表題
A high-throughput sequence analysis of Japanese patients revealed 11 candidate genes associated with type 1 autoimmune pancreatitis susceptibility

(High-throughput sequencing による 自己免疫性膵炎疾患感受性遺伝子の同定に関する研究)

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A high-throughput sequence analysis of Japanese patients revealed 11 candidate genes associated with type 1 autoimmune pancreatitis susceptibility

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Abstract
The pathogenesis of autoimmune pancreatitis is unknown. In the present study we used high-throughput sequencing with next generation sequencing to identify the candidate genes associated with AIP. A total of 27 type 1 AIP patients and 30 healthy blood donors were recruited, and DNA samples were isolated from their mononuclear cells. A high-throughput sequencer with an original custom panel of 1031 genes was used to detect the genetic variants in each sample. Polymorphisms of CACNA1S (c.4642C>T), rs41554316, rs2231119, rs1042131, rs2838171, P2RX3 (c.195delG), rs75639061, SMAD7 (c.624delC) and TOP1 (c.2007delG), were identified as candidate genetic variants in patients with type 1 AIP. P2RX3 and TOP1 were significantly associated with AIP, even after adjusting bay means of Bonferroni’s correction. In addition, we also identified eight candidate genetic variants that were associated with the relapse of type 1 AIP, namely: rs1143146, rs1050716, HLA-C (c.759_763delCCCCCinsTCCCG), rs1050451, rs4154112, rs1049069, CACNA1C (c.5996delC) and CXCR3 (c.630_631delGC). Finally polymorphisms of rs1050716 and rs111493987 were identified as candidate genetic variants associated with extra-pancreatic lesions in patients with type 1 AIP. These candidates might be used as markers of AIP susceptibility and could contribute to the pathogenesis of type 1 AIP.

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1. Introduction
Autoimmune pancreatitis (AIP) is a condition which involves the chronic inflammation of pancreas. It is characterized radiologically by pancreatic enlargement with delayed enhancement and strictures or narrowing of the main pancreatic duct without marked upstream dilation; serologically by elevation of serum immunoglobulin G fraction 4 (IgG4); histologically by lymphoplasmacytic infiltration and fibrosis; and therapeutically by a dramatic response to steroids [1]. The International Consensus Criteria for AIP [1] proposed two subtypes of the disease. The majority of Japanese patients with AIP are classified as type 1; type 2 AIP is more common in Western countries. Type 1 AIP is recognized as a pancreatic manifestation of IgG4-related disease [2]. Conversely, type 2 AIP is frequently complicated by inflammatory bowel diseases [1]. The pathogenesis of type 1 AIP is unknown but it is considered to be a multifactorial disease which is associated with genetic and environmental factors. Haruta et al. reported that a mouse model of AIP was established by persistent exposure to heat-killed Escherichia coli, suggesting that the chronic activation of the innate immune system, triggered by intestinal flora, might cause AIP [3]. In addition, gastric infection by Helicobacter pylori was suspected to contribute to the pathogenesis of AIP, because it shows significant homology with human carbonic anhydrase 2 and the alpha-carbonic anhydrase of Helicobacter pylori [4].

On the other hand, the genetic factors associated with AIP have also been reported. Kawa et al. reported that the human leukocyte...
antigen (HLA) DRB1*04:05-DQB1*0401 haplotype was associated with AIP [5]. Subsequently, some of the genes that are associated with AIP susceptibility were identified by direct sequencing or a Taqman assay, these include: ATP-binding cassette sub-family F1 [6], Fc receptor-like 3 [7], cytotoxic T lymphocyte antigen 4 [8], KCNA3 [9] and PPR51 [10].

Because multiple genes are usually associated with susceptibility to a multifactorial disease (including unknown genes), a high-throughput sequence analysis is necessary for the investigation of genes that confer AIP susceptibility. Oguchi et al. performed a genome-wide association study to investigate genes that conferred susceptibility to the triggering of dacrocyoadenitis or sialadenitis in Japanese AIP patients; however, they did not show the genes that were associated with AIP susceptibility [11]. In the present study, we performed high-throughput sequencing with next generation sequencing, which targeted more than 1000 genes.

2. Material and methods

2.1. Patients and clinical diagnosis

A total of 27 patients with type 1 AIP and 30 healthy blood donors were consecutively diagnosed and recruited from January 2013 to September 2014 at Asahikawa Medical University or its affiliated institutions. All of the AIP patients and healthy blood donors were Japanese and provided written informed consent for inclusion in the high-throughput sequencing analysis. The patients with a definite or probable diagnosis of AIP based on the Clinical Diagnostic Criteria for Autoimmune Pancreatitis 2011 [12] were enrolled in this study.

2.2. Primer design for custom amplicon sequencing

Because the present study aimed to identify new genetic variants that reflect AIP susceptibility, the genes associated with inflammatory and autoimmune diseases, hematological and metabolic disorders, and oxidative stress in the gastrointestinal tract, liver, pancreas and biliary tract were selected as candidates. Consequently, 883 genes that are associated with inflammatory and autoimmune diseases and 209 genes that are associated with metabolic disorders and oxidative stress were identified. After excluding the overlapping genes, 1031 genes were investigated in the present study. We designed multiple primer sets which targeted 1031 genes (total of 12,609 amplicons) using the Ion AmpliSeq™ Designer software program (https://www.ampliseq.com/browse.action) (Life Technologies, Carlsbad, CA, USA). These primer sets were designed as five primer pools. The 12,609 amplicons and the targeted lesions are described in Supplemental Table 1.

2.3. Sample preparation for amplicon sequencing

Peripheral blood samples from each of the patients or healthy volunteers (HVs) were processed for mononuclear cell isolation by Ficoll gradient centrifugation. The genomic deoxyribonucleic acid (DNA) was then extracted and purified using DNeasy Blood & Tissue Kits (Qiagen, Venlo, Netherlands). The DNA concentrations were determined by a QubitTM Fluorometer (Life Technologies, Carlsbad, CA, USA). The quality of the genomic DNA was assessed by agarose gel electrophoresis.

2.4. High-throughput sequencing

Using 50 ng of each DNA sample, an ultra-high multiplex polymerase chain reaction (PCR) was performed and a DNA fragments library (5 primer pools per sample) was generated, using an Ion AmpliSeq™ Library Kit 2.0 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions in order to perform custom amplicon sequencing. The concentration and quality of the DNA fragments library was evaluated with an Agilent 2200 Tape station (Agilent Technologies, Santa Clara, CA, USA). The DNA fragment libraries were then processed for an emulsion PCR using an Ion OneTouchTM System and an Ion OneTouch 200 Template Kit v3 (Life Technologies, Carlsbad, CA, USA). Template-positive Ion SphereTM Particles from the sequencing reaction were enriched and purified with an Ion OneTouchTM ES system (Life Technologies, Carlsbad, CA, USA). The template-positive Ion SphereTM Particles were then applied on Ion PTM Chips (Life Technologies, Carlsbad, CA, USA), and the high throughput sequencing reaction was carried out using an Ion Proton™ Semiconductor sequencer (Life Technologies, Carlsbad, CA, USA).

2.5. The data analysis to detect genetic variants

All of the sequencing data were mapped on a human reference genome sequence (GRCh37/hg19) using the Torrent Suite Software program (Life technologies, Carlsbad, CA, USA). The genetic variants were then detected by a Torrent Variant Caller plug-in for the software program (Life technologies, Carlsbad, CA, USA). In this program, alleles with frequencies of (the percentage of reads which possessed a variant) > 10%, with a coverage (the number of reads which had a variant) of > 5 and a quality score of > 15 were regarded as significant variants. The variant information for each sample was imported into the CLC Genomics Workbench software system (CLC bio, Aarhus, Denmark), and Fisher’s exact test was performed to determine the significance of the differences among the samples. Strand bias was defined according to the following numerical formula: strand bias = max [(VpCm × VmCp)]/VpCm + VmCp (Cm, the number of reads from the minus direction in the known sequence; Cv, the number of reads from the minus direction in the known sequence; Vp, the number of reads from the plus direction in the variant sequence;Vm, the number of reads from the minus direction in the variant sequence).

2.6. Statistical analysis

In the amplicon sequencing analysis, the candidate genetic variants were filtered using the P-values determined by Fisher’s exact test. The age between AIP patients and HVs was compared using the Mann-Whitney U test. P values of < 0.05 were considered to indicate statistical significance.

2.7. Ethics statement

The present study was approved by the institutional review board of Asahikawa Medical University. Written informed consent was obtained from all of the subjects after a full explanation of the study.

3. Results

3.1. The demographics of the AIP patients and HVs

A total of 57 participants, including 27 patients with type 1 AIP (male, n = 22; female, n = 5) and 30 HVs (male, n = 17; female, n = 13) were enrolled in this study. The median age of the AIP patients at the time of blood collection was 73 years (range: 55–87). The median age of the HVs at the time of blood collection was 29.5 years (range: 22–49). The HVs were significantly younger.
value after adjustment by Bonferroni’s correction.
ed candidates of genetic variants associated with type 1 AIP.
ized by patients with AIP and HVs.

<table>
<thead>
<tr>
<th>Chr</th>
<th>Position type (SNP rs)</th>
<th>A</th>
<th>Frequency</th>
<th>Candidate Gene</th>
<th>Gene description</th>
<th>P value (Pc value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIP%</td>
<td>HVs%</td>
<td>N=27</td>
<td>N=30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>201,016,269 SNP (new)</td>
<td>G&gt;A</td>
<td>7 (25.9)</td>
<td>0(0)</td>
<td>CACNA1S</td>
<td>Calcium channel in skeleton muscle</td>
</tr>
<tr>
<td>6</td>
<td>29,912,315 SNP (rs14554316)</td>
<td>A&gt;C</td>
<td>11 (40.7)</td>
<td>4(15.3)</td>
<td>HLA-A</td>
<td>The class 1 major histocompatibility complex</td>
</tr>
<tr>
<td>6</td>
<td>29,912,856 SNP (rs2231119)</td>
<td>A&gt;T</td>
<td>23 (85.2)</td>
<td>18(60)</td>
<td>HLA-A</td>
<td>The class 1 major histocompatibility complex</td>
</tr>
<tr>
<td>6</td>
<td>33,048,602 SNP (rs1042131)</td>
<td>C&gt;A</td>
<td>24 (88.9)</td>
<td>18(60)</td>
<td>HLA-DPB1</td>
<td>The class 2 major histocompatibility complex</td>
</tr>
<tr>
<td>17</td>
<td>151,945,051 SNP</td>
<td>A&gt;G</td>
<td>12 (44.4)</td>
<td>2(6.7)</td>
<td>ML3</td>
<td>Methylation of histon, Altering antibody effector function</td>
</tr>
<tr>
<td>11</td>
<td>57,114,093 Deletion</td>
<td>G&gt; -</td>
<td>11 (40.7)</td>
<td>0(0)</td>
<td>P2RX3</td>
<td>Purinergic receptor of pain perception</td>
</tr>
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<td>15</td>
<td>78,789,672 SNP (rs10739061)</td>
<td>A&gt;G</td>
<td>9 (33.3)</td>
<td>3(10)</td>
<td>IREG2</td>
<td>Regulation of iron homeostasis</td>
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<td>18</td>
<td>46,474,797 Deletion</td>
<td>G&gt;-</td>
<td>5 (18.5)</td>
<td>0(0)</td>
<td>MAD7</td>
<td>Inhibitory protein of TGBF1 signaling</td>
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<tr>
<td>20</td>
<td>39,750,392 Deletion</td>
<td>G&gt;-</td>
<td>12 (44.4)</td>
<td>0(0)</td>
<td>STIP1</td>
<td>DNA topoisomerases 1 catalyze the breaking DNA</td>
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</table>
Identified candidate genetic variants associated with relapse of type 1 AIP

<table>
<thead>
<tr>
<th>Chr</th>
<th>Position type (SNP rs) Allele</th>
<th>Candidate gene</th>
<th>Gene description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>259183534 SNP (rs11431466) C &gt; G</td>
<td>HLA-A</td>
<td>The class 1 major histocompatibility complex</td>
</tr>
<tr>
<td>6</td>
<td>312392802 SNP (rs11431466) G &gt; C</td>
<td>P2RX3</td>
<td>The class 3 major histocompatibility complex</td>
</tr>
<tr>
<td>6</td>
<td>312392802 SNP (rs11431466) C &gt; T</td>
<td>P2RX3</td>
<td>The class 3 major histocompatibility complex</td>
</tr>
<tr>
<td>6</td>
<td>312392802 SNP (rs11431466) A &gt; G</td>
<td>P2RX3</td>
<td>The class 3 major histocompatibility complex</td>
</tr>
<tr>
<td>6</td>
<td>312392802 SNP (rs11431466) 4 bp deletion</td>
<td>P2RX3</td>
<td>The chemokine receptor expressed by helper T cell</td>
</tr>
</tbody>
</table>

AIP relapse occurred in seven patients. Next, we profiled the difference in the frequencies of genetic variants in the AIP patients with/without relapse. Among a total 2631 variants, 13 variants were extracted as significant candidates by Fisher’s exact test \( (P < 0.05) \) after excluding the variants without amino-acid modifications in which the strand bias was \( > 0.60 \). Among these 13 candidates, there were ten known single nucleotide polymorphisms (SNPs, rs4973986, rs1050716, rs1143146, rs2231119, rs1042131, rs2838171, rs75639061, rs17576) and four new variants (CACNA1S \( (c.624C \rightarrow T) \), TOP1 \( (c.2007delC) \), SMAD7 \( (c.624delC) \), TOP1 \( (c.2007delC) \)). Rs4973986 and rs17576 were excluded because almost all Japanese individuals are known to have these variants \[15\]. Finally, we identified nine candidate variants associated with type 1 AIP (Table 3).

3.3. The candidate genetic variants identified as being associated with the relapse of type 1 AIP

Among the 24 AIP patients, 18 had extra-pancreatic lesions. Finally, we investigated the difference in the frequencies of genetic variants in the AIP patients with/without extra-pancreatic lesions. Among a total 3319 variants, five known SNPs were extracted as significant candidates by Fisher’s exact test \( (P < 0.05) \) after excluding the variants without amino-acid modifications or in which the strand bias was \( > 0.60 \). Among these five SNPs, P2RX3 \( (c.195delG) \), SMAD7 \( (c.624delC) \), TOP1 \( (c.2007delC) \), CACNA1S \( (c.5996delC) \), CXCR3 \( (c.630_631delGC) \). Rs328, rs238238, rs16027, rs2071747 and three new variants (HLA-C \( (c.759_763delC) \), CACNA1C \( (c.5996delC) \), CXCR3 \( (c.630_631delGC) \)). Rs328, rs238238, rs16027, rs2071747 were excluded because almost all Japanese individuals are known to have these variants \[15\]. Finally, we identified eight candidate variants associated with the relapse of AIP (Table 4).

4. Discussion

The high-throughput sequencing analysis using next generation sequencing identified nine, eight and two candidate variants associated with the development of type 1 AIP, AIP relapse and extra-pancreatic lesions in type 1 AIP patients, respectively. Most of the previous studies to investigate the genes associated with susceptibility to AIP \[6–10\] did not involve comprehensive analyses, rather, they focused on target lesions that were reported to be associated with autoimmune disease or which were extracted by a microsatellite genotyping analysis. This might have been caused by the classical sequencing procedures. In contrast, our
study used high-throughput sequencing. A recent genome-wide association study did not reveal the genes that are associated with AIP susceptibility [11]. The present study is therefore the first comprehensive analysis of the genes related to AIP susceptibility.

AIP is considered to be an autoimmune disorder. Indeed, carbonic anhydrase, lactoferrin, pancreatic secretory trypsin inhibitor, amylase-alpha, ubiquitin-protein ligase E3, SPINK and TRY1 have been identified as the autoimmune antigens associated with AIP [16,17]. We therefore hypothesized that there might be commonality in the genetic background of AIP patients with regard to the genes associated with the immune system.

Among the nine candidate genetic variants that were found to be associated with type 1 AIP, P2RX3 (c.195delG) and TOP1 (c.2007delG) showed a highly significant association with AIP, even after adjustment by Bonferroni’s correction. P2RX3, which constitutes a positive autocrine signal for insulin release in the pancreatic beta cells [18,19] and contributes to the pancreatic pain caused by chronic pancreatitis [20], is associated with the immune system. A recent study reported that an enlarged spleen, which corresponds to an increase in the numbers of lymphocytes and macrophages, and hypocellularity of the thymus and bone marrow were observed in P2RX2 and P2RX3 knockout mice, suggesting that they resulted from compensatory changes in a compromised immune system [21]. TOP1, which encodes DNA topoisomerase 1 that catayzes the breaking and rejoining of single-strand DNA and which functions normally during transcription [22], is also associated with various autoimmune diseases, including scleroderma, systemic lupus erythematosus and rheumatoid arthritis [23,24]. Besides these two candidates, CACNA1S and MLL3 also play a role in the immune system. CACNA1S, which encodes the L-type calcium channel alpha 1 subunit, is expressed on T-lymphocytes and regulates the calcium current, which can induce the activation of T cell antigen receptors [25]. MLL3 forms a complex with MLL4 and subsequently initiates the transcription of downstream switch regions at the immunoglobulin heavy-chain locus in B-lymphocytes, leading to defective immunoglobulin class switching [26]. Although, these candidates might contribute to the pathogenesis of AIP, the influence of these candidate genetic variants on the immune system remains unknown. Further studies to investigate the relationship between these candidate genetic variants and the autoantibodies associated with AIP are needed.

Transforming growth factor beta (TGF-β) is known to be an important regulating factor in the maintenance of immune homeostasis. A previous study suggested that a loss of TGF-β signaling contributed to AIP [27]. In the present study, we identified a deletion in SMAD7 as a candidate genetic factor associated with the pathogenesis of AIP. Thus SMAD7 could be a suitable marker of AIP; however, it is necessary to further evaluate TGF-β signaling under the genetic condition of SMAD7 deletion (c.624delC).

We also identified eight candidate genetic variants that are associated with the relapse of type 1 AIP. Six candidates were HLAs, which are closely associated with some autoimmune diseases, including AIP [5]. In particular, HLA-DQB1 was reported to be associated with the relapse of AIP in a Korean study [28], which suggests that HLA-DQB1 could be a strong candidate gene associated with susceptibility to AIP relapse.

In this study, we also explored the genes that were associated with susceptibility to extra-pancreatic lesions, including sclerosing cholangitis, sialadenitis, dacryoadenitis, interstitial nephritis and lymph node swelling, and identified two candidates. Oguchi et al. performed a genome-wide association study and extracted ten candidates associated with susceptibility to dacryoadenitis or sialadenitis in the Japanese patients with AIP, while no genes associated with all extra-pancreatic lesions was identified [11]. In this study, because sialadenitis and dacryoadenitis were only observed in two and three patients, respectively, we could not identified the genes that were specifically associated with dacryoadenitis or sialadenitis susceptibility in AIP patients. Indeed, AIP is a rare disease with a prevalence rate of 4.6 per 100,000 population and an annual incidence rate of 1.4 per 100,000 population [13]. A nationwide or worldwide study will therefore be needed to validate the candidates associated with susceptibility to AIP.

5. Conclusion

In the present study, we performed a high-throughput sequencing analysis using next generation sequencing to comprehensively investigate 1031 genes. Our analysis identified nine, eight and two candidate variants associated with type 1 AIP, the relapse of AIP and extra-pancreatic lesions, respectively. These candidates might be used as markers for the diagnosis of AIP and extra-pancreatic lesions as well as for predicting the relapse of AIP.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jbbr.2016.03.005.

References


