Accurate clinical genetic testing for autoinflammatory diseases using the next-generation sequencing platform MiSeq

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ABSTRACT

Autoinflammatory diseases occupy one of a group of primary immunodeficiency diseases that are generally thought to be caused by mutation of genes responsible for innate immunity, rather than by acquired immunity. Mutations related to autoinflammatory diseases occur in 12 genes. For example, low-level somatic mosaic NLRP3 mutations underlie chronic infantile neurologic, cutaneous, articular syndrome (CINCA), also known as neonatal-onset multisystem inflammatory disease (NOMID). In current clinical practice, clinical genetic testing plays an important role in providing patients with quick, definite diagnoses. To increase the availability of such testing, low-cost high-throughput gene-analysis systems are required, ones that not only have the sensitivity to detect even low-level somatic mosaic mutations, but also can operate simply in a clinical setting. To this end, we developed a simple method that employs two-step tailed PCR and an NGS system, MiSeq platform, to detect mutations in all coding exons of the 12 genes responsible for autoinflammatory diseases. Using this amplicon sequencing system, we amplified a total of 234 amplicons derived from the 12 genes with multiplex PCR. This was done simultaneously and in one test tube. Each sample was distinguished by an index sequence of second PCR primers following PCR amplification. With our procedure and tips for reducing PCR amplification bias, we were able to analyze 12 genes from 25 clinical samples in one MiSeq run. Moreover, with the certified primers designed by our short program—which detects and avoids common SNPs in gene-specific PCR primers—we used this system for routine genetic testing. Our optimized procedure uses a simple protocol, which can easily be followed by virtually any office medical staff. Because of the small PCR amplification bias, we can analyze simultaneously several clinical DNA samples with low cost and can obtain sufficient read numbers to detect a low level of somatic mosaic mutations.

1. Introduction

There are many similar diseases that present clinically with fever and inflammation, and many of these occur due to gene mutations. Traditionally, identifying disease-causing gene mutations has been time-consuming and costly, requiring patient samples to be analyzed by a specialized laboratory employing sophisticated equipment. In recent years, however, a push is being made to make genetic testing available in clinical practice, in settings wherein doctors can quickly determine whether the patients in front of them have a mutation of genes known to underlie certain diseases. This type of clinical genetic testing plays an indispensable role in providing patients with quick, definite diagnoses. In order to reduce the economic burden on patients, clinical genetic testing must be performed accurately and quickly at a relatively low cost.

A general trend in Next Generation Sequencing (NGS) platforms is a move from the Roche GS 454 (its manufacturer no longer offers technical support) to the Illumina HiSeq. Development of a simple, accurate, and high-throughput method is embodied by the MiSeq platform. Presently, many kits are commercially available, but they are still too expensive for routine sequencing. A significant contributor to the cost of genetic testing is labor costs. Most currently used sequenc-
Clinical genetic testing should be performed carefully because autosomal dominant gain of function mutations are reported in many of these autoimmune diseases. Moreover, it is not known exactly whether somatic mutations other than those in NLRP3 confer one of the autoimmune diseases. We have chosen two-step nested PCR amplification and ampiclon sequencing for library preparation for the MiSeq platform for five reasons. Firstly, around 10 genes have been generally estimated as being candidate genes responsible for autoimmune diseases based on symptoms associated with these diseases. Secondly, new target genes will likely be added as this field of study continues to progress. Even if one made custom-ordered probes or capturing probes for all target genes simultaneously, researchers will want additional probes for newly discovered responsible genes in the future. It is costly to custom order a new set of probes again, because commercially available systems generally do not support small additions and changes to existing probes. Thirdly, high sensitivity and sufficient read depth are necessary in order to detect somatic mosaic mutations. Fourthly, as simple a procedure as possible is desirable. Lastly, costs need to be reduced. Motivated by these five reasons, we attempted to simultaneously analyze several clinical DNA samples on one MiSeq run, aiming to decrease PCR amplification bias as much as possible.

2. Materials and methods

2.1. Patients and clinical diagnosis

A total of 108 patients having an autoimmune disease diagnosis were consecutively diagnosed and recruited at the Department of Pediatrics, Kyoto University Graduate School of Medicine. All of the patients were Japanese and provided written informed consent (below) for inclusion in the high-throughput sequencing analysis.

2.2. Ethics statement

All patients provided written informed consent after we gave them a full explanation of the study. All patients gave us explicit permission to analyze their DNA sequencing data for genes responsible for autoimmune diseases. This study was approved by both the Human Research Ethics Committee of the University of Kyoto and the Kazusa DNA Research Institute.

2.3. DNA samples

DNA samples were de-identified with regard to subjects’ personal information. DNA from patients’ blood was purified using a QIAamp DNA blood kit (QIAGEN, Venlo, Netherlands). Before use, DNA samples were quantified by Qubit Fluorometric quantitation (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Design PCR primers

Primers were basically designed by ExonPrimer Perl script (https://ihg.helmholtz-muenchen.de/ihg/ExonPrimer.html). The design input parameters were 30 bp, 150 bp, and 20 bp for the minimal distance between the primer and exon/intron boundary, maxima target size, and overlap, respectively. The standard values were selected according to the parameters of Primer3Web options (http://bioinfo.ut.ee/ primer3/) [6].

ExonPrimer suggested candidate paired PCR primers. We wrote a short custom program to detect common SNPs in the DNA sequence of candidate PCR primers. This program called the UCSC In Silico PCR database (http://roshdb.cmb.ucsc.edu/G8shape/cgi-bin/hgPcr) and the dbSNPs database (dbSNP 135). We named this short program “Primer-dbSNP search” (i.e., SNPs finder for PCR primers). After checking common SNPs using Primer-dbSNP search, we re-designed undesirable primers to avoid common SNPs using commercially available oligo 6.0 Primer Analysis Software (Molecular Biology Insights, Inc., Colorado Springs, CO, USA). M13FW (5′-TGTTAAAAACGACGCGCC-3′) and M13RV (5′-GGAAACAGCTATGAC-3′) were added at the 5′ end of each forward and reverse primer, respectively. Primers were synthesized by Eurofins Genomics K.K. (Tokyo, Japan).

2.5. Library preparation following multiplex PCR

Supplemental Table 1 lists 234 PCR primer pairs used to target the protein coding region and a minimum of 20 bp of untranslated, flanking intronic region of the genes. Dual-indexed secondary PCR primers are described in Fig. 1. For the first PCR amplification, amplification was performed in a 0.2 ml 8-strip PCR tube (Corning, Corning, NY, USA) in a final volume of 50 μl. It had 25 μl of 2 X Multiplex PCR Buffer (Mg2+, dNTP plus) (TAKARA, Multiplex PCR Assay Kit Ver. 2); 0.25 μl of Multiplex PCR Enzyme Mix; each gene-specific primer pool (0.05 μM); and 400 ng of human genomic DNA. The following amplification steps were performed: 94 °C for 1 min, 10 cycles at 94 °C for 30 s, and 60 °C for 1 min, followed by incubation at 72 °C for 10 min. The resulting PCR products were purified twice with AMPureXP beads (Beckman Coulter Inc., Brea, CA USA). To minimize accidental cross-contamination between samples, we purified the PCR product using a low-binding tube, not a 96-well plate. Typically, 10 ng of purified PCR product was used for each PCR step. This is equivalent to using only one-third of the eluate from AMPureXP purification. For secondary PCR amplification, amplification was performed in a final volume of 50 μl. It had 25 μl of 2 X Multiplex PCR Buffer (Mg2+, dNTP plus) (TAKARA, Multiplex PCR Assay Kit Ver. 2); 0.25 μl of Multiplex PCR Enzyme Mix; each index primer (10 μM; each D501-D508-like and D701-D712-like Dual-indexed secondary PCR primers); and 10 ng of purified PCR product. The following amplification steps were performed: 94 °C for 1 min, 5 cycle at 94 °C for 30 s, 55 °C for 10 s, and 72 °C for 30 s, followed by incubation at 72 °C for 10 min. The PCR product was purified twice with AMPureXP beads using non-skirted thin-wall 96-well 0.2 ml plates. The purified PCR product was quantified with the Kapa Library Quantification Kit for the Illumina NGS (Kapa Biosystems, Wilmington, MA, USA), using an ABI 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). After quantification, we typically mixed equal molar concentrations of PCR product from 25 individual DNA samples, applied it to MiSeq using the MiSeq Reagent Kit for the Illumina NGS (Illumina, San Diego, CA, USA).
kit v3 600 cycles (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. In line with the so-called DarkCycle procedure, we did not collect data for the first 15 cycles, because the first 15 bp are common sequences containing M13FW and M13RV DNA sequences in R1 and R2 reads, respectively.

### 2.6. Data analysis

Each PCR primer sequence at the 5′ terminus of the read sequence was trimmed from the sequence read data, and each PCR primer and additional M13FW or M13RV 15-mer sequence in the 3′ terminus of the read sequence were also trimmed from the sequence read data by the software Cutadapt [7]. Low quality sequences, namely low quality 25 (Q25) from the 3′ terminus of the read sequence were trimmed from the sequence read data without the primer sequence using the software sickle [8]. The software's quality filter removes the R1 and R2 reads, which contain low quality sequences at a high ratio. The software Sommelier, described previously [5], is a variant caller program that includes Blat software [9] as a component. Trimmed sequence read data were mapped onto DNA sequence data of the amplicon derived from the human reference genome (hg19) using Blat and default parameters. Variants, which were identified by Sommelier, were annotated using a short program (proc_exome_mutation_b37.v4.pl). After manual curation, all missense, nonsense, and frame-shift mutations, as well as other severe mutations, were confirmed by Sanger DNA sequencing.

![Schematic diagram illustrating our MiSeq approach to identifying genes responsible for autoinflammatory diseases.](image)
PCR primers contained an additional common 5′-15 bp of M13FW and M13RV sequence in the 5′ terminus to be completely overlapped with some amplicons derived from split exons. These gene-specific primers contained 88 coding exons. Since large exons need multiple primer pairs, we made 320 primers for PCR amplification and deviation of read number of each amplicon by analyzing several DNA samples simultaneously.

First, we selected nine genes (IL1RN, MEFV, MVK, NLRP12, NLRP3, NOD2, PSMB8, PSTPIP1, and TNFRSF1A) associated with autoinflammatory diseases, and made a total of 160 primer pairs. These nine genes contain 88 coding exons. Because PCR amplification bias increases during each step of the PCR cycle, in our protocol we performed 10 cycles for the first PCR amplification and 5 cycles for the second PCR amplification. Various PCR amplification parameters were assessed during preliminary experiments in order to determine the optimal conditions (i.e., the number of PCR cycles, the concentration of each PCR primer in the reaction, and the annealing temperature) for the gene-specific primer sets for the nine genes responsible for autoinflammatory diseases (see Methods). We noticed that performing the AMPureXP purification produced better results for MiSeq DNA sequencing, since small PCR products derived from primer dimers produce many empty sequence reads, which waste large volumes of limited and valuable read numbers in MiSeq.

Secondary PCR amplification was performed to add an index sequence distinguishing each PCR product derived from each clinical DNA sample. Typically, the indexed PCR product derived from the 25 individual DNA samples were mixed and applied to one MiSeq run. After a MiSeq run, sequencing data were demultiplexed by using the index sequence, PCR primer sequences and low quality regions were trimmed, and sequence reads containing low-quality sequences were removed. This was accomplished using the variant call software Sommelier, which calls variants from the trimmed sequence read data and writes a user-friendly file containing a variant list with annotation information in Excel format.

Supplemental File 2 shows typical clinical genetic testing results for autoinflammatory diseases. Sheet 1, “Read Count” of Supplementary File 2 shows the read number of each amplicon. These indicate that there are enough read numbers in each amplicon. The average read number for forward and reverse orientation is 4583 and 4469, respectively. The rate of over 20 reads for both orientations is 100%. The rate of over 30 and 100 reads in both orientations are 99.79% and 98.95%, respectively. Moreover, total read numbers, or the sum of forward and reverse read numbers for each amplicon, are shown in Fig. 2. Average total read number of each amplicon was 9052. The average minimum read number and maximum read number was 405 and 32434, respectively. The ratio of the average maximum read number to average minimum read number was 79.94. We observed that the total read number in some amplicons was relatively small, and they contained an unusually high GC-rich region. PCR amplification bias is unavoidable for such extremely GC-rich regions. In conclusion, in our multiplex experimental condition, PCR amplification bias is low, which permits parallel analysis of many clinical samples.

The read number for each amplicon in Fig. 2 and Supplemental File 2 relates to the average depth of each exon; that is, the quotient is total base number derived from all read numbers divided by base number of the exon. Because large exons were covered by sequence reads of some overlapping amplicons and gaps in exons, special attention was required, especially for large exons. The read depth for each base was completely checked for all coding exons of the targeted genes. When no gaps are present in the coding exons, MiSeq achieves sufficient read depth for each base. For regions sequenced at a depth greater than 25, the depth coverage was 100%; for those sequenced at a depth greater than 30, the depth coverage was 99.98%. These results showed that amplicon sequencing with sufficient depth was performed.

Sheet 2 of Supplementary File 2 (Final Results sheet) shows a list of mutations identified using the MiSeq platform. The list contains...
The presence of an accidental SNP in a gene-specific template. In heterozygotes, the DNA template from one allele hybridizes with the primer theoretically can occur during PCR amplification. However, we believe this is not relevant in the case of our approach for the following reasons. First of all, we used 400 ng of human DNA, which is equivalent to 1.3x10^6 copies of the haplotype, which translates to an average read number of about 9000 per amplicon. This read depth is sufficient enough so that one can ignore any rare, artificial mutations that theoretically can occur during PCR amplification. Because our PCR products were derived from amplification of an independent template of 1.3x10^6 copies, even if misincorporation did occur during the first PCR amplification step, its effect would have been extremely small or negligible.

Frequency of variants is an important numerical aspect for determining whether a mutation is a homozygous or heterozygous mutation. In the early stages of this study, we noticed a case that had a variant frequency of less than 12% in one amplicon of a sample from one patient. Sanger DNA sequencing revealed that genomic DNA corresponding to the gene-specific PCR primer, by chance contained a SNP. The presence of an accidental SNP in a gene-specific PCR primer causes a one-base mismatch between the PCR primer and the DNA template. In heterozygotes, the DNA template from one allele hybridizes with the primer completely. On the other hand, a DNA template with a SNP hybridizes with the mismatch. Therefore, PCR amplification efficiency is much lower in cases of mismatch than it is when the PCR primer and DNA template complete match. Fig. 3 shows the relationship between the location of a mismatch in a PCR primer and the detection frequency of a variant allele containing a SNP. As expected, a SNP near the 3' end of the primer greatly affects the detection frequency of the variant. Especially having a SNP at the first and second base position in a PCR primer significantly decreases the detection frequency by 1–2%.

Although SNPs very rarely occur in gene-specific PCR primers, because we plan to analyze genes of several hundred patients, we took countermeasures to reasonably guard against this problem. We wrote a short program to detect SNPs in gene-specific PCR primers. When common SNPs (> 1% frequency) were detected in a PCR primer, the PCR primer was re-designed to avoid SNP sites. Completely new PCR sets void of common SNPs were used in subsequent MiSeq experiments.

Our genetic testing using MiSeq also detected large deletions in X-chromosome-linked genes from a patient (data not shown; manuscript in preparation) on other similar panel sets for primary immunodeficiency diseases, but not on the panel we used for the present study to examine autoinflammatory-disease-causing genes. The significant decrease in read number of the amplicon indicates the deletion of the exon(s). In fact, we did confirm that this was due to a large deletion in the patient's genome.

Next, we tried to detect mosaic mutations using our MiSeq detection system. In the first model experiments, two DNA samples with different SNPs were mixed at a rate of 5% and 10%, respectively. This mixed DNA sample was used for our MiSeq experiment. When a SNP in the material was a heterozygote, as expected we detected nearly 2.5% and 5% of variant frequency. This demonstrates that variant frequency values derived from our system are extremely precise. In the next experiment involving the detection of mosaic mutations, we tested three real DNA samples, which were identified to have somatic mosaic mutations. As shown in Table 1, our MiSeq experiment detected 35.3%, 7.0%, and 6.3% of the variant frequency of these samples.

Next, we tested whether we could add the primers of three other genes onto the nine-gene panel used for our MiSeq experiment. The gene-specific primers of three genes, NLR54C, PLCG2, and HMOX1, were designed according to the procedure described above. We took special precautions to avoid accidental incorporation of common SNPs into the designed candidate primers. PCR primer pairs of 74 amplicons derived from 45 coding exons in these three genes were added to the pool of PCR primers used for the previously identified nine genes responsible for autoinflammatory diseases. Addition of the three new primer pairs did not negatively affect the accuracy of genetic testing using our MiSeq system. Indeed, our system is sufficiently robust and flexible that it could accommodate changes, such as the addition of these three genes. One strong advantage this system has in terms of clinical genetic testing is its flexibility, which would be especially useful for relatively “new” syndromes like autoinflammatory diseases. These diseases have only recently been recognized, and as they receive more attention from the scientific community, likely more responsible genes will be identified and will need to be characterized.

The mutations that we found in this study are summarized in Table 2. Twenty-seven missense mutations emerged from the 108 patients examined. The most notable of these were Gly566Ala in NLRP3, Lys34Thr and Asp369Gly in PSTPIP1, Arg410His and Pro113Arg in MEFV, and Gln902Lys in NOD2, which have not been reported previously. All missense mutations identified in this study were confirmed by Sanger sequencing. Independent DNA sequencing using gold-standard methods, such as Sanger sequencing, validates the reliability of genetic testing using the MiSeq system in identifying mutations. In addition, independent DNA sequencing would identify any errors that may have arisen from, for example, a technician inadvertently mixing up patient samples.

In one of the samples we analyzed, we did not detect any mutations in genes that were identified previously to cause autoinflammatory diseases. This suggests that not all responsible genes were included in our PCR-primer sets, because some genes responsible for autoinflammatory diseases have not been identified yet. Our aim, therefore, is to broaden our ability to test for more autoinflammatory disease-associated genes simultaneously using our MiSeq platform.

In the present study, we noticed that MiSeq had some tendencies that could lead to inaccurate results if not fixed via silico analysis. First, in the eight serial thymine (T) cluster, we often observed artificial T deletions, at an approximate frequency of 2%. Second, mis-synthesized PCR primers were often detected as noise in the analysis. The DNA synthesizer we used never produced 100% exact oligomers. Rather, it mis-synthesized a very small volume of product. Moreover, cutadapt software hardly ever detected and trimmed PCR primers containing a mutation. Thus, the noise derived from mis-synthesized primers should be removed during silico analysis. Fourth, pseudo genes, gene families, and repeat sequences located extremely close to an exon can lead to the production of false amplicons during PCR, which, when amplified, produce noise reads. False mapping was detected as noise. Such false mapping should also be removed by in silico analysis.

In our MiSeq approach, the primer design step is one of the most important steps to ensure successful multiplex PCR amplification with minimal PCR amplification bias. PCR primers that overlap each other must be avoided, above and beyond the need to make good PCR primers.

Exome analysis can survey mutations in all exons without knowing ahead of time the genes responsible for any given disease. At the present time, however, exome analysis remains expensive and has a relatively low read number. Indeed, with a small read depth, even the MiSeq platform cannot distinguish true mutations from misreads and surely cannot detect low frequencies of somatic mosaic mutations.

Our MiSeq approach is suitable for routine clinical genetic testing. If our MiSeq approach does not detect a significant mutation, if necessary, additional analysis (e.g., exome analysis) should be per-
formed. We are currently developing another gene panel for the MiSeq platform in order to analyze another category of primary immunodeficiency disease. Along this line, we are developing a system to analyze simultaneously over one thousand amplicons of 57 genes that confer primary immunodeficiency diseases, although we need to reduce PCR amplification bias and differences in read number in NGS.

### Table 1: Detection of somatic mosaic mutations in patient DNA using our MiSeq analysis system.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Genome Position</th>
<th>Gene Symbol</th>
<th>Fwd Freq</th>
<th>Rev Freq</th>
<th>Fwd Read</th>
<th>Rev Read</th>
<th>Region</th>
<th>CDS Level Change</th>
<th>AA Variation</th>
<th>dbsNP</th>
</tr>
</thead>
<tbody>
<tr>
<td>11028IS</td>
<td>chr1:247587751</td>
<td>NLRP3'</td>
<td>35.767</td>
<td>34.922</td>
<td>4985</td>
<td>2643</td>
<td>exon3e</td>
<td>c.1000 A &gt; G</td>
<td>p. Ile334Val</td>
<td>–</td>
</tr>
</tbody>
</table>

In “Regions” having large exons, the exons were overlapped by amplicons. Letter after exon number indicates the name of each amplicon. The names of the amplicons are identical to the names of the corresponding primers, as shown in Additional File 1. Amplicons, exon3i, and exon3j were independently amplified, sequenced, and analyzed.

1 In NLRP3, the clinical genetics community typically uses the second Met as the initial start site, not the first Met. Thus, in this study, NLRP3 had two additional amino acids because the first Met was used for the annotation of the NLRP3 gene.
was used for the annotation of the NLRP3 gene in this study.

4. Conclusions

In this study, our MiSeq approach using multiplex PCR can perform genetic testing with high sensitivity and accuracy to detect even low-level somatic mosaic mutations that cause autoinflammatory diseases. This simple procedure can be both time- and cost-effective, and can be conducted by technicians with an average skill level and without special training. We routinely analyze several clinical DNA samples in one MiSeq run every two weeks. This simple procedure will also minimize inevitable human errors and guarantee the accuracy required for diagnostic application.

Conflict of interest

The authors declare that there are no conflicts of interest.

Authors’ contributions

M.N. carried out the MiSeq experiments; M.N., H.O., and O.O. conducted the bioinformatics analyses; K.N., T.Y., T.K., K.I., R.N., and O.O. conceived the study, and participated in its design and coordination. All authors helped to draft the manuscript. All authors read and approved the final manuscript.

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Appendix A. Transparency document

Transparency data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.12.002.

References


