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Gene Mosaicism Screening Using Single-Molecule Molecular Inversion Probes in Routine Diagnostics for Systemic Autoinflammatory Diseases

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Diagnosis of systemic autoinflammatory diseases (SAIDs) is often difficult to achieve and can delay the start of proper treatments and result in irreversible organ damage. In several patients with dominantly inherited SAID, postzygotic mutations have been detected as the disease-causing gene defects. Mutations with allele frequencies <5% have been detected, even in patients with severe phenotypes. Next-generation sequencing techniques are currently used to detect mutations in SAID-associated genes. However, even if the genomic region is highly covered, this approach is usually not able to distinguish low-grade postzygotic variants from background noise. We, therefore, developed a sensitive deep sequencing assay for mosaicism detection in SAID-associated genes using single-molecule molecular inversion probes. Our results show the accurate detection of postzygotic variants with allele frequencies as low as 1%. The probability of calling mutations with allele frequencies ≥3% exceeds 99.9%. To date, we have detected three patients with mosaicism, two carrying likely pathogenic *NLRP3* variants and one carrying a likely pathogenic *TNFRSF1A* variant with an allele frequency of 1.3%, confirming the relevance of the technology. The assay shown herein is a flexible, robust, fast, cost-effective, and highly reliable method for mosaicism detection; therefore, it is well suited for routine diagnostics. *(J Mol Diagn 2019, 21: 943—950; https://doi.org/10.1016/j.jmoldx.2019.06.009)*

Systemic autoinflammatory diseases (SAIDs) are a group of clinical conditions characterized by systemic sterile inflammation, caused by an abnormal overactivation of the innate immune system.[1—3] Some of the most common symptoms are fever, rash, abdominal pain, and arthritis. Many different SAIDs have been described, but as their symptoms often overlap, it is challenging to achieve the definitive diagnosis clinically. Moreover, up to 50% of SAID patients do not clearly fit one of the described conditions.[4,5] In the past 25 years, >30 SAID-associated genes have been discovered,[6] the majority causing a monogenic SAID.[7,8] Unexpectedly, conventional DNA analyses fail to find a disease-causing mutation in >80% of SAID patients.[9—12] This further complicates and postpones the patients' definitive diagnosis, which may also delay receiving the proper treatment. Therefore, irreversible organ damage can occur. Major causes of the low diagnostic yield are the large phenotypic spectrum, genetic diversity, and intrinsic limits of used methods of genetic analyses.[4,13]

In 2005, the first postzygotic mutation causing SAID was detected in the *NLRP3* gene.[14] Since then, additional patients with mosaicism have been found, mainly in the *NLRP3* gene but also in other SAID-associated genes like *NOD2*, *TNFRSF1A*, and *NLRC4*.[15—18] Previous reports have shown that even mutations with an allele frequency

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<5% in DNA isolated from whole blood have been found to result in severe SAID.[19–21]

With next-generation sequencing (NGS) deep sequencing techniques, low-grade mosaicism is detected by generating high coverage. However, these results are generally not corrected for PCR bias and DNA polymerase–induced artifacts. Therefore, true postzygotic variants with low allele frequencies cannot be reliably distinguished from background noise, leading to false-negative results. To date, 2.4% was the lowest allele frequency of a postzygotic SAID–associated mutation detected in whole blood.[21] Mutations with similar or even lower allele frequencies were detected, but only when examining a previously detected variant in specific cell populations or when testing parents of a mutation carrier for recurrence risk.[21–23]

Molecular inversion probes (MIPs) were originally developed for time- and cost-effective resequencing of candidate genes in large patient cohorts.[24] The addition of a series of randomly built-in nucleotides, the unique molecular identifier (UMI), also described as a single-molecule tag, made the probes suitable for detection of postzygotic variants.[25] Because of the UMI, all PCR duplicates originating from a single DNA molecule are marked with the same molecular barcode, facilitating deduplication into a single consensus read. This enables assessment of true library complexity and strongly reduces the number of false-positive results due to sequencing errors. Hereby, we present a highly sensitive single-molecule MIPs (smMIP) assay for mosaicism detection and demonstrate its added value for routine SAID diagnostics.

Materials and Methods

Sample Inclusion

For all included patients, genetic SAID analysis was requested by a clinician and performed by whole exome sequencing. Analysis of 33 SAID-associated genes showed no (likely) pathogenic variants. For smMIP assay performance analysis and patient mosaicism screening, 325 samples from 158 patients were used. For each patient, at least two independent DNA samples were tested.

Sample Preparation

Genomic DNA was isolated from whole blood using the Chemagic DNA Blood Kit (PerkinElmer, Waltham, MA) and sheared into 350-bp fragments on a Covaris LE220 (Covaris, Woburn, MA), according to the manufacturer's protocol with the following settings: water bath temperature, 5°C to 8°C; sample volume, 130 μL; duration, 110 seconds; peak incident power, 450 W; duty factor, 15%; and cycles per burst, 200.

smMIP Pool Preparation

smMIPs were designed using MIPgen,[26] targeting the following regions of interest in genome build GRCh37 (hg19): *NLRC4* exon 4 (NM_021209.4), *NLRP3* exon 3 (NM_001243133.1), *NOD2* exon 4 (NM_022162.2), *PSTPIP1* exons 10 and 11 (NM_003798.4), and *TNFRSF1A* exons 2, 3, 4, and 5 (NM_001065.3). Each region of interest includes 20 bp of flanking intronic sequences on both sides of the exon. In total, this panel covers 6632 bp.

All smMIPs are 78 nucleotides in length, consisting of a 30-nucleotide common backbone, two hybridizing arms with a sum of 40 nucleotides, and an 8-nucleotide UMI. All smMIPs capture a 112-bp target sequence. If one of the hybridizing arms covered a single-nucleotide polymorphism with >1% minor allele frequency in the gnomAD[27] or GoNL[28] database, two smMIPs were designed to target both alleles. After manufacturing (Integrated DNA Technologies, Coralville, IA), all smMIPs were equimolarly pooled and tested. Rebalancing of the pool was considered per smMIP, dependent on its performance, location within the regions of interest, and overlap with other probes.

The smMIP pool was phosphorylated by adding 1 μL of T4 Polynucleotide Kinase (New England Biolabs, Ipswich, MA) for every 25 μL of 100 μmol/L smMIPs in 1× T4 DNA ligase buffer with 10 mmol/L ATP (New England Biolabs). The mixture was incubated at 37°C for 45 minutes, followed by kinase inactivation at 60°C for 20 minutes.

Library Preparation

Capture was performed on 200 to 500 ng of fragmented DNA in a 25-μL reaction volume by adding 1 unit of Ampligase DNA ligase (Epicentre, Madison, WI), 1× Ampligase Buffer (Epicentre), 3.2 units of Hemo Klentaq (New England Biolabs), 8 pmol dNTPs, and an aliquot of smMIP pool to achieve a molecular ratio DNA/smMIP of 1:800 for each individual smMIP. The mixture was denatured at 95°C for 10 minutes and then incubated at 60°C for 21 hours to enable correct smMIP hybridization, extension, and ligation. After cooling, exonuclease treatment was performed in 1× Ampligase buffer by adding 10 units of Exonuclease I (New England Biolabs) and 50 units of Exonuclease III (New England Biolabs) to the capture product. The mixture was incubated at 37°C for 45 minutes, followed by inactivation at 95°C for 2 minutes. PCR was performed in a 50-μL volume containing 50 pmol common forward primer, 50 pmol barcoded reverse primer,[24] 1× iProof high-fidelity master mix (Bio-Rad, Hercules, CA), and 20 μL of the exonuclease treated product. The mixture was denatured at 98°C for 30 seconds, followed by 19 cycles with 10 seconds of denaturing at 98°C, 30 seconds of hybridization at 60°C, and 30 seconds of elongation at 72°C, and a final elongation step at 72°C for 2 minutes. After cooling, the PCR products were measured with

TapeStation 4200 (Agilent, Santa Clara, CA), and up to 96 samples were equimolarly pooled. The pool was purified by bead-based size selection with a 0.5% and a 0.8% volume of Agencourt Ampure XP (Beckman Coulter, Brea, CA).

Sequencing

The pool was denatured and diluted to a concentration of 1.0 pmol/L and loaded on a NextSeq500 sequencer (Illumina, San Diego, CA), according to the manufacturer's protocol. The following sequencing primers were used: forward, 5′-CATACGAGATCCGTAATCGGGAAGCTGAAG-3′; reverse, 5′-ACACGCACGATCCGACGGTAGTGT-3′; and index, 5′-ACACTACCGTCGGATCGTGCGTGT-3′. The sequencer was run with a 300-cycles Mid Output sequencing kit (Illumina), resulting in 2 × 150 bp paired-end reads.

Data Analysis

Conversion of raw sequencing data to FASTQ files and simultaneous barcode demultiplexing were performed with bcl2fastq2 Conversion Software version 2.20 (Illumina). The FASTQ files were transferred to a server running the SeqNext module of the Sequence Pilot commercial analysis software version 4.3.1 (JSI Medical Systems, Ettenheim, Germany). The designfile was used to generate a mapping target, according to the SeqNext manual. Two minor adjustments were made to rule out recurrent false-positive results in analysis (Supplemental Table S1). Read mapping, deduplication of reads with the same UMI (consensus calling), and variant calling were performed semi-automatically in SeqNext with the following settings: required coverage, minimum absolute coverage, 200 per direction; mutations, minimum absolute coverage, 5 combined; minimum percentage coverage, 1% per direction; mutation sorting, distinct/other percentage coverage, 40% per direction; tags enabled, yes; tag length R1, 8 bp; ignore tags with N bases, yes; ignore tags with low Qs, yes; minimum absolute coverage, 1; minimum percentage coverage, 50%; and ignore consensus read threshold, 30%. For each UMI, a minimum of two reads is required for consensus calling. SeqNext performs consensus calling and further analysis separately for forward reads and reverse reads (per direction). This results in a forward and a reverse consensus read derived from each captured molecule. A variant call will be made when the requirements are met by both forward consensus reads and reverse consensus reads. With the settings used, all variants with label other are considered candidate postzygotic variants and need to be further examined.

Statistical Analysis

A cumulative binomial distribution function was used to determine the variant call probability, with p being the true variant allele frequency in the tested material, N the consensus read depth per direction at the particular nucleotide position, and x the number of variant reads needed for a variant call to be made. The standard function calculates the probability of x or less variant reads:

$$P(X \leq x) = \sum_{i=0}^{x} p^i \binom{N}{i} (1-p)^{N-i} \quad (1)$$

When performing the assay on two independent DNA samples, a variant call has to be made in at least one for a postzygotic mutation to be detected. In case a variant is detected in one sample and not in the other, further examination is required. With the settings used, a variant call is made when at least 1% of consensus reads per direction contain the variant and when at least five consensus variant reads (combined, similar to three reads per direction) are detected. Hence, the function was adjusted to calculate the probability of a variant being called in at least one sample, with N^* equaling N rounded up to the next hundred:

$$\begin{aligned} &1 - P(X \leq x)^2 = 1 - \left(\sum_{i=0}^{x} p^i \binom{N}{i} (1-p)^{N-i} \right)^2 \\ &\text{at} \geq 300X : x = \frac{N^*}{100} - 1 \\ &\text{at} < 300X : x = 2 \end{aligned} \quad (2)$$

Herein, it was assumed that every forward consensus read has a mate reverse consensus read originating from the same captured molecule.

ddPCR

The postzygotic c.269C>A p.Thr90Asn variant in the *TNFRSF1A* gene was not detected by Sanger sequencing or whole exome sequencing; therefore, droplet digital PCR (ddPCR) was performed. All available samples containing the postzygotic variant were tested together with positive and negative control samples. Multiple water control samples were tested for determining background noise. The following primers and probes were designed using Primer3 software version 2.4.0, manufactured by Integrated DNA Technologies, and mixed with primers to probe ratio 1.8: forward primer, 5′-CCCATTCACAGGAACCTACTTG-3′; reverse primer, 5′-ACTCACCCTTTCGGCATTTG-3′; reference allele probe, 5′-CAGGGAGTGTGAGAGCGGCTCCTTCACCGC-3′ (FAM-labeled and double quenched); and variant allele probe, 5′-CAGGGAGTGTGAGAGCGGCTCCTTCAACGC-3′ (HEX labeled and double quenched). ddPCR was performed by the QX200 Droplet Digital PCR system (Bio-Rad). A PCR mixture was prepared containing 20 ng of DNA, 1× ddPCR supermix for probes (no dUTP; Bio-Rad), 1× variant allele primers/probe, and 1× reference allele primers/probe in a total volume of 20 μL. The droplet generator was used to partition the PCR mixture in 8000 to 22,000 droplets. The droplets were incubated at 95°C for 10 minutes for polymerase activation, followed by 40 cycles with 30 seconds of

denaturation at 95°C and 1 minute of annealing and extension at 62°C. The enzyme was then deactivated at 98°C for 10 minutes. After cooling down, fluorescent signals of individual droplets were read by the droplet reader. Analysis was performed using QuantaSoft software version 1.7.4.0917 (Bio-Rad).

Results

smMIP Pool Balance and Performance

Supplemental Table S1 shows all used smMIPs and their rebalancing factor in the final pool. This pool contains 111 smMIPs for 109 amplicons. Of the nucleotides within the regions of interest, 100% are covered by at least one smMIP, with 66.4% (4402 of 6632 nucleotides) being targeted by two or more smMIPs.

For performance analysis, 325 samples were evaluated from 158 SAID patients tested in six independent preparation and sequencing runs. For each patient, at least two independent samples were tested. After deduplication, the mean coverage of all nucleotides per sample was 4926× per direction (range, 1057× to 10,076×) (Figure 1A). The mean of lowest nucleotide coverages per sample was 696× per direction (range, 206× to 2158×) (Figure 1B). With all samples having ≥206× coverage on all nucleotide positions, all patients have a >99.9% probability for calling variants with ≥3% allele frequency on any nucleotide position in this panel. For variants with 2%, 1.5%, 1.2%, and 1% allele frequency, having 206× coverage, a variant call is made with 95%, 84%, 70%, and 56% probability, respectively. For the obtained mean nucleotide coverage (4926×), probabilities are 100%, 100%, 99%, and 73%, respectively.

Positive Control Samples

Four samples from positive control subjects, containing postzygotic variants with allele frequencies ranging from 2% to 20%, were tested. All variants were called with this assay. In addition, samples with different genotypes at common single-nucleotide polymorphism positions were selected, mixed in ratios varying from 1:1 to 1:255, and tested as single samples in three independent validation runs. These runs had lower coverage than those used for performance analysis and patient screening. All variants with ratios of 1:1 to 1:31 were called, including 32 variants with a ratio of 1:31 (±3.1% allele frequency). Results for 32 variants with a ratio of 1:63 (±1.6% allele frequency) are listed in Table 1.

Patient Screening Results

A total of 158 patients with SAID for mosaicism and 3 postzygotic variants (1.9%) have been detected (Table 2). All variants were present in at least two independent samples with similar allele frequencies. Variant 1 was captured by only one smMIP, whereas variants 2 and 3 were captured by two overlapping smMIPs and were detected by both with similar allele frequencies. Also, variants 2 and 3 were detected with similar allele frequencies in analyses from multiple runs and in DNA samples isolated from whole blood extracted in different years.

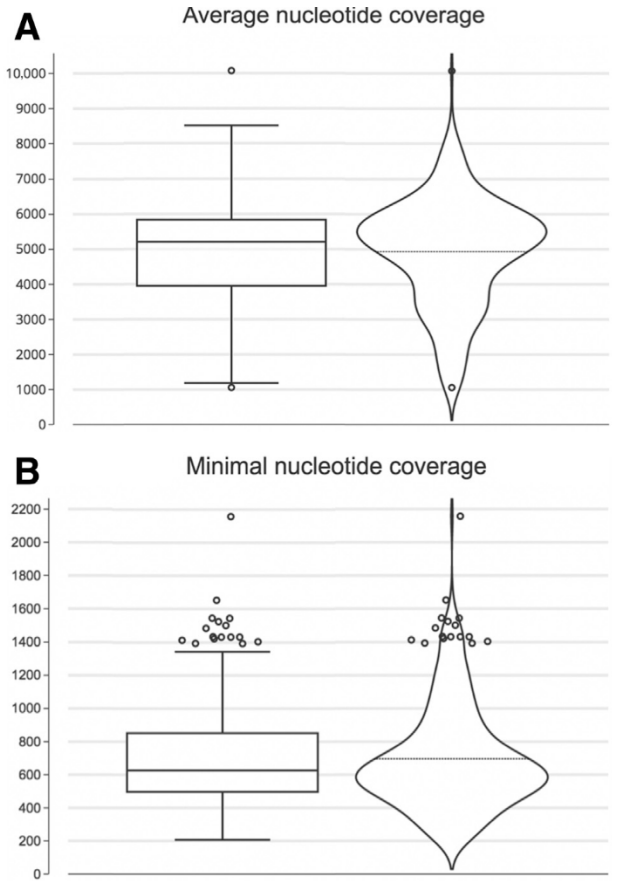

Figure 1 Distribution of mean (**A**) and minimal (**B**) nucleotide coverages of 325 samples. **Dotted lines** in the violin plots represent the mean values. All coverages are per direction.

Confirmation of Postzygotic Variants

The three detected postzygotic variants (Table 2) were assessed and confirmed by alternative methods. Variant 1 was confirmed by whole exome sequencing. Although this variant did not pass variant calling, it was present in the processed binary alignment map (BAM) file. Variant 2 was confirmed by Sanger sequencing. Variant 3 was not detected in the patient's whole exome sequencing data. Therefore, ddPCR was performed, and this variant was detected with similar allele frequency as detected with the smMIP assay (Table 3).

Discrimination of False Positives from True Variants

In 158 patients analyzed, 15 positive results were detected. Three of these corresponded to the aforementioned samples with confirmed postzygotic variants. The remaining 12 positive results, found at four different nucleotide positions,

Table 1 Results of Variants with ±1.6% Allele Frequency from Three Independent Validation Runs

Run	Mean coverage at variant position	Variants, *n*	Calls expected, *n*	Calls made, *n*
1	217×	8	5—6	7
2	629×	12	10—11	10
3	4891×	12	12	12

Coverages are per direction. Call expectations are based on the used statistics and adjusted for testing single samples.

were called with <1.2% allele frequency. Three variants were called once, whereas one variant was called in nine samples from the same run. Three of the four nucleotide positions were captured by overlapping smMIPs, with variant reads found only in one smMIP. None of these variants was called in the other analyzed sample from the same patient or in another analysis of the same patient's sample. Moreover, for all variants detected in positive control and patient samples, background noise was checked in 210 to 218 samples from four runs (Figure 2). The mean background noise was highest in variants 1 through 4. For these variants, many samples had background noise >0.5%; however, no values >0.2% were found for variants 5 through 11. Also, background noise of variants 1 through 4 was significantly higher in the run with a positive result compared with the other runs. For these reasons, variants 1 through 4 were regarded as false-positive results.

Discussion

We described an smMIP-based assay, adapted and optimized from previously published protocols,[24,29] for gene mosaicism screening in SAID patients. Our smMIP panel includes nine exons from five genes, all being associated with dominantly inherited SAID, caused by a gain-of-function mechanism. A few postzygotic variants have been detected in genes associated with recessively inherited SAID,[18] but by far most SAID-related postzygotic variants have been detected in the regions targeted. To target new regions of interest, new probes can be added to an existent smMIP pool without affecting capture performance of the existing smMIP pool.

Most SAID-causing mutations are single-nucleotide variants. To date, only a single deletion in the *TNFRSF1A* gene has been described.[16] Although our positive control samples and detected variants are single-nucleotide variants, it is well known that small deletions and insertions can reliably be detected by smMIP technology.[29]

In early SAID-related mosaicism screening, Tanaka et al[20] suggested a detection limit of 5% allele frequency for their subcloning approach. They expected to miss variants with lower allele frequencies and mentioned the need for NGS techniques to overcome this issue. Izawa et al[30] first described a highly sensitive NGS-based method for SAID mosaicism screening, although their studies were restricted to the *NLRP3* gene. They were able to reliably call postzygotic variants with 1% allele frequency by correcting for sequencing errors. However, on error-prone positions, variants with up to 5% allele frequency could not be detected with similar reliability. As the NGS error rates have been greatly reduced in recent years, NGS deep sequencing techniques have become the method of choice regardless of sequencing artifacts.

With this assay, variants with allele frequencies as low as 1% could be called reliably. On the basis of the performance of this assay in six independent runs with a total of 325 analyzed samples, the probability of calling a variant with 1.2% allele frequency on any position was 99%. Moreover, the probability of missing a postzygotic variant with allele frequency ≥3% on any nucleotide position in this panel was <0.1%. And for all variants called, it could easily be determined whether it was a false-positive result or a true postzygotic variant.

With this assay, three patients carrying postzygotic variants, two in the *NLRP3* gene and one in the *TNFRSF1A* gene, have been detected. One of the detected *NLRP3* variants had increasing allele frequency over time, as has been described before.[23] It appears that the *TNFRSF1A* variant also has a slowly increasing allele frequency over time. Over 30 different *NLRP3* postzygotic variants have been found,[18] but only two have been reported in the *TNFRSF1A* gene.[16,31] Interestingly, those have allele frequencies high enough to be detected by Sanger sequencing, whereas the postzygotic *TNFRSF1A* variant detected by this assay has a much lower allele frequency (1.3%). By our knowledge, this is the SAID-causing postzygotic variant with lowest allele

Table 2 Postzygotic Variants Detected in This Study

Patient no. (year)	Gene	Variant	Protein effect	Allele frequency, %	Mean coverage*
1	*NLRP3*	c.918A>T	p.Gln306His	10.4	6768× (2)
2 (2005)	*NLRP3*	c.1706 G>C	p.Gly569Ala	18.8	5228× (4)
2 (2013)				34.6	4499× (4)
3 (2011)	*TNFRSF1A*	c.269C>A	p.Thr90Asn	1.1	14,797× (4)
3 (2013)				1.3	9828× (6)
3 (2018)				1.4	4744× (4)

For variants 2 and 3, DNA samples isolated from whole blood extracted in different years were tested.
*Coverages are per direction. The number inside parentheses represents the number of independent measurements used for calculating the mean coverage.

Table 3 Results of ddPCR Testing of the *TNFRSF1A* Postzygotic Variant and Control Samples

Sample	Gene	Variant	Protein effect	Allele frequency, %	Mean coverage*
PC	*TNFRSF1A*	c.269C>A	p.Thr90Asn	48.9	2437× (2)
NC				0.0	2184× (2)
2011				1.2	2585× (4)
2013				1.4	3453× (4)
2018				1.5	2706× (4)

*The number inside parentheses represents the number of independent measurements used for calculating the mean coverage.

ddPCR, droplet digital PCR; NC, negative control sample from a healthy subject; PC, positive control sample from a systemic autoinflammatory disease patient with germline p.Thr90Asn variant.

frequency found to date in whole blood. This variant was present in two smMIPs with similar allele frequencies. Subsequently, it was confirmed in an independent run as well as by ddPCR; therefore, the variant was regarded as a true postzygotic variant. With ddPCR, the detected allele frequencies were similar to those found with the smMIP assay. Our results underline ddPCR as a suitable confirmation method for all postzygotic variants that cannot be detected by routine laboratory techniques.[32]

In recent years, myeloid-restricted postzygotic *NLRP3* variants have been found in mild and late-onset cryopyrin-associated periodic syndrome and variant-type Schnitzler syndrome.[33–35] Greater correlation is expected between disease severity and variant allele frequency in myeloid cells than in whole blood. This might explain why a 2.4% postzygotic variant was detected in whole blood of an asymptomatic carrier,[22] whereas patients with severe SAID might have postzygotic variants with even lower allele frequencies.[21] Although this assay demonstrates to be sensitive, low-grade variants restricted to certain specific cell populations might be missed as only whole blood is tested in routine diagnostics.

The presence of a DNA variant does not necessarily imply its pathogenicity. The p.Gly569Ala variant in the *NLRP3* gene and the p.Thr90Asn variant in the *TNFRSF1A* gene have been found before as germline variants in SAID patients and were classified by experts as likely pathogenic mutations.[36] The p.Gln306His variant in the *NLRP3* gene

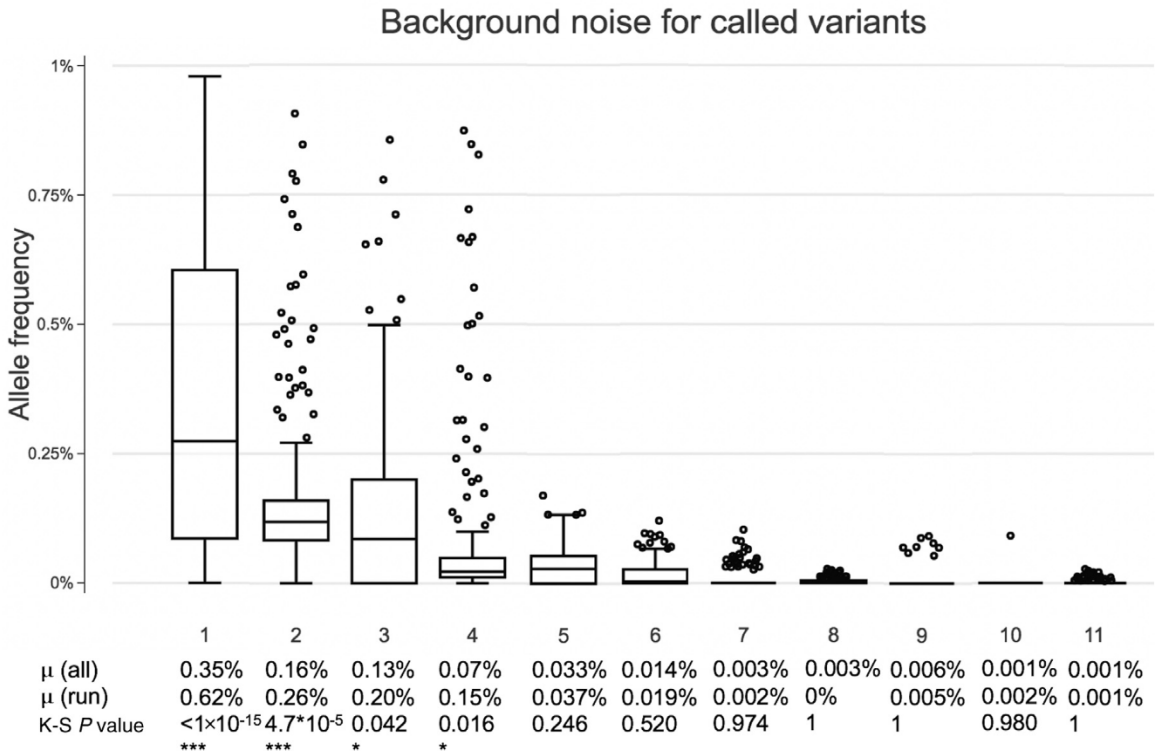

Figure 2 Distribution of background noise in >200 independent samples at all nucleotide positions where a variant was called. Lanes 1 through 4 correspond to the results considered as false positive; lanes 5, 8, 9, and 11 represent variants called in samples from positive control subjects; and lanes 6, 7, and 10 correspond to results considered as true postzygotic variants. μ (all), mean background noise in all samples; μ (run), mean background noise in samples in the run with the positive result (positive result excluded); K-S *P* value, significance using the Kolmogorov-Smirnov test; H_0, background noise values of samples in the run with the positive result are from the same distribution as those of samples in other runs. $*P < 0.05$, $***P < 0.001$ versus the null hypothesis that background noise values of samples in the run with the positive result are from the same distribution as those of samples in other runs.

has recently been detected as a postzygotic variant.[37] Variants detected in the same and adjacent amino acids are classified as likely pathogenic variants, making it plausible that this variant would be classified accordingly.[38] Both *NLRP3* variants detected in this study are located in mutational hotspots.[18,36] For the p.Gly569Ala variant, a different postzygotic pathogenic variant on the same amino acid residue has been described.[23] Strikingly, the *TNFRSF1A* variant is located only 4 bp from the uniquely known missense postzygotic variant,[31] suggesting the presence of a hotspot region for postzygotic variants in the *TNFRSF1A* gene as well.

With this highly sensitive assay, routine diagnostic screening for mosaicism could be performed in large cohorts of patients with SAID. The described combination of smMIP design, laboratory protocol, and software analysis can be used for any disease involving low-grade mosaicism. Many postzygotic variants have been described in SAID patients; and with this assay, more are likely to be identified. With the detection, confirmation, and, if relevant, functional follow-up of a postzygotic variant, the patient might finally be definitively diagnosed and receive proper medication. This reliable assay is of great importance to properly diagnose SAID and of added value to routine SAID diagnostics.

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Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2019.06.009*.

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