulative malignancy rate for the IgG4-RD group was higher than that for the Japanese general population during the first 12 years after diagnosis, according to log-rank testing (Figure 2).

Analysis of malignancy type. SIR calculations for each malignancy type uncovered no significant results. Pancreatic cancer demonstrated an SIR of 5.48 (95% CI 0.11–10.85), which suggested a non-significant association with IgG4-RD. Comparable results were obtained for each malignancy type for the AIP group, in which the SIR for pancreatic cancer was markedly higher (Table 3).

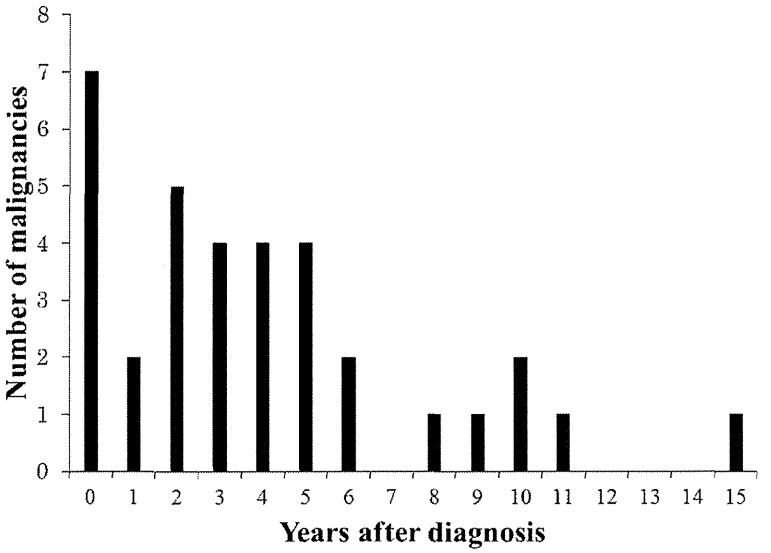

Figure 1. Incidence of malignancies according to time after IgG4-RD diagnosis. Twenty-six patients experienced malignancies within the first 5 years after diagnosis versus 8 patients afterward, indicating that the occurrence of malignancies tended to be most frequent soon after IgG4-RD diagnosis. IgG4-RD: immunoglobulin G4–related disease.

Table 2. SIR of malignancies in IgG4-RD and AIP.

	IgG4-RD		AIP	
	SIR	95% CI	SIR	95% CI
Overall	2.01	1.34–2.69	2.08	1.32–2.85
Without concurrent diagnosis	1.60	1.07–2.13	1.71	1.01–2.41
Years after IgG4-RD diagnosis				
< 1	3.53	1.23–5.83	3.91	1.02–6.80
≥ 1	1.48	0.99–1.98	1.57	0.90–2.23

SIR: standardized incidence ratio; IgG4-RD: immunoglobulin G4–related disease; AIP: autoimmune pancreatitis.

Risk factors associated with malignancy development. To identify the risk factors associated with the formation of malignancies, we compared several clinical variables between IgG4-RD patients with malignancies and those without (Table 4). Serum concentrations of IgG, IgG4, sIL-2R, and CIC were significantly higher in patients with malignancies ($p = 0.002$, 0.005, 0.005, and 0.019, respectively). In contrast, a history of corticosteroid treatment, number of recurrences, alcohol intake, smoking, and DM all showed no significant associations with risk (Table 4). Similar results were obtained for the AIP group apart from a negative result for CIC (Table 4).

DISCUSSION

Our present study showed that IgG4-RD and AIP were significantly associated with the occurrence of malignancies, which was consistent with reports by Yamamoto, *et al* and Shiokawa, *et al*[15,16]. However, Hirano, *et al* found that the incidence of total malignancies in IgG4-RD was similar to that of the Japanese general population[17]. This discrepancy may be attributed to differences in study protocols; whereas Hirano excluded patients who were concomitantly diagnosed as having IgG4-RD and malignancies to avoid selection bias, the Yamamoto and Shiokawa studies included those subjects. We were able to confirm that IgG4-RD (AIP) was significantly associated with malignancy even in patients without a concurrent diagnosis of malignancy. In Kaplan-Meier testing, the cumulative malignancy rate for the IgG4-RD group was significantly higher than that for the Japanese general population up to 12 years after diagnosis, according to log-rank testing. These results indicated that IgG4-RD was significantly associated with the occurrence of malignancies within this period. This significant association disappeared afterward, likely due to a higher malignancy rate attributed to older age.

There may have been detection bias in our present study for the diagnosis of malignancy because patients with IgG4-RD were likely followed more closely than the general population, which could have resulted in a more timely and frequent detection of malignancy. In addition, many patients were referred to a tertiary care center that promptly made the diagnosis of malignancy using intensive imaging examination. To mitigate this selection bias, we also examined subjects after excluding those with a concurrent diagnosis of malignancy. This group showed a significant association with malignancy as well. On the other hand, Hart, *et al* compared the occurrence of malignancies between type 1 AIP and control subjects from a primary care clinic, in which the risk of detection bias could be ignored, and found no significant

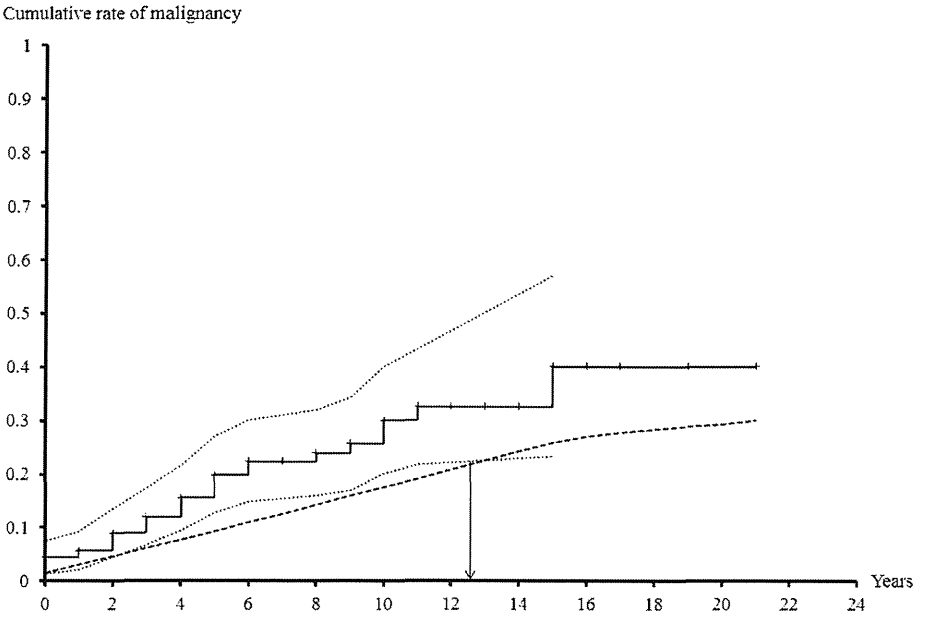

Figure 2. Cumulative malignancy rate for patients with IgG4-RD (solid line) and for the Japanese general population (broken line). The development rate of malignancy was higher for patients with IgG4-RD until 12 years after IgG4-RD diagnosis, according to log-rank testing. IgG4-RD: immunoglobulin G4–related disease.

Table 3. SIR for each malignancy in IgG4-RD and AIP.

Type of Malignancy	IgG4-RD		AIP	
	SIR	95% CI	SIR	95% CI
Lung	2.09	0.26–3.93	2.10	0.26–3.94
Colon	1.75	0.22–3.29	1.33	0.17–2.48
Prostate	2.05	0.25–3.85	2.07	0.26–3.88
Stomach	1.43	0.03–2.83	1.35	0.03–2.66
Pancreas	5.48	0.11–10.8	6.81	0.13–13.5

SIR: standardized incidence ratio; IgG4-RD: immunoglobulin G4–related disease; AIP: autoimmune pancreatitis.

association of malignancy occurrence between the groups[18]. However, the control subjects from the primary care clinic likely carried the risk of more frequent complications of malignancies than the general population because their visit may have been due to malignancy-related complaints.

The question arises of whether some cases of IgG4-RD and AIP should be regarded as types of paraneoplastic syndrome. The occurrence of malignancies was significantly more frequent within the first year after IgG4-RD diagnosis compared with that of subsequent years. This was in agreement with the study by Shiokawa, *et al*, showing that IgG4-RD may be considered as a type of paraneoplastic syndrome, which was found to be the case for AIP[16]. Paraneoplastic syndrome is described as systemic inflammatory diseases provoked by inflammatory mediators generated by malignancies[30]. If IgG4-RD is indeed a paraneoplastic syndrome, treatment of the underlying malignancy may result in amelioration of IgG4-RD[31]. In our present study, 8 patients whose malignancies were successfully treated by surgery, chemotherapy, or radiotherapy experienced no relapse during or after subsequent corticosteroid therapy, thus strengthening the hypothesis that IgG4-RD can be regarded as a type of paraneoplastic syndrome, although we have encountered no cases of genuine paraneoplastic syndrome that were apparently improved following surgery and/or chemotherapy alone. Several cases of pancreatic cancer complicated with AIP have been described as showing abundant IgG4-bearing plasma cell infiltration, suggesting that pancreatic cancer could initiate an immune response with subsequent plasma cell infiltration in pancreatic and peripancreatic tissue, a hallmark of AIP[32,33,34]. However, because not all of the cases of IgG4-RD were associated with malignancies, it is possible that another mechanism apart from paraneoplastic syndrome, such as a failure in immune surveillance, leads to a loss in inflammation control or tumor growth suppression and subsequent systemic inflammation and malignancy[35,36].

We uncovered 36 malignancies in 158 patients with IgG4-RD, among which 5 cases each of lung, colon, and prostate cancer and 4 cases each of gastric and pancreatic cancer were noted. In Shiokawa, *et al*'s analysis of 18 malignancies of 108 patients with AIP, gastric (7 cases) and lung (5 cases) cancers were most commonly seen, but pancreatic cancer was not observed[16]. In the Hirano, *et al* study of 14 malignancies of 113 patients with IgG4-RD, lung cancers were most frequently detected (5 cases), followed by

Table 4A. Comparison of clinical variables between IgG4-RD patients with malignancies and those without.

Variables	Malignancies (+)	Malignancies (–)	p
Onset age of IgG4-RD, yrs, mean	67.6	65.0	0.211
Sex, male/female	29/5	90/34	0.194
Number of involved organs, mean	3.03	2.56	0.054
Corticosteroid use, ±	26/8	85/39	0.467
Recurrence of IgG4-RD, ±	7/26	20/58	0.799
Alcohol intake, ±	10/22	36/79	0.834
Smoking, ±	20/14	69/46	0.939
Diabetes mellitus, ±	8/26	31/93	0.860
IgG, median, mg/dl	2420	1986	0.002
IgG4, median, mg/dl	749	430	0.005
C3, median, mg/dl	100	107	0.638
C4, median, mg/dl	19.3	22.8	0.229
CIC, median, μg/ml	8	5.1	0.019
sIL-2R, median, U/ml	1250	755	0.005

Table 4B. Comparison of clinical variables between AIP patients with malignancies and those without.

Variables	Malignancies (+)	Malignancies (–)	p
Onset age of AIP, yrs, mean	67.0	64.1	0.226
Sex, male/female	24/4	60/21	0.316
Number of involved organs, mean	3.14	2.95	0.404
Corticosteroid use, ±	22/6	62/19	0.968
Recurrence of IgG4-RD, ±	6/21	23/57	0.682
Alcohol intake, ±	7/19	26/48	0.601
Smoking, ±	17/11	45/31	0.931
Diabetes mellitus, ±	7/21	24/57	0.640
IgG, median, mg/dl	2419	1997	0.019
IgG4, median, mg/dl	749	442	0.027
C3, median, mg/dl	99.5	103	0.705
C4, median, mg/dl	20.6	22.2	0.451
CIC, median, μg/ml	7.7	5.35	0.097
sIL-2R, median, U/ml	1233	755	0.011

IgG4-RD: immunoglobulin G4–related disease; AIP: autoimmune pancreatitis; CIC: circulating immune complex; sIL-2R: soluble interleukin 2 receptor.

pancreatic and gastric cancers (2 cases each)[17]. Similarly to these reports, our results revealed a higher prevalence of gastric and lung cancers, both of which are also more common in the Japanese general population. Pancreatic malignancies are considerably rare among the Japanese. However, their relatively high occurrence in Hirano's study and ours suggests that pancreatic cancer may be closely associated with IgG4-RD or AIP[17].

The high prevalence of pancreatic cancer in patients with IgG4-RD may have been due to the large proportion of AIP (109 of 158 patients) in our cohort. Four cases of pancreatic cancer were found in the AIP cohort at 3, 5, 5, and 9 years after IgG4-RD diagnosis, suggesting that an inflammatory state of pancreatic tissue induced malignancy and possibly accounted for the association between AIP and pancreatic cancer. Because IgG4 values are generally not measured for typical pancreatic cancer cases, instances of pancreatic cancer with an AIP background may have largely been ignored[16].

We examined whether the chronic inflammatory state of IgG4-RD causes malignancies. It is well known that chronic inflammation induces malignancies under some circumstances[37]. Mediators of the inflammatory response, such as cytokines, free radicals, prostaglandins, and growth factors can induce genetic and epigenetic changes, including point mutations in tumor suppressor genes, DNA methylation, and posttranslational modifications, and lead to the development of cancer[38]. Chronic pancreatitis was reported to be a significant risk factor for pancreatic cancer[39], and some cases of AIP were prone to transformation into chronic pancreatitis after calcification[40]. Further, Kamisawa, *et al* found that *K-ras* mutations occurred significantly more frequently in the pancreatobiliary regions of patients with AIP, suggesting a close association between AIP and pancreatic cancer[41]. Our present study showed that the SIR of malignancies occurring after 1 year following IgG4-RD diagnosis was 1.48 (95% CI 0.99–1.98), indicating a possible association between a

chronic inflammatory state of IgG4-RD and malignancy. Considering that a period of more than a decade is often needed for chronic inflammation to lead to carcinogenesis, we cannot conclude with certainty that a chronic inflammatory state in IgG4-RD induces malignancies because most of the tumors in our cohort were detected within 5 years after IgG4-RD diagnosis. In addition, the fact that the majority of malignancies occurred in organs different from those affected by IgG4-RD inflammation indicated a weak correlation between chronic inflammatory state and malignancy in IgG4-RD, although pancreatic cancer demonstrated a high SIR in the AIP subgroup.

As far as risk factors for developing malignancies in patients with IgG4-RD, our study showed that among various clinical variables, serum concentrations of IgG, IgG4, sIL-2R, and CIC were significantly higher in patients with malignancies than in those without, indicating that such activity markers may be associated with the development of tumors. Shiokawa, *et al* found a similar result, in which serum IgG4 was significantly higher in patients with a malignancy compared with those without[16]. Such findings imply that patients with IgG4-RD may harbor a weakened defense system, such as an immunodeficient state, or a paraneoplastic condition in affected organs, such as deranged oncogenes, and that a highly active disease state may exacerbate weakened immune defenses to result in the development of cancer. Accordingly, IgG4-RD patients with high serum concentrations of activity markers should be carefully followed for early detection of complicating malignancies.

IgG4-RD is believed to have a close association with the development of malignancy within 12 years after diagnosis, most notably during the first year. High concentrations of activity markers are a risk factor for cancer onset and should be monitored closely.

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RESEARCH ARTICLE

Investigation of Susceptibility Genes Triggering Lachrymal/Salivary Gland Lesion Complications in Japanese Patients with Type 1 Autoimmune Pancreatitis

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Abstract

Autoimmune pancreatitis (AIP) is a unique form of chronic pancreatitis characterized by high serum IgG4 concentration and a variety of complicating extra-pancreatic lesions. In particular, lachrymal/salivary gland lesions tend to manifest in a highly active AIP disease state, and several genes are speculated to be associated with the onset of this complication. We therefore searched for candidate susceptibility genes related to lachrymal/salivary gland lesions in a genome-wide association study (GWAS) with the GeneChip Human Mapping 500k Array Set (Affymetrix, CA) that was followed by fine mapping of additional single nucleotide polymorphisms (SNPs) in strongly significant genes with TaqMan assays. Venous blood samples were obtained from 50 type 1 AIP patients with lachrymal/salivary gland lesions (A group) and 53 type 1 AIP patients without (B group). The mean values of IgG and IG4 were both significantly different ($P<0.05$) between the groups. SNPs that showed a significant association with the A group at the genome-wide level ($P<0.0001$) were identified and subsequently used in fine SNP mapping of candidate genes. In total, five SNPs had a positive association with complicated AIP (most notably rs2284932 [$P=0.0000021$]) and five SNPs possessed a negative association (particularly rs9371942 [$P=0.00000039$]). Among them, *KLF7*, *FRMD4B*, *LOC101928923*, and *MPPED2* were further examined for complication susceptibility using additional SNPs that were not included in the GWAS. Individual genotyping of *KLF7* rs2284932 revealed that the frequency of the minor C allele was significantly increased ($P=0.00062$, $Pc=0.0018$, OR=2.98, 95%CI=1.58-5.65) in group A. The minor T allele of rs4473559 in *FRMD4* demonstrated a significant association in the A group ($P=0.00015$, OR=3.38, 95%CI=1.77-7.65). In the *LOC101928923* gene, the frequency of the minor C allele of rs4379306 was significantly decreased in group A in both TaqMan and GWAS analyses. Lastly, the minor C allele of *MPPED2* rs514644 carried a significantly

increased risk of complications. These four genes may be linked with the onset of lachrymal/salivary gland lesions in type 1 AIP patients and require further study.

Introduction

Autoimmune pancreatitis (AIP) is a unique form of chronic pancreatitis characterized by the imaging findings of irregular narrowing of the main pancreatic duct, pancreatic swelling, and obstructive jaundice, all of which mimic the clinical signs of pancreatic cancer [1]. AIP patients also exhibit high serum IgG4 concentration, abundant lymphoplasmacytic and IgG4-bearing plasma cell infiltration in pancreatic lesions, and a favorable response to steroid therapy. Although such features indicate that specific autoimmune mechanisms associated with IgG4 are present in AIP [2, 3], its precise pathogenesis has not been fully elucidated.

AIP is complicated with a variety of extra-pancreatic lesions, such as dacryoadenitis/sialadenitis [4, 5], lung lesions [6], sclerosing cholangitis [7, 8], retroperitoneal fibrosis [3], and tubulointerstitial nephritis [9]. Since AIP and these lesions often share the pathological features of prominent IgG4-positive plasma cell infiltration in affected organs and a positive response to corticosteroids, a common pathogenic background is suspected [3]. IgG4-related disease (IgG4-RD) is a newly proposed systemic disorder that encompasses both of these conditions. Accordingly, AIP is now recognized as pancreatic manifestation of IgG4-RD [1].

Dacryoadenitis/sialadenitis occurring in AIP was previously considered to represent Mikulicz disease. However, it is now considered to be a principal member of IgG4-RD and is referred to as IgG4-related dacryoadenitis and sialadenitis [10]. IgG4-related dacryoadenitis and sialadenitis is characterized by symmetrical swelling of the lacrimal and submandibular glands, high serum IgG4 concentration, and abundant IgG4-positive plasma cell infiltration in the affected tissues [4, 11]. Unlike Sjögren's syndrome, which also exhibits dacryoadenitis and sialadenitis, the IgG4-related variety has no relation to disease-specific autoantibodies, such as anti-SSA or anti-SSB, shows mild or absent exocrine insufficiency, and reacts well to corticosteroid therapy. Moreover, complicating lachrymal/salivary gland lesions tend to manifest in a highly active AIP disease state [11].

Since many autoimmune disorders are associated with multiple genetic and environmental factors, it is generally considered that the development of AIP is influenced by several susceptibility genes, including *HLA DRB1*04:05-DQB1*04:01*, *FCRL3*, *CTLA4*, *KCNA3*, and *TLR4* [12–16]. Among them, HLA class II genes have been genetically characterized as primary predisposition [12, 17] and relapse [18] factors in AIP. However, disease susceptibility remains poorly understood, especially the relationship between relapse and a substitution of aspartic acid at codon 57 of DQ β1 [19]. These, and other, genes are also speculated to be linked to the induction of AIP complicated with dacryoadenitis/sialadenitis and have important clinical significance.

The genome-wide association study (GWAS) method is a powerful and widely-used technique for exploring the relationships among common sequence variations and disease susceptibility or resistance throughout the entire genome. This approach has demonstrated numerous common variants that contribute to disease predisposition and complex traits [20]. To our knowledge, no GWAS has been done on AIP complicated with lachrymal/salivary gland lesions to date. Proper consideration for small sample sizes and sample collection biases is needed to reliably identify disease-susceptible loci using a GWAS [21, 22]. Major autoimmune diseases, such as rheumatoid arthritis, type I diabetes mellitus and systemic lupus erythematosus, have large patient populations from which to sample. Although AIP is a rare disease, we expect that the

collection of a well defined cohort using specific clinical diagnostic criteria will enable adequate GWAS analysis. In the present study, we first screened for susceptibility genes of lachrymal/salivary gland lesions in type 1 AIP using the GeneChip Human Mapping 500k Array Set (Affymetrix, CA). Next, fine-tuned mapping of specific single nucleotide polymorphisms (SNPs) was performed for candidate genes that showed a strong statistical significance ($P<0.0001$).

Materials and Methods

1. Patients and Samples

One hundred and nine patients with type 1 AIP (82 men and 26 women, median age at AIP onset: 66 years) were examined and treated at Shinshu University Hospital or its affiliated institutions between August 1992 and August 2012. Among them, we recruited 103 patients who provided consent for inclusion in the GWAS and collected venous blood samples from 50 AIP patients with lachrymal/salivary gland lesions (i.e., the A group) and 53 patients without (i.e., the B group). Collected samples were immediately frozen and stored at minus 80°C until analysis.

The A group consisted of 41 men and 9 women who ranged from 49 to 85 years of age (average: 63.5 years). The B group included 40 men and 13 women who ranged from 38 to 84 years of age (average: 65.1 years). Dacryoadenitis and sialadenitis were defined as symmetrical swelling of the lachrymal and salivary glands as confirmed by physical examination, CT and MRI findings, and gallium scintigraphy.

2. Methods

2–1. Comparison of activity state between type 1 AIP with and without lachrymal/salivary gland lesions. To confirm whether type 1 AIP with lachrymal/salivary gland lesions was at a higher disease activity state than AIP without the involvement of lesions, we performed a comparative study between the groups using several activity markers, including IgG, IgG4, circulating immune complex (CIC), β 2-microgloburin (β2MG), soluble interleulkin-2 receptor (sIL2R), and complement C3 and C4, as well as estimation of other organ involvement, such as lung disease, sclerosing cholangitis, kidney disease, or retroperitoneal fibrosis.

2–2. Genetic analysis.

2-2-1. Preparation of genomic DNA. Genomic DNA was isolated and purified from venous whole blood samples using a commercially available kit (QuickGene DNA whole blood kit L, Kurabo, Osaka, Japan). All procedures were performed according to the manufacturer's instructions under standardized conditions to prevent variation in DNA quality.

2-2-2. Genome-wide genotyping. Genotyping with the GeneChip Human Mapping 500K Array Set was carried out according to the manufacturer's protocol for our first stage of analysis. Samples with a <93% genotype call rate were excluded from the study, as were SNPs with a call rate of <95% or a minor allele frequency of <5% overall.

2-2-3. SNP genotyping. To specifically identify possible susceptibility genes of AIP-complicating dacryoadenitis and sialadenitis, SNPs that showed a strongly significant association in the A group at the genome-wide level ($P<0.0001$) were assessed. Among them, we selected four candidate genes (Kruppel-like factor 7 [*KLF7*], FERM domain containing 4B [*FRMD4B*], uncharacterized LOC1928923 [*LOC101928923*], and metallophosphor esterase domain-containing protein [*MPPED2*]) (Table 1) and examined tagging SNPs in these genes as the second stage of our analysis. The selection criteria for the SNPs were based on information from the NCBI dbSNP database (build 37.3, http://www.ncbi.nlm.nih.gov/projects/SNP/), HapMap database (http://hapmap.ncbi.nlm.nih.gov/downloads/index.html.en), and SNP database of Applied

Table 1. Single nucleotide polymorphisms showing the strongest associations (P<0.0001) in the genome-wide association study.

dbSNP ID	Chrom. location	Position	Candidate gene	MA	*P* value	OR (95% CI)
rs2284932	2q33.3	207720754	KLF7	C	<0.000003	4.35 (2.32–8.16)
rs9831516	3p14.1	69312751	FRMD4B	G	<0.00002	3.11 (1.72–5.62)
rs2407212	5q23.2	121912400	SNCAIP	G	<0.00009	6.20 (2.28–16.91)
rs524762	6q13	75112962	COL12A1	T	<0.00003	0.31 (0.17–0.53)
rs9371942	6q25.3	156276214	LOC101928923	G	<0.0000004	0.20 (0.10–0.42)
rs4735508	8q22.1	98986987	MATN2	A	<0.0001	8.42 (2.43–29.14)
rs1536067	9p22.2	17727893	SH3GL2	G	<0.00004	0.24 (0.12–0.48)
rs4878053	9q21.33	89102948	FLJ45537	T	<0.00001	0.29 (0.17–0.51)
rs514644	11p14.1	30408757	MPPED2	C	<0.00006	3.06 (1.77–5.30)
rs7170215	15q25.3	86299145	NTRK3	A	<0.00009	0.27 (0.13–0.53)

dbSNP ID: SNP database identification, Chrom: chromosome, Position: distance from the short-arm telomere, MA: minor allele, OR: odds ratio, CI: confidence interval.

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Biosystems (http://bioinfo.appliedbiosystems.com/genome-database/snp-genotyping.html) as: 1) location within the candidate gene; 2) minor allele frequency >5% in Japanese populations; 3) call rate ≧95%; and 4) Hardy-Weinberg equilibrium P≧0.001. Genotyping of all SNPs was performed using the ABI TaqMan allelic discrimination kit and the ABI7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA) following the manufacturer's instructions.

2–3. Statistical analysis. Fisher's exact and Pearson's chi-square tests were adopted to test for differences in clinical data between the patient subgroups. The Mann-Whitney *U* test was employed to compare continuous data. All tests were performed using Statflex ver. 6 (Artech Co., Ltd., Japan). *P* values of less than 0.05 were considered to be statistically significant.

All association analyses between group A and group B for GWAS data were carried out using HelixTree SVS 7 software (Golden Helix, Inc., Bozeman, MT). The statistical significances of allele frequencies between AIP with and without lachrymal/salivary gland lesions in second stage analyses were calculated using the chi-square test. A *P* value of less than 0.05 was considered to be statistically significant after adjustment by Bonferroni's correction. The Hardy-Weinberg equilibrium of all SNPs was confirmed.

3. Ethics statement

The present study was approved by the Ethics Committee of Shinshu University School of Medicine (Matsumoto, Japan). The protocol of this investigation was in accordance with the principals outlined in the Declaration on Helsinki of the World Medical Association and was approved by the Ethics Committee of Shinshu University School of Medicine. Written informed consent was obtained from each subject after a full explanation of the study.

Results

1. Comparison of activity state in type 1 AIP with and without lachrymal/salivary gland lesions

Serum IgG and IgG4 concentrations were significantly higher in the A group than in the B group. No remarkable associations were observed for CIC, β2MG, sIL2R, C3, or C4 between the groups. Significantly higher prevalences of kidney disease and retroperitoneal fibrosis were detected in the A group. Taken together, type 1 AIP with lachrymal/salivary gland lesions appeared to be in a more highly activated state (Table 2).

Table 2. Comparison of activity state between type 1 AIP with and without lachrymal/salivary gland lesions.

	A group median (range)	B group median (range)	*P* value
IgG	2437.5 (1199–6408)	1865 (892–4661)1865(892–4661)1865(892–4661)	0.00035
IgG4	773 (33–2970)	379 (4–1950)	0.00062
CIC	6.20 (2–41.6)	5 (1.4–58.4)	0.42
β2MG	2.315 (1.3–8.9)	2.165 (1.2–15.3)	0.23
sIL2R	869 (345–4695)869(345–4695)	755 (257–2260)	0.10
C3	102.5 (16–218)	104.5 (12–238)	0.74
C4	21.8 (1.1–152)	23.3 (1.0–162)	0.74
lung disease (+/-)	18/31	10/40	0.10
Sclerosing cholangitis (+/-)	5/42	7/44	0.64
Kidney disease (+/-)	15/35	4/48	0.0038
Retroperitoneal fibrosis (+/-)	18/32	9/42	0.043

A group: with lachrymal/salivary gland lesions, B group: without lachrymal/salivary gland lesions.

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2. Genetic analysis

We preliminarily conducted a GWAS screening analysis of Japanese type 1 AIP patients with and without lachrymal/salivary gland lesions. Of the total of 12,033 SNPs that passed the internal quality control, 242 exhibited a statistical significance ($P<0.001$) in allele-based tests. The results for strong signals ($P<0.0001$) are shown in Table 2. Five SNPs demonstrated a positive association with complicated AIP: rs2284932 (OR = 4.35), rs9831516 (OR = 3.11), rs2407212 (OR = 6.20), rs4735508 (OR = 8.42), and rs514644 (OR = 3.06). Five SNPs were negatively associated with complicated AIP: rs524762 (OR = 0.31), rs9371942 (OR = 0.20), rs1536067 (OR = 0.24), rs4878053 (OR = 0.29), and rs7170215 (OR = 0.27). The strongest associations with the pathogenesis of lachrymal/salivary gland lesions were rs9371942 ($P = 0.00000039$) and rs2284932 ($P = 0.0000021$).

The candidate genes containing the highly significant SNPs are listed in Table 1 according to data from the NCBI Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/). Among the 10 genes, *KLF7* (2q33.3), *FRMD4B* (3p14.1), *LOC101928923* (6q25.3), and *MPPED2* (11p14.1) were further examined for conferring susceptibility to complications using SNPs that resided in the genes but were not tested in the GWAS. Individual genotyping of *KLF7* rs2284932 using a TaqMan assay showed that the frequency of the minor C allele was significantly increased ($P = 0.00062$, OR = 2.98) in the A group as in the GWAS (Table 3).

The minor T allele of rs4473559 in *FRMD4* also had a significant association in the A group ($P = 0.00015$, OR = 3.38) (Table 4).

In the *LOC101928923* gene, the frequency of the minor C allele of rs4379306 was significantly decreased ($P = 0.00017$, OR = 0.30) in both TaqMan and GWAS analyses in the A group (Table 5).

Lastly, the minor C allele for rs514644 of *MPPED2* carried a significantly increased risk for complicating lachrymal/salivary gland lesions ($P = 0.0075$, OR = 2.14) (Table 6).

Discussion

AIP is believed to be a pancreatic manifestation of a systematic IgG4 disorder that is subclassified as either IgG4-related (type 1) or non-IgG4-related (type 2) [1]. Type 1 AIP tends to

Table 3. Association analysis of single nucleotide polymorphisms in the KLF7 gene.

dbSNP ID	Chrom. location	Typing method	Alleles		Frequency (%)		*P* value		*Pc* value	OR (95% CI)
					A group	B group				
rs2287505	207655331	GWAS	C>A				A	0.027		2.74(1.09–6.90)
rs1263615	207667483	TaqMan	A>G	A	74.0	68.6	G	0.758	3.032	1.10(0.61–1.96)
				G	26.0	31.4	GG+GA/AA	0.567	2.268	1.26(0.57–2.81)
rs768090	207711824	TaqMan	A>T	A	74.0	83.3	T	0.091	0.364	1.80(0.91–3.57)
				T	26.0	16.7	TT+TA/AA	0.047	0.188	2.28(1.01–5.16)
rs10195536	207715065	GWAS	T>A				A	0.016		3.79(1.21–11.92)
rs2284932	207720754	TaqMan	T>C	T	90.0	95.1	C	0.00062	0.003	2.98(1.58–5.65)
				C	10.0	4.9	CC+AC/AA	0.00037	0.002	4.37(1.90–10.02)
		GWAS					C	0.0000021		4.35(2.32–8.16)
rs12466923	207721800	TaqMan	A>C	A	60.0	81.4	C	0.039	0.156	2.16 (1.03–4.54)
				C	40.0	18.6	CC+AC/AA	0.0093	0.037	3.10(1.30–7.38)

A group: with lachrymal/salivary gland lesions, B group: without lachrymal/salivary gland lesions, dbSNP ID: SNP database identification, Chrom: chromosome, Pc: corrected P.

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exhibit lesions in various other organs, including the lachrymal and salivary glands, lungs, retroperitoneum, and prostate.

We observed in the present study that approximately 50% of patients had lachrymal/salivary lesion involvement despite other reports showing the prevalence of these lesions to be approximately 20% in Japan [23, 24]. Since lachrymal/salivary lesions are considered to be a major member of the IgG4-RD family along with Mikulicz's disease, their incidence in AIP is presumably high. Although the reason for such a discrepancy in positivity rates for these lesions is not precisely clear, it may be attributable to differences in diagnostic procedures, such as physical examinations or imaging tests. In our previous study, imaging analysis of AIP by an

Table 4. Association analysis of single nucleotide polymorphisms in the FRMD4 gene.

dbSNP ID	Chrom. location	Typing method	Alleles		Frequency (%)		*P* value		*Pc* value	OR (95% CI)
					A group	B group				
rs12637416	69302498	GWAS	T>A				A	0.00062		2.73(1.52–4.89)
rs6763046	69302897	TaqMan	C>A	A	40.0	18.6	A	0.00084	0.0034	2.91(1.54–5.52)
				C	60.0	81.4	AA+AC/CC	0.00050	0.002	4.25(1.85–9.76)
		GWAS					A	0.00035		2.91(1.60–5.29)
rs4473559	69305553	TaqMan	G>T	T	42.0	17.6	T	0.00015	0.0006	3.38(1.77–6.45)
				G	58.0	82.4	TT+TG/GG	0.000045	0.00018	5.62(2.39–13.21)
rs4464459	69306951	TaqMan	C>A	A	36.0	16.7	A	0.0018	0.0072	2.81(1.45–5.45)
				C	64.0	83.3	AA+AC/CC	0.00048	0.0019	4.31(1.86–9.98)
rs11128118	69308278	GWAS	G>T				T	0.00019		3.02(1.67–5.45)
rs9831516	69312751	GWAS	A>G				G	0.00013		3.11(1.72–5.62)
rs9836305	69313491	TaqMan	A>G	G	42	18.6	A	0.00030	0.0012	3.16(1.67–5.98)
				A	58	81.4	AA+AG/GG	0.00050	0.002	4.25(1.85–9.76)

A group: with lachrymal/salivary gland lesions, B group: without lachrymal/salivary gland lesions, dbSNP ID: SNP database identification, Chrom: chromosome, Pc: corrected P.

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Table 5. Association analysis of single nucleotide polymorphisms in the LOC101928923 gene.

dbSNP ID	Chrom. location	Typing method	Alleles		Frequency (%)		*P* value		*Pc* value	OR (95% CI)
					A group	B group				
rs9371942	156276214	GWAS	A>G				G	0.0000039		0.20(0.10–0.42)
rs4379306	156276633	GWAS	A>T				T	0.0000089		0.21(0.10–0.44)
		TaqMan		T	17.0	32.4	T	0.011	0.033	0.43(0.22–0.83)
				A	83.0	67.6	TT+TA/AA	0.0065	0.020	0.33(0.14–0.74)
rs9397861	156279496	TaqMan	A>G	G	16.0	36.3	G	0.0011	0.0033	0.33(0.17–0.65)
				A	84.0	63.7	GG+GA/AA	0.0019	0.0057	0.28(0.12–0.63)
rs4428513	156288830	TaqMan	T>C	C	8.0	7.8	C	0.97		1.02(0.37–2.84)
				T	92.0	92.2	CC+CT/TT	0.75		1.20(0.40–3.59)
rs9371408	156307788	GWAS	A>G				G	0.000029		0.22(0.11–0.47)
rs9384400	156323446	GWAS	A>T				T	0.000014		0.22(0.11–0.45)

A group: with lachrymal/salivary gland lesions, B group: without lachrymal/salivary gland lesions, dbSNP ID: SNP database identification, Chrom: chromosome, Pc: corrected P.

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experienced radiologist disclosed the presence of extra-pancreatic lesions in 92% of AIP patients and lachrymal/salivary lesions in 47.5% of cases [25].

Although type 1 AIP complicated with lachrymal/salivary gland lesions can be clearly diagnosed using recent clinical, immunological, radiological, and morphological characterization criteria [11], little is known on the pathogenesis of these complications. Therefore, we investigated whether genetic factors affected the development of lachrymal/salivary gland lesions in type 1 AIP using a GWAS followed by fine mapping of additional SNPs and uncovered four novel candidate susceptibility genes.

Table 6. Association analysis of single nucleotide polymorphisms in the MPPED2 gene.

dbSNP ID	Chrom. location	Typing method	Alleles		Frequency (%)		*P* value		*Pc* value	OR (95% CI)
					A group	B group				
rs10835665	30406319	TaqMan	G>A	A	26.0	19.6	A	0.28		1.44(0.74–2.79)
				G	74.0	80.4	AA+AG/GG	0.49		1.33(0.59–2.96)
rs514644	30408757	TaqMan	T>C	C	59.0	40.2	C	0.0075	0.045	2.14(1.22–3.75)
				T	41.0	59.8	CC+CT/TT	0.017	0.102	2.63(1.01–6.82)
		GWAS					C	0.000054		3.06(1.77–5.30)
rs487742	30410971	TaqMan	G>A	A	66.0	45.1	A	0.0028	0.017	2.36(1.34–4.17)
				G	34.0	54.9	AA+AG/GG	0.051	0.306	2.77(0.97–7.94)
rs808182	30411818	TaqMan	G>A	A	33.0	20.6	A	0.046	0.276	1.90(1.01–3.59)
				G	67.0	79.4	AA+AG/GG	0.036	0.216	2.35(1.05–5.25)
rs11031087	30415467	TaqMan	A>T	T	7.0	4.9	T	0.53		1.46(0.45–4.76)
				A	93.0	95.1	TT+AT/AA	0.51		1.50(0.44–5.08)
rs11031093	30424076	TaqMan	G>A	A	7.0	4.9	A	0.53		1.46(0.45–4.76)
				G	93.0	95.1	AA+AG/GG	0.51		1.50(0.44–5.08)
rs537944	30434891	GWAS	G>A				A	0.000052		3.07(1.77–5.32)
rs521436	30449780	GWAS	T>A				A	0.000099		2.92(1.69–5.04)

A group: with lachrymal/salivary gland lesions, B group: without lachrymal/salivary gland lesions, dbSNP ID: SNP database identification, Chrom: chromosome, Pc: corrected P.

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The initial GWAS revealed 10 candidate genes possibly influencing the pathogenesis of lachrymal/salivary gland lesions in AIP. As several genes contained multiple SNPs that were strongly associated with complications, we selected four (positive association: *KLF7*, *FRMD4*, and *MPPED2*; negative association: *LOC101928923*) according to *P* values and OR for ensuing minor allele analysis.

The *KLF7* gene encodes a member of the Kruppel-like factors among DNA-binding transcriptional regulators that play diverse roles during cell proliferation and differentiation [26–28]. KLF7 is reportedly related to neurogenesis [29], progression of type 2 diabetes [30], obesity [31], and regulation of thymocyte development [32]. Determining the specific role of *KLF7* polymorphisms in the onset of lachrymal/salivary gland lesions appears challenging at present. However, they may contribute to disease complications as shown in the individual genotyping results in Table 3 (C allele at rs2284932 [$P = 0.00037$] with a dominant model and C allele at rs12466923 [$P = 0.0093$] with a dominant model).

The *FRMD4B* gene is ubiquitously expressed and encodes a GRP1-binding protein (GRSP1) that contains a FERM protein domain [33]. FRMD4B might be involved in the establishment of epithelial cell polarity and play a role as a scaffolding molecule [34]. This protein also participates in activated insulin receptor signaling complexes and performs functions in insulin receptor, growth factor receptor, and other phosphatidylinoshitol (3,4,5)-trisphosphate (PIP3) signaling events [35]. To date, few disease associations have been made with *FRMD4B* polymorphisms. Our study showed that all SNPs had strongly significant associations ($P<0.001$) with lachrymal/salivary gland lesions in both TaqMan and GWAS typing. The precise involvement of *FRMD4B* remains unknown, but we speculate that polymorphisms may affect complication onset based on previous functional information [35].

All statistically associated SNPs (rs9371942, rs4379306, and rs9397861) were located in the *LOC101928923* gene on chromosome 6p25.3. This little known gene resides in the short intergenic region between *NOX3* and *MIR1202*. NOX3 is a member of the NOX family of NADPH oxidases. NOX enzymes are a potential source of reactive oxygen species (ROS) production that transport electrons across the plasma membrane [36]. NOX3-derived ROS appear to be associated with numerous biological functions, including insulin action, host defense, cellular signaling, regulation of gene expression, and cell differentiation [37]. *NOX3* polymorphisms may be in high linkage disequilibrium with the three candidate SNPs uncovered in *LOC101928923* and influence the induction of complications. Further association studies using SNPs in *NOX3* might determine whether *NOX3* polymorphisms affect the pathogenesis of complications.

The frequencies of two minor alleles (C at rs514644 and A at rs487742) in *MPPED2* were significantly increased in the A group over the B group ($P = 0.0075$ and $P = 0.0028$, respectively). The *MPPED2* gene (also known as *c11orf8* or *239FB*) is located on human chromosome 11p13 between the *FSHB* and *PAX6* genes. The upregulation of *MPPED2* reduces cell proliferation, induces apoptosis, and stimulates the differentiation of neuronal precursors [38]. Particularly in papillary thyroid carcinomas and breast cancer, MPPED2 expression has been reported to affect the malignancy of lesions [39–41]. Therefore, *MPPED2*-regulated anti-tumorigenesis may play an important role in the induction or regulation of lachrymal/salivary gland lesions.

The number of enrolled subjects for this rare disease was too small to overcome type I statistical error. However, the false positive report probability (FPRP) values as calculated by Wacholder's method (http://jnci.oxfordjournals.org/content/96/6/434/suppl/DC1) [42] support the significant findings revealed in the present study, i.e., when a prior probability of 0.05 was set, FPRP values were 0.018 (dominant model) with rs2284932 in *KLF7*, 0.003 (dominant model) with rs4473559 in *FRMF4*, 0.0049 (additive model) with rs9397861 in *LOC101928923*, and 0.106 (additive model) with rs487742 in *MPPED2*.

In conclusion, we identified four novel candidate genes (*KLF7*, *FRMD4B*, *NOX3*, and *MPPED2*) that might be linked to the development of lachrymal/salivary gland lesions in type 1 AIP patients using a GWAS followed by fine mapping of highly significant genes. Further studies using larger sample sizes and functional analysis of genes associated with AIP complications are needed to confirm the present results.

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Author Contributions

Conceived and designed the experiments: TO MO SK. Performed the experiments: TO YK AM. Analyzed the data: TO MO YK AM. Contributed reagents/materials/analysis tools: TO TI HH NA SK. Wrote the paper: TO MO SK.

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