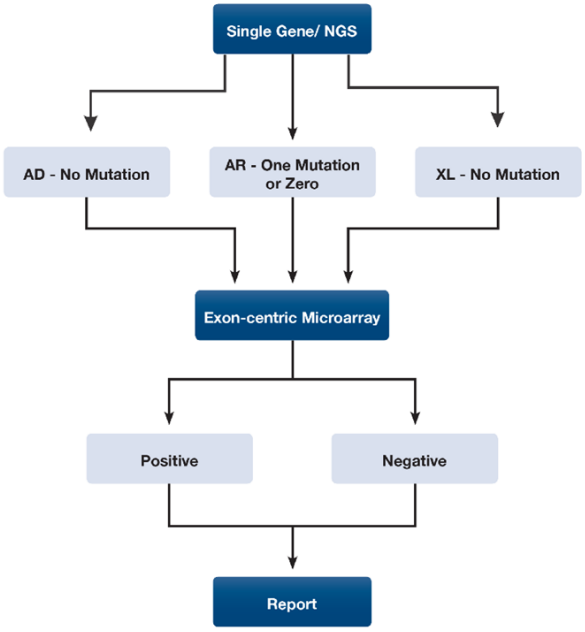

Figure 1: CNV testing algorithm for Mendelian disorders. The diagram demonstrates the diagnostic work-up of a Mendelian disorder using next generation sequencing as the first step followed by deletion/duplication testing to find an allele or second allele. CNV: copy number variant; NGS: next-generation sequencing

performed on 1048 patient samples referred to the Molecular Genetics Laboratory at Cincinnati Children's Hospital Medical Center (CCHMC). ECM analysis was performed on only the gene(s) of interest. Reference DNA was obtained from Promega (Madison, WI). This study was approved by the Institutional Review Board at CCHMC.

The custom 4X180K microarray was developed on the Agilent Technologies (Santa Clara, CA) platform using the Genefficiency service (Oxford Gene Technology, Oxford, UK). OGT used proprietary ink-jet *in situ* printer technology (IJISS) developed by Rosetta InPharmatics (Kirkland, WA) and Agilent Technologies that allows *in situ* synthesis of long oligonucleotides. The probes were 60 bp in length and annotated against NCBI build 37 (UCSC hg19, March 2006). Included were 364,591 probes that targeted 1508 genes (exons and introns), up to 300 bp flanking the exon/intron boundaries and backbone coverage.

DNA was extracted from whole blood collected in EDTA (purple-top) collection tubes using the Puregene DNA extraction kit (Qiagen, Valencia, CA) or the Chemagic magnetic separation module I (PerkinElmer, Baesweiler, Germany) according to the manufacturer's recommendations. Comparative genomic hybridization was performed following the manufacturer's protocol (Agilent Technologies, Santa Clara, CA). The reference DNA was used from two pools (male and female) from normal individuals, run as a same-sex control. DNA was sonicated using a Branson Sonifier 450 with cup horn (Danbury, CT) and visualized on a two-percent agarose gel prior to labeling, as a quality control measure. Each patient and reference DNA was labeled with Cy3 and Cy5 9-mer primers, respectively. Purification of labeled products, hybridization, and post-wash of the array was carried out according to Agilent's recommendation and with their proprietary solutions. Microarray slides were scanned with Innopsys Innoscan 710 and raw data was generated with extraction software (Chicago, Illinois).

CytoSure Interpret software version 4.4 (OGT) was used for analysis of microarray data (referred to as CytoSure). The program uses the circular binary segmentation algorithm to generate segments along the chromosomes that have similar copy number relative to reference chromosome. Averaging of the segments is with median value of all segments on a chromosome as the baseline. Deletion or duplication CNV calls were made using the log2 ratio of each segment with a minimum of 4 unique and consequent probes. The threshold factor for deletions was set as a log2 ratio of -0.6 that is less stringent than the theoretical log2 score of -1 for heterozygous deletions [heterozygous deletion log2(1/2) = -1; no change in allele number log2(2/2) = 0; heterozygous duplication log2(3/2) = 0.59; hemizygous deletion log2(0/1)= -1; homozygous deletion log2(0/2)= -2]. The software uses the standard deviation of the log2 ratio to calculate a deviation log ratio (DLR), which is used as a quality control check. A DLR of 0.08-0.19 is acceptable, 0.20-0.29 is borderline, and ≥ 0.30 is rejected. The DLR for all microarrays shown was scored by this scale. Data was analyzed only for the gene(s) ordered for testing.

We used the high-resolution ECM to process 1048 of samples that were reflex testing from negative or equivocal targeted NGS panel testing or ordered for single gene assessment. We identified 23 CNVs in rare immunodeficiency and related genes [Table 1]. Of the 23 CNVs detected, 21 deletions were identified. Significantly, of these 23 CNVs, 11 (48%) of them were novel, for which intragenic deletions and duplications are not a commonly reported etiology, either due to the low mutation burden of these events or the lack of recognition by an appropriate testing methodology. Six of the 23 CNVs in immunodeficiency and related genes were identified in dominantly inherited genes (26%), 7 hemizygous CNVs were identified in male patients in X-linked recessive genes (30%), 5 CNVs were identified as the second allele in 3 autosomal recessive genes(17%), and surprisingly,

Table 1: List of patients with CNVs identified by exon-centric microarray testing

Patient	Gene	OMIM phenotype: inheritance	CNV coordinates (min)	Min size (Max size)	Min region affected	Protein domain affected	Sequencing results	Reference
1	*FAS*	Autoimmune lymphoproliferative syndrome: AD	arr[hg19] 10q23.31 (90,770,398-90,777,298) x1	6.9 Kb (53.59 Kb)	exons 6-9	Death-like	No variant identified	This study
2	*FAS*	Autoimmune lymphoproliferative syndrome: AD	arr[hg19] 10q23.31 (90,748,451-91,780,017) x1	1.03 Mb (1.19 Mb)	whole gene		No variant identified	This study
3^	*FAS*	Autoimmune lymphoproliferative syndrome: AD	arr[hg19] 10q23.21(90774157-90774381)x0	224bp (403bp)	exon 9	Death-like	No variant identified	This study
4	*RPS26*	Diamond-Blackfan anemia: AD	arr[hg19] 12q13.2q13.3 (56,175,892-56,691,772) x1	515.88 Kb (688.55 Kb)	whole gene		N/A	[6]
5	*RPL35A*	Diamond-Blackfan anemia: AD	arr[hg19] 3q29 (197,456,033-197,844,960) x1	388.93 Kb (855.23 Kb)	whole gene		N/A	[6]
6	*TERT*	Dyskeratosis congenita: AD	arr[hg19] 5p15.33 (1,226,629-1,286,091)x1	59.46 Kb (60.16 Kb)	exon 3-16	Reverse transcriptase	No variant identified	This study
7*	*WAS*	Wiskot-Aldrich syndrome: XLR	arr[hg19] Xp11.23 (48,533,049-48,542,539) x0	9.49 Kb (147.2 Kb)	exon 1	EVH1	No variant identified	This study
8*	*WAS*	Wiskot-Aldrich syndrome: XLR	arr[hg19] Xp11.23 (48,533,302-48,613,923) x0	80.62 Kb (110.45 Kb)	whole gene		No variant identified	[7]
9	*WAS*	Wiskot-Aldrich syndrome: XLR	arr[hg19] Xp11.23 (48,533,049-48,550,909) x0	17.86 Kb (220.16 Kb)	whole gene		No variant identified	[7]
10	*WAS*	Wiskot-Aldrich syndrome: XLR	arr[hg19] Xp11.23 (48,533,049-48,551,398) x1	18.35 Kb (282.83 Kb)	whole gene		No variant identified	[7]
11	*SH2D1A*	X-linked recessive lymphoproliferative disorder: XLR	arr[hg19] Xq25 (123,481,887-123,501,318) x0	19.43 Kb (20.58 Kb)	exon 2	SH2	No variant identified	[8,9]
12	*XIAP*	X-linked recessive lymphoproliferative disorder: XLR	arr[hg19] Xq25 (123,007,906-123,021,550) x0	13.64 Kb (15.61 Kb)	exon 2	Baculoviral inhibition of apoptosis protein repeat	No variant identified	This study
13	*MAGT1*	Immunodeficiency: XLR	arr[hg19]Xq21.1 (77,088,396-77,150,089) x0	63 Kb (63 Kb)	Exons 2-7	Oligosaccharyl transferase complex	No variant identified	This study
14	*IL7R*	Severe combined immunodeficiency, T-cell -, B-cell +: AR	arr[hg19] 5p13.2 (35,867,205-35,867,604) x1	399 bp (3.94 Kb)	exon 3		c.669_688del20 (p.S223fs)	[10]
15	*FANCA*	Fanconi Anemia: AR	arr[hg19] 16q24.3 (89,880,335-89,928,824) x0	48.49 Kb (107.96 Kb)	exons 1-3		No variant identified	[11]
16	*FANCA*	Fanconi Anemia: AR	arr[hg19] 16p24.3 (89,874,512-89,884,589) x1	10.08kb (56.89 Kb)	exons 1-6		c.862G>T(p.E288X)	[12]
17	*FANCA*	Fanconi Anemia: AR	arr[hg19] 16q24.3 (89,880,335-89,928,824) x1	48.49 Kb (107.96 Kb)	exons 1-3		c.3918dupT(p.Q1307fs)	[11]
18	*FANCC*	Fanconi Anemia: AR	arr[hg19] 9q22.32 (98,007,056-98,011,765) x1	4.71 Kb (5.77 Kb)	exons 2-3		c.1642C>T(p.R548*)	[13,14]
19	*RAD51C*	Fanconi Anemia: AR	arr[hg19] 17q22 (56,765,347-56,790,584) x3	25.24 Kb (156.62 Kb)	exons 1-5	DNA recombination and repair, ATP binding domain	No variant identified	This study

20	*LRBA*	Immunodeficiency: AR	arr[hg19] 4q31.3 (151,198,604-151,199,333) x0	729 bp (7.59 Kb)	exon 57	WD40 repeat	No variant identified	This study
21^	*STXBP2*	Hemophagocytic lymphohistiocytosis: AR	arr[hg19] 19p13.2 (7,706,766-7,781,506) x0	74.74 Kb (426.44 Kb)	exons 8-19	Sec-like protein	No variant identified	This study
22	*RAB27A*	Griscelli syndrome, type 2: AR	arr[hg19] 15q21.3 (55,499,521-55,562,873) x1	63.35 Kb (67.75 Kb)	exons 1-5	GTPase	No variant identified	[15]
23	*RAB27A*	Griscelli syndrome, type 2: AR	arr[hg19] 15q21.3 (55,514,892-55,550,411) x3	35.52 Kb (40.86 Kb)	exons 2-5	GTPase	No variant identified	This study

*Maternal inheritance confirmed; ^parental carrier status confirmed. CNV: copy number variant; AD: autosomal dominant; AR: autosomal recessive; XLR: X-linked recessive

3 homozygous deletions were identified (13%).

Meaningfully, we identified 3 novel CNVs in the *FAS* gene. Heterozygous mutations in the *FAS* gene cause autoimmune lymphoproliferative syndrome (ALPS), an autosomal dominant disorder, where only three CNVs have been previously described[1-4]. In patient 1, we identified a 6.9 Kb heterozygous deletion that encompassed exons 6-9 and included the intracellular domain and the death domain of the FAS protein. Single gene CNV assessment of *FAS* was ordered for this patient after negative sequencing of *FAS*, *FASLG*, and *CASP10* genes. This patient's phenotype included splenomegaly, thrombocytopenia, history or acute cerebellitis and failure to thrive. Flow cytometry studies from an external laboratory identified an increased population of CD4/CD8 double negative T cells and reduced FAS protein expression. Similarly, in patient 2 and 3, we identified a 1.03 Mb whole gene heterozygous deletion and a 224 bp homozygous deletion in exon 9, respectively. ECM testing was ordered as reflex testing from Sanger sequencing in all 3 patients. The identification of the *FAS* gene deletions in these patients confirmed a diagnosis of ALPS. In addition, the CNV mutation spectrum of FAS was doubled, showing that ECM is a powerful tool for CNV discovery in rare Mendelian disorders.

Other rare hematology and immunodeficiency genes for which novel CNVs were identified included *TERT*, *WAS*, *XIAP*, *MAGT1*, *RAD51C*, *LRBA*, *STXBP2*, and *RAB27A*. Deletions in X-linked genes showed high clinical utility in males due to their hemizygous status. In several cases, a lack of amplification by PCR suggested a deletion, but results were inconclusive in clinical interpretation due to lack of direct evidence and insufficient controls. WAS-related disorders are X-linked recessive and include Wiskott-Aldrich syndrome, X-linked thrombocytopenia, and X-linked congenital neutropenia. Sequencing of the *WAS* gene identifies up to 95% of pathogenic variants and the remaining 5% of variants are detectable by deletion/duplication testing and 15 gross deletions have been reported. We identified 3 CNVs in 3 male patients involving the *WAS* gene. In patient 7, we identified a previously unreported 9.49 Kb intragenic deletion of exon 1, and in patients 8, 9 and 10, we identified previously reported whole gene deletions in *WAS* of size 80.62 Kb, 17.86 Kb, and 18.35 Kb, respectively. Autosomal dominant mutations in *TERT* cause dyskeratosis congenital, a rare multisystem disorder caused by defective telomere maintenance. We identified a novel 59.46 Kb heterozygous deletion of exons 3-16 and included the reverse transcriptase domain of the TERT protein, the catalytic component of the enzyme. To date, only one CNV in TERT had been identified in a family with adult-onset pulmonary fibrosis[4]. In addition, the LRBA protein is involved in regulating recycling of the cytotoxic T lymphocytes-associated protein 4, which helps regulate autoimmune responses. Biallelic deleterious mutations in *LRBA* have been associated with common variable immunodeficiency type 8 and to date, only 6 mutations have been identified in *LRBA*. The biological role of *LRBA* is currently being elucidated and recent publications have suggested targeted therapies for patients with *LRBA* deficiency[5]. We identified a novel 729 bp homozygous deletion of exon 57 of *LRBA* in a patient from consanguineous parents.

In our cohort, we recognized a high diagnostic yield for the ECM assays for immunodeficiencies and related conditions. However, functional studies will be necessary to confirm the causative relationship to disease. This report emphasizes the importance of appropriate ECM testing for immunodeficiency disorders and helps further define gene mutation spectra and our understanding of disease mechanisms in this understudied group of genetically heterogeneous disorders.

DECLARATIONS

Authors' contributions

Conceptualized and initiated the study, collected, analyzed, and interpreted the data, wrote and reviewed the manuscript: C.A. Valencia

Analyzed and interpreted the data, wrote and reviewed the manuscript: L. Dyer, X. Li, K. Zhang

Developed the test and reviewed the manuscript: A. Mathur, J. Denton

Performed the experiments, collected, analyzed and interpreted the data and reviewed the manuscript: B. Jones, E. Liston, D. Hilton

Approved the final manuscript as submitted and agree to be accountable for all aspects of the work: all authors

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Conflicts of interest

The authors declare that they have no relevant conflicts of interest.

Patient consent

Not applicable.

Ethics approval

This study was approved by the Institutional Review Board at Cincinnati Children's Hospital Medical Center.

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