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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.

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 also 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

Abbreviations: AIP, autoimmune pancreatitis; IgG4, immunoglobulin G fraction 4; HLA, human leukocyte antigen; HV, healthy volunteer; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism

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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 PPRS1 [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 dacryoadenitis 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 AmpliSeqTM Designer software program (https://www.ampliseq.com/browse.action) (Life Technologies, Carlsbad, CA, USA). These primer sets were provided 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 AmpliSeqTM 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 PI™ 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 (Cp, the number of reads from the plus direction in the known sequence; Cm, 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