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Targeted high-throughput sequencing technique for the molecular diagnosis of primary immunodeficiency disorders

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Abstract

The aim of this study was to investigate the usefulness of targeted high-throughput sequencing (HTS) for the molecular diagnosis of primary immunodeficiency diseases (PID).

A total of 56 clinically diagnosed or suspected PID patients were divided into 4 groups according to the International Union of Immunological Societies Expert Committee for Primary Immunodeficiency 2015 and their chief clinical presentations. Patients and their biological family members were examined by targeted HTS, which sequenced the exons and ±10 bp flanking introns of 171 PID-related genes panel. All significant variants were confirmed by PCR-Sanger sequencing. Pathogenicity of the variants was evaluated by using bioinformatics.

A total of 117 variants in 73 genes were found in 56 patients. Accurate molecular diagnosis of PID was made in 13 (23.2%) patients, and 12 novel mutations were detected in these patients. Twenty-seven patients carried heterozygous variants that are probably pathogenic in ≥2 genes; 16 patients had only 1 missense variant, or had several variants but not >1 variant was deleterious as evaluated by bioinformatics. The meaning of the targeted HTS results of these patients remains to be studied.

Targeted HTS can make a precise molecular diagnosis of PID and detect more novel pathogenic mutations. More and more variations with ambiguous significance are discovered and explanation of these variations is a challenge to the clinicians.

Abbreviations: HTS = high-throughput sequencing, IUIS = International Union of Immunological Societies, PIDs = primary immunodeficiency diseases.

Keywords: high throughput sequencing, molecular diagnosis, primary immunodeficiency disease, targeted HTS

1. Introduction

Primary immunodeficiency diseases (PIDs) are a heterogeneous group of heterogeneous diseases caused by inheritable defects in immune molecules, resulting in a susceptible status to infections, malignancies, lymphoproliferation, or autoimmune diseases.[1] PIDs are difficult to be diagnosed because of the diverse clinical manifestations and various related genes. In the past years, the main method to diagnose the molecular defects in PID patients is the PCR-Sanger sequencing, but it is time-consuming, low efficient, and expensive, and can hardly meet the needs for clinical requirements.[2,3] Following the development of the high-throughput sequencing (HTS) technology (the second generation sequencing), more PID genes have been identified. The International Union of Immunological Societies (IUIS) Expert Committee for Primary Immunodeficiency 2015 has reported mutations in nearly 300 genes that are responsible for PIDs.[1] Targeted HTS uses a large set of primers for sequencing the exons and their flanking introns of most PID-related genes, and enables us to detect mutations in these genes simultaneously. This method is sensitive and cost-effective,[4] and renders fast and precise diagnosis of PID feasible.[5] In this article, we used targeted HTS including a panel of 171 PID-related genes to detect 56 clinical diagnosed PID patients and their biological family members, to evaluate its clinical value for the molecular diagnosis of PIDs.

2. Methods

2.1. The patients and their family members

The study was initiated from December 2014 to January 2016, and a total of 56 patients (37 males and 19 females with a median age of 2 years) clinically diagnosed with PID and their biological family members were involved. These patients came from the departments of pediatrics, respirology, and hematology of 12 hospitals including Peking University First Hospital and Zhengzhou University First Hospital in China. Their clinical data, routine examinations, and family history were recruited.

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ZHC and WW contributed equally to this study.

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For comparison between the highly variable clinical symptoms of PIDs and gene mutations, we divided these patients into 4 groups based on IUIS and their chief clinical presentations: group I (unclassified), patients with recurrent fever, cough, and pneumonia (n=26, 46.4%); group II (congenital defects of phagocyte number, function,or both), patients with severe leukocytopenia (n=11, 19.6%); group III (predominantly antibody deficiencies, PADs), patients with hypoimmuno-globulinemia, recurrent fever and infection (n=9, 16.1%); and group IV (immune dysregulation), patients with hemophagocytic lymphohistiocytosis, fever, lymphadenopathy, or hepato-splenomegaly (n=10, 17.9%). They were followed up for >7 months. Written consent was obtained from the patients or their guardians. Ethical permission for the study was obtained from the Medical Ethics Committee of Peking University First Hospital.

2.2. DNA extraction

Peripheral blood samples with EDTA anticoagulant were collected from all patients and their biological family members. DNA was isolated by using the FlexiGene DNA Kit (#512206, Qiagen, Germany) and stored at −80°C.

2.3. Targeted HTS

A panel of sequencing primers were designed for the sequencing of the exons and ±10 bp flanking introns of 171 PID-related genes (supplementary 1, http://links.lww.com/MD/C534) using the Agilent SureDesign online design tool. Ten nanograms of a DNA sample were used to prepare the targeted gene captured library. The DNA library was then sequenced in the NEXTSEQ500 sequencer (Illumina). The quality controls bioinformatics filters were ≥30 and the coverage reached 99.9%.

2.4. Analysis of HTS results and confirmation of mutations

The raw data from NEXTSEQ500 sequencer were analyzed by RTA (real-time analysis, Illumina), BWA, Genome Analysis Toolkit (GATK) bioinformatics software. Sequencing qualities were satisfied with the requirements. Variation interpretation after use of the bioinformatics software and databases (PolyPhen-2, Human Splicing Finder, SIFT, MutationTaster, NetGenes, ANNOVAR, HGMD, dbSNP, 1000 Genomes) was in accordance with the interpretation guidelines of the American College of Medical Genetics and Genomics[6,7] gene description in NCBI, and disease and inheritance in OMIM. Mutations and variants in patients and family members listed in this article were all validated by PCR-Sanger sequencing method.

3. Results

3.1. A number of variants in various genes detected in the PID patients

A total of 117 variants in 73 PID-related genes were identified in the 56 PID patients. In the 73 genes, variants were found in 6 patients/gene in 1 gene (*MUC5B*), in 4 patients/gene in 2 genes (*TNFRSF13B* and *ADA*), in 3 patients/gene in 10 genes (*JAK3*, *PDGFRA*, *C3*, *RAG1*, *CYBB*, *IL2RG*, *AK2*, *NCF2*, *IL17F*, and *STXBP2*), in 2 patients/gene in 7 genes (*AP3B1*, *NLRP3*, *VPS13B*, *BMPR2*, *ARMC4*, *DNAH5*, and *AIRE*), and in 1 patient/gene in 53 genes. In the 117 variants, 93 were missense variants (79.5%), 13 were splicing variants (11.1%), 7 were nonsense variants (6%), and 4 were small indels causing frame shift (3%). Large indels and copy number variations were not included because of the limitation of the targeted HTS.

3.2. A complicated relationship between genotype and phenotype in the PID patients

Patients were arbitrarily divided into 4 groups according to the clinical manifestations to evaluate the relationship between genotype and phenotype (Supplementary 2, http://links.lww.com/MD/C534). The variants were highly variable among the 4 groups. Group I (n=26) with recurrent fever, cough, and pneumonia had the variants in *CYBB*, *JAK3*, *NLRP3*, *TNFRSF13B*, *FOXN1*, *NCF2*, *DOCK8*, *MASP1*, *VPS13B*, *UNC13D*, *SP110*, *MUC5B*, and so on. Group II (n=11) with leukocytopenia had the variants in *ARMC4*, *C3*, *CCDC114*, *DOCK8*, *FERMT3*, *MUC5B,* and so on. Group III (n=9) with hypoimmunoglobulinemia, recurrent fever, and infection had the variants in *ADA*, *RAG1*, *BTK*, *IL17R*, *CD40LG*, *IL2RG*, *AK2*, *ITGB2*, *NOD2*, *CARD9*, *NLRP3*, *IL17F*, and so on. Group IV (n=10) with hemophagocytic lymphohistiocytosis had the variants in *MASP1*, *IFNGR1*, *UNC13D*, *RAB27A*, *LPIN2*, *IL17RA*, *MUC5B*, *C6*, *STXBP2*, and *SH2D1A*.

On the contrary, the variants in the same gene resulted in various clinical presentations. For example, 3 patients diagnosed as having chronic granulomatous disease carried *CYBB* gene mutations (Table 1), one (case #8) showed multiple abscesses in lung, liver, and peri-anus and died of severe infection at 2 months of age, one (case #28) presented as having persistent fever and pulmonary nodules after birth, and another case (case #7) was susceptible to respiratory tract infection having bronchial pneumonia and mycoplasma pneumonia.

3.3. Molecular diagnosis of PID made by targeted HTS in 13 patients

Thirteen of the 56 patients (23.2%) met the criteria for the molecular diagnosis of PID[1] (Table 1). These cases were all males with the median age of 2 years (2 months–9 years). Parental analysis indicated that 8 males with X-linked inheritance were inherited from their carrier mothers, and 5 patients with autosomal recessive inheritance were inherited from their carrier parents (Figs. 1 and 2). These patients were followed up for >7 months, and 6 of the 13 patients died of severe infections (case #28, 17, 13, 51, 26, and 42).

Seventeen mutations in 9 genes (*CYBB, IL2RG, CD40LG, SH2D1A, BTK, JAK3, ADA, IL7RA, RAG1*) were detected in the 13 PID patients (Table 1). Five of them (*CYBB* c.676C>T,[8] *CYBB* c.607G>T,[9] *ADA* c.703C>T,[10] *RAG1* c.2210G>A,[11] and *RAG1* c.322C>T[12]) had been reported previously in related diseases, and 12 of them were novel mutations after search for databases and evaluated by the software of HGMD database, dbSNP database, 1000 Genome database, and PolyPhen-2.

3.4. Heterozygous variants in two or more genes probably related to PID in 27 patients

Most of the remaining 43 patients were found to have heterozygous variants in 2 or >2 genes. All of these variants detected by targeted HTS were confirmed by PCR-Sanger

Table 1

Results of targeted HTS confirmed the clinical diagnosis of PID in 13 patients.

Case no.	Sex/age, y	Gene	mRNA no.	Mutation	Effect	Novel mutation	Carrier father/mother	Clinical diagnosis	Inheritance
7	M/2.4	*CYBB*	NM_000397	c.45+4A>G	Splicing †	Yes	M	CGD (macrophage gp91 phox deficiency)	XL
8	M/0.2	*CYBB*	NM_000397	c.676C>T	p.R226‡	No[9]	M	CGD (macrophage gp91 phox deficiency)	XL
28	M/0.3	*CYBB*	NM_000397	c.607G>T	p.E203‡	No[10]	M	CGD (macrophage gp91 phox deficiency)	XL
17	M/0.2	*IL2RG*	NM_000206	c.865_866insC	P.289fs	Yes	M	T^-B^+ SCID (common γ chain deficiency)	XL
24	M/1	*IL2RG*	NM_000206	c.944dupA	p.K315Rfs‡51	Yes	M	T^-B^+ SCID (common γ chain deficiency)	XL
31	M/5	*CD40LG*	NM_000074	c.763T>C	p.S255P*	Yes	M	Combined immunodeficiencies (CD40 ligand deficiency)	XL
13	M/0.3	*SH2D1A*	NM_002351	c.201+1G>T	Splicing †	Yes	M	FHL syndrome (SH2D1A deficiency)	XL
37	M/9	*BTK*	NM_000061	c.83G>A	p.R28H *	Yes	M	Agammaglobulinaemia (BTK deficiency)	XL
51	M/0.25	*ADA*	NM_000022	c.532del G	p.V178‡	Yes	F	T^-B^-SCID (ADA deficiency)	AR
		ADA	NM_000022	c.533del G	p.V178‡	Yes	M		
23	M/0.5	*JAK3*	NM_000215	c.1205G>A	p.R402H *	Yes	M	T^-B^+ SCID (JAK3 deficiency)	AR
		JAK3	NM_000215	c.3019C>T	p.Q1007‡	Yes	F		
26	M/0.4	*ADA*	NM_000022	c.703C>T	p.R235W *	No[11]	F	T^-B^- SCID (ADA deficiency)	AR
		ADA	NM_000022	c.221G>A	p.G74D *	Yes	M		
33	M/2	*IL7RA*	NM_002185	c.314G>A	p.S105N *	Yes	F	T^-B^+ SCID (IL7Rα deficiency)	AR
		IL7RA	NM_002185	c.536C>T	p.T179M *	Yes	M		
42	M/0.6	*RAG1*	NM_000448	c.2210G>A	p.R737H *	No[12]	F	T^-B^- SCID (RAG1 deficiency)	AR
		RAG1	NM_000448	c.322C>T	p.R108‡	No[13]	M		

AR = autosomal recessive inheritance, CGD = chronic granulomatous disease, FHL = familial hemophagocytic lymphohistiocytosis, HTS = high-throughput sequencing, PID = primary immunodeficiency disease, SCID = severe combined immunodeficiency, XL = X-linked inheritance.

* Probably damaging or possibly damaging missense mutation evaluated by "PolyPhen-2" (http://genetics.bwh.harvard.edu/pph2/index.shtml).

† Splicing mutation evaluated by "Human Splicing Finder" (http://www.umd.be/HSF3/index.html).

‡ Nonsense mutation.

sequencing. It is difficult to determine whether these variants are the genetic causes of PID in these patients. To reduce the ambiguous significance of these variants, we only considered small indels, nonsense variants, possible or probable damaging missense changes evaluated by PolyPhen-2, and splicing variants evaluated by Human Splicing Finder, as the possibly pathogenic variants, and listed in Table 2. A total of 27 patients had multiple variants in multiple genes (variants in 2 genes in 17 patients, in 3 genes in 6 patients, in 4 genes in 4 patients). In the 20 patients whose parents were also examined for the abnormal alleles, heterozygous variants in 1 or >1 genes with normal phenotype were also found in parents of 11 patients (case #1, 18, 20, 35, 46, 50, 54, 53, 55, 11, and 52), indicating that the variants were inherited from their parents; 4 patients (case #20, 46, 55, and 52) had the same variants as their unaffected father or mother, suggesting that these patients may have other factors inducing the PID phenotype. Some patients (case #2, 6, 12, 15, 40, 48, 56, 4, and 21) may have novel variants. The molecular mechanism of the 27 PID patients remains to be studied.

The accumulation of insufficient doses of several gene products may relate to the PID at least in some of these patients. For example, case #35 had typical hemophagocytic lymphohistiocytosis syndrome (Fig. 3). She carried a heterozygous missense variant of c.736C>T in *NCF2* from her unaffected father, and a splicing variant of c.451+3G>A in *CORO1A* inherited from her unaffected mother. Mutations in *NCF2* cause recessive chronic granulomatosis,[13] and mutations in *CORO1A* have been reported in recessive lymphoproliferative disease.[14] Both genes are in the phagosome pathway (KEGG Hsa04145). However, the targeted HTS we used only includes a large part of the important PID-related genes, and mutations in other genes relating to phagosome pathway cannot be excluded in this patient.

3.5. Variations of ambiguous significance in 16 PID patients

Sixteen of the 56 patients had only 1 missense variant, or had several variants but no >1 variant was deleterious evaluated by PolyPhen-2 Human Splicing Finder software or SIFT (Supplementary 3, http://links.lww.com/MD/C534). The meaning of the targeted HTS results in these patients is controversial. More studies should be carried out to disclose the molecular basis of PID in these patients.

4. Discussion

In this study we used the targeted HTS technology to search for mutations in a panel of 171 PID genes in 56 clinically diagnosed PID patients. A total of 117 variants in 73 genes were detected in the 56 patients, with the higher frequency of variant (≥2 patients/gene) in 20 genes. The major mutation type was missense mutation, and more than half of the missense variants were deleterious predicted with bioinformatics software.[15] PID is a group of genotypically and phenotypically heterogeneous diseases. We divided the patients into 4 groups according to IUIS and their chief clinical presentations. We found that variants in many genes were detected in a group and the variants in the same gene were detected in several groups, suggesting a complicated relationship between genotype and phenotype. In X-linked chronic granulomatous disease, the genotype correlated with phenotype,[16] but other researchers[17] showed that the 2 patients in the same kindred with the same genotype had different clinical manifestations. Our findings also demonstrated that mutations in different genes could lead to a similar phenotype and the same gene mutation could present as a different phenotype, leading to the molecular diagnosis of PID more complex.[18,19] Consequently, it is essential to detect a large number of related genes simultaneously.

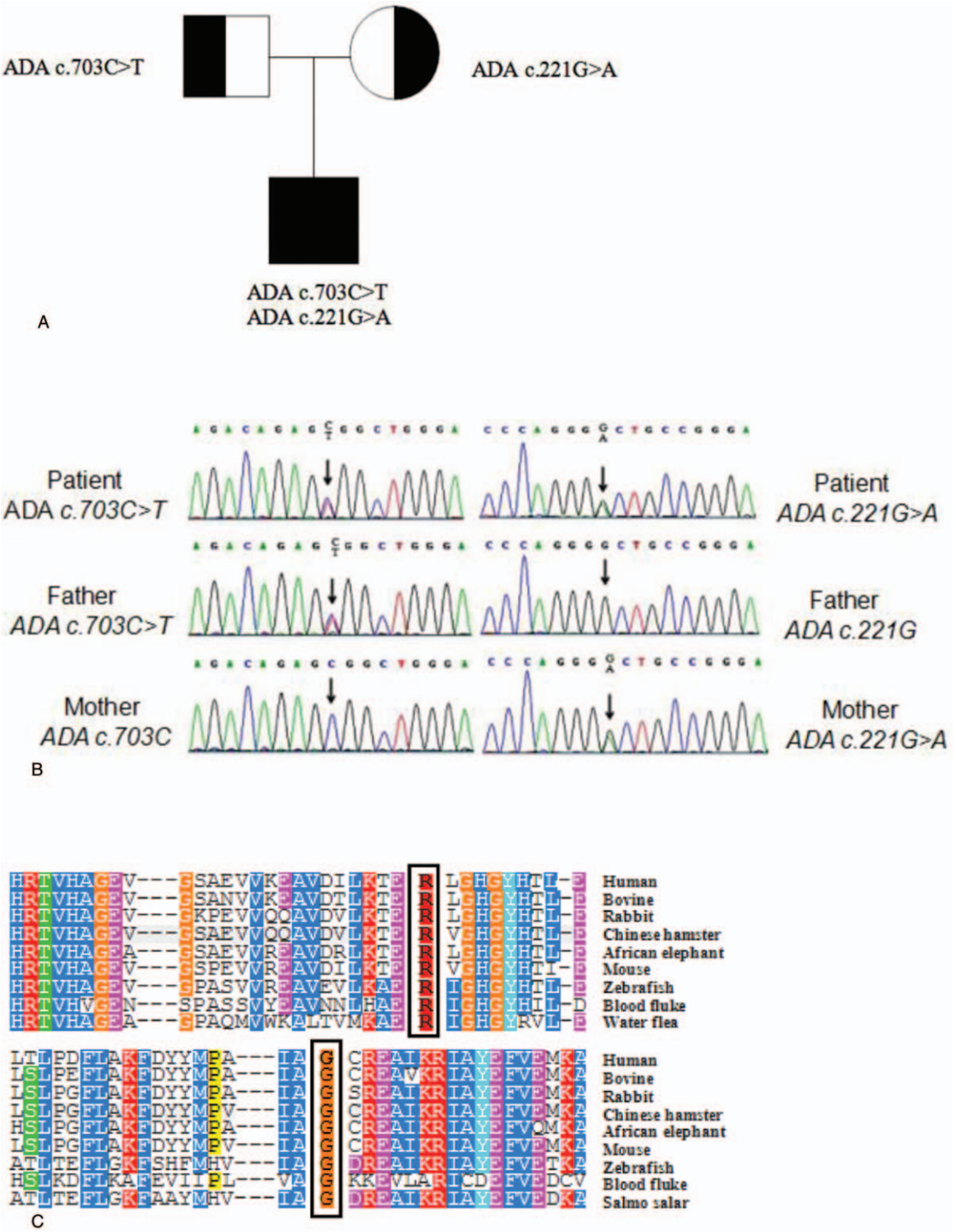

Figure 1. Case #26, a boy of aged 4 months clinically diagnosed as having $T^{-}B^{-}$ severe combined immunodeficiency. (A and B). His unaffected father has a heterozygous mutation of c.703C>T in *ADA*. His unaffected mother has a heterozygous mutation of c.221G>A in *ADA*. The patient has compound heterozygous mutations in *ADA* inherited the c.703C>T allele from his father and the c.221G>A allele from his mother. (C). The 2 missense mutations of p.R235W and p.G74D locate at the highly conservative sites among vertebral and nonvertebral organisms.

The molecular diagnosis of PID was accurately made in 13 of the 56 patients (23.2%) based on targeted HTS. Most of these patients had been tested several PID-related genes by PCR-Sanger sequencing method because of recurrent infections or hypoimmunoglobulinemia but without positive findings. Targeted HTS has the advantage of determining mutations in hundred genes at 1 time. Al-Mousa et al[19] used targeted HTS containing 162 PID-related genes and known mutation cases as

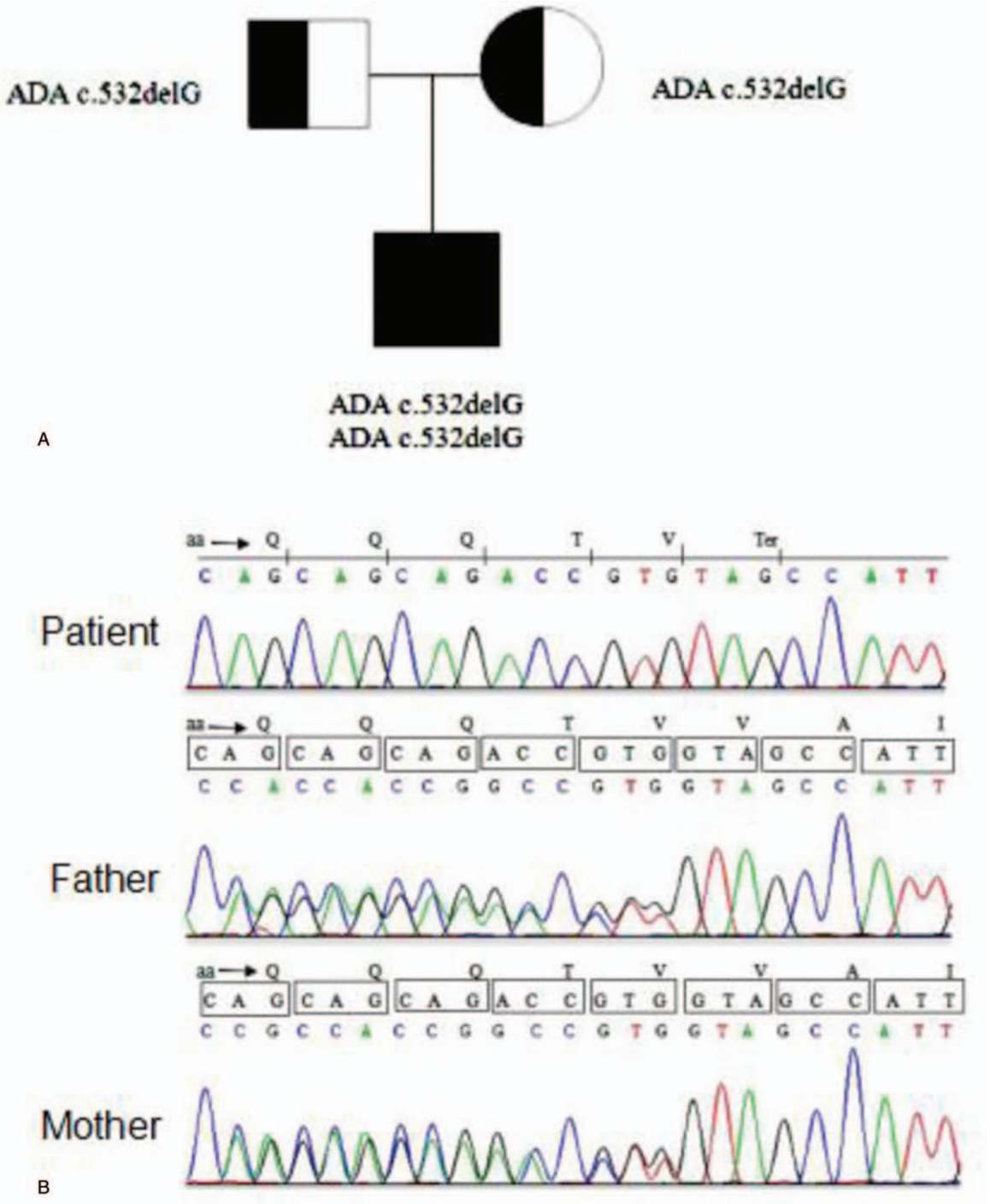

Figure 2. Case #51, a boy aged 3 months clinically diagnosed as having $T^{-}B^{-}$ severe combined immunodeficiency. (A). His unaffected parents are heterozygous of the frameshift mutation c.532delG in *ADA*. (B). The patient has homozygous mutation of c.532delG (p.V178*) inherited from his father and mother.

the positive control, and showed that HTS was able to detect 96% of positive control subjects and the diagnosis rate for 139 cases with atypical clinical presentations was 25%. Yang et al[20] provided a molecular diagnostic rate of 25% in 250 patients by whole-exome sequencing, a HTS technique covering more genes useful to find new genes for the diseases. Targeted HTS is cost-effective and the targeted gene panel can be updated.[21] Moens et al[18] proposed a step-by-step strategy for molecular diagnosis of PID, first targeted sequencing, then whole-exome sequencing or whole genome sequencing.

It is extraordinary that 27 patients (48.2%) of the 56 PID patients had the variants in 2 or >2 genes. The significance of these variants in the pathogenesis of PID is undetermined. Case #35 in Figure 3 demonstrates that the 2 heterozygous variants in *NCF2* and in *CORO1A* may relate to the hemophagocytic lymphohistiocytosis syndrome in this patient. Some scholars

Table 2

Heterozygous mutations in 2 or >2 genes in 27 PID patients.

Case no.	Sex/age, y	Symptom	Gene	mRNA no.	Heterozygous mutation	Carrier father/mother
1	F/1.6	Recurrent cough and fever after birth	*NCF2*	NM_000433	c.305G>A, p.R102Q*	F
			C1R	NM_001733	c.424+3G>C Splicing †	M
			MEFV	NM_000243	c.548C>A, p.P183Q*	F
			TNFRSF13B	NM_012452	c.260T>A, p.I87N *	F
2	M/0.7	Recurrent fever and cough after birth	*PDGFRA*	NM_006206	c.3098A>T, p.D1033V*	M
			AP3B1	NM_003664	c.1393A>G, p.I465V *	UN/UN
6	M/0.2	Fever, pulmonary fungal infection after birth, and poor therapeutic responses	*FOXN1*	NM_003593	c.713G>A, p.G238D*	UN/UN
			JAK3	NM_000215	c.567-5T>A, Splicing †	UN/UN
12	M/1.2	Recurrent respiratory tract infections and severe neutropenia	*VPS13B*	NM_017890	c.5119A>G, p.K1707E*	M
			ADA	NM_000022	c.1079-7C>T, Splicing x	UN/UN
			FERMT3	NM_031471	c.158T>G, p.I53S b	UN/UN
			CD79B	NM_021602	c.107G>A, p.R36Q b	UN/UN
15	F/1	Recurrent fever for 1 month, severe neutropenia	*DNAH11*	NM_001277115	c.8361C>A, p.C2787‡	UN/UN
			ARMC4	NM_018076	c.383-6delT, Splicing x	UN/UN
16	F/2.3	Recurrent fever shortly after birth, rashes and high IgE level	*AK2*	NM_013411	c.422G>C, p.G141A*	NS/NS
			RAG1	NM_000448	c.1186C>T, p.R396C*	NS/NS
			RAG1	NM_000448	c.1400C>T, p.P467L*	NS/NS
			JAK3	NM_000215	c.2062A>T, p.I688F *	NS/NS
18	F/0.2	Axillary abscess after birth, low IgG, IgA, and IgM	*CARD9*	NM_052814	c.808-8C>T, Splicing x	F
			NLRP3	NM_001079821	c.157G>A, p.D53N *	M
20	M/3.3	Recurrent infections and poor dental and hair development	*ADA*	NM_000022	c.757C>T, p.R253W*	F
			IL17F	NM_052872	c.377A>G, p.E126G *	F
40	M/7	Recurrent cough and fever for 5 years	*PDGFRA*	NM_006206	c.3098A>T, p.D1033V*	UN/UN
			AP3B1	NM_003664	c.1393A>G, p.I465V *	UN/UN
46	F/3	Fever and cough for 2 years	*CD46*	NM_172359	c.574G>C, p.D192H*	M
			NLRP3	NM_001079821	c.2398C>A, p.L800M*	M
			PDGFRA	NM_006206	c.1998G>C, p.K666N *	M
54	M/2	Recurrent cough and fever since birth	*VPS13B*	NM_017890	c.3865A>G, p.T1289A*	M
			SLC37A4	NM_001467	c.839C>T, p.A280V*	F
			SCNN1G	NM_001039	c.652A>C, p.T218P*	F
			CXCR4	NM_001008540	c.277A>G, p.I93V*	M
9	M/1	Susceptible to infection, severe neutropenia	*C9*	NM_001737	c.1337G>C, p.R446P*	NS/NS
			MUC5B	NM_002458	c.239G>C, p.W80S *	NS/NS
19	F/2.5	Recurrent supurative tonsillitis, severe neutropenia	*IL10*	NM_000572	c.507A>C, p.E169D*	UN/NS
			IL17F	NM_052872	c.274C>T, p.R92W*	F
			ZBTB24	NM_014797	c.755G>A, p.R252Q*	UN/NS
			STX11	NM_003764	c.799G>A, p.V267M *	UN/NS
35	F/2	Recurrent fever, cytopenia, lymph node enlargement in neck, hepatomegaly, and phagocytes in bone marrow smear	*NCF2*	NM_000433	c.736C>T, p.R246C*	F
			CORO1A	NM_001193333	c.451+3G>A, Splicing x	M
38	F/3	Recurrent cough, fever, and pneumonia for 1 year	*CD81*	NM_001297649	c.67-4C>T, Splicing x	NS/NS
			IL2RG	NM_000206	c.266A>G, p.Y89C*	NS/NS
			IL12RB1	NM_001290023	c.1781G>A, p.G594E b	NS/NS
48	M/1.5	Neutropenia and recurrent respiratory tract infections	*AK2*	NM_013411	c.73G>A, p.G25R*	UN/UN
			IKZF1	NM_001291839	c.630_632del, p.210_211del	UN/UN
			STXBP2	NM_001272034	c.184A>G, p.N62D *	M
50	F/1.6	Recurrent fever, cough, and severe pneumonia after birth	*ZAP70*	NM_001079	c.889+7G>A, Splicing x	F
			C3	NM_000064	c.1846-4G>A, Splicing x	M
			MUC5B	NM_002458	c.9517G>A, p.V3173M*	F
			DCLRE1C	NM_001289076	c.995C>A, p.T332K b	F
53	M/3	Recurrent upper respiratory tract infections and neutropenia	*AIRE*	NM_000383	c.595G>A, p.V199I*	F
			BMPR2	NM_001204	c.1481C>T, p.A494V*	M
			SERPING1	NM_001032295	c.1474A>G, p.M492V *	F

Case no.	Sex/age, y	Symptom	Gene	mRNA no.	Heterozygous mutation	Carrier father/mother
55	M/8	Neutropenia, thrombocytopenia for 5 years, and poor therapeutic responses	*DOCK8* *IL17RA*	NM_001193536 NM_014339	c.832G>A, p.V278I* c.1403G>A, p.R468H *	F F
56	M/1.5	Neutropenia and recurrent respiratory tract infections	*AK2* *STXBP2*	NM_013411 NM_001272034	c.73G>A, p.G25R* c.1210C>A, p.L404M *	UN/UN M
11	M/0.4	Fever, diarrhea, skin, and soft tissue infections and low IgG level	*AIRE* *RNF168* *IL17F*	NM_000383 NM_152617 NM_052872	c.1322C>T, p.T441M* c.1612A>G, p.N538D* c.377A>G, p.E126G *	M M F
4	F/0.9	Fever and lymphadenopathy	*FCN3* *PSTPIP1*	NM_173452 NM_003978	c.637A>C, p.S213R* c.1115C>T, p.A372V *	UN/UN M
10	M/6	Recurrent fever, lymphadenopathy, splenomegaly since 2 years' old	*C6* *NOD2* *STXBP2*	NM_000065 NM_001293557 NM_001272034	c.1075G>A, p.D359N* c.1027C>T, p.H343Y* c.1492G>A, p.V498M *	UN/NS UN/NS UN/NS
21	F/9	Recurrent fever, lymphadenopathy, splenomegaly, EBV (+)	*C8B* *LPIN2*	NM_001278543 NM_014646	c.93G>T, p.R31S* c.622_624del, p.208_208del	UN/UN UN/UN
27	F/57	Recurrent fever, lymphadenopathy, and leukopenia	*MUC5B* *RAB27A*	NM_002458 NM_004580	c.3095A>T, p.N1032I* c.551G>A, p.R184Q *	NS/NS NS/NS
36	M/5	Recurrent fever and lymphadenopathy	*ITK* *TNFRSF13B*	NM_005546 NM_012452	c.527T>C, p.V176A* c.460A>T, p.K154‡	NS/NS NS/NS
52	M/0.3	Fever, skin rashes, lymphadenopathy, and hemophagocyte in bone marrow smear	*CCDC39* *DNAL1*	NM_181426 NM_031427	c.1331C>T, p.T444I* c.42+9A>G, Splicing †	F F

EBV = Epstein-Barr virus, PID = primary immunodeficiency disease, NS = no sample, UN = undetected.
* Probably damaging or possibly damaging missense mutation evaluated by "PolyPhen-2" (http://genetics.bwh.harvard.edu/pph2/index.shtml).
† Splicing mutation evaluated by "Human Splicing Finder" (http://www.umd.be/HSF3/index.html). x: has probably no impact on splicing by "Human Splicing Finder" (http://www.umd.be/HSF3/index.html).
‡ nonsense mutation.
b: Benign change evaluated by "PolyPhen-2".

propose that PID is probably not limited to be Mendelian monogenic disease,[22] perhaps also has di-genic or multi-genetic inheritance. Zhang et al[23] revealed that 28 familial hemophagocytic lymphohistiocytosis patients carried heterozygous mutations in double genes in the cytotoxin pathway and thought that it should be classified as a double gene inheritance model. How to explain the gene variations with undetermined significance is a challenge to the clinicians in face of more patients examined with HTS. At present, the knowledge about molecular mechanisms of PID is just the tip of an iceberg, and a large number of unknown mutations relating to PID need to be investigated further.[24]

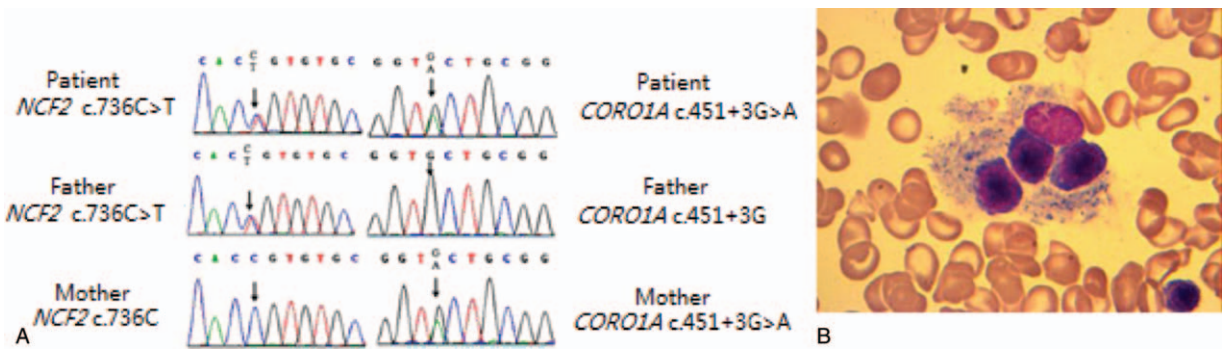

Figure 3. Case #35, a girl aged 2 years with 2 variants in *NCF2* and *CORO1A*. (A). She carried heterozygous variant of c.736C>T in *NCF2* inherited from her unaffected father and splicing variant of c.451+3G>A in *CORO1A* inherited from her unaffected mother. (B). Phagocytosis of 3 nucleated erythrocytes in a cell in her bone marrow smear. She also had recurrent fever, lymph node enlargement, hepatomegaly, low white blood cell, and platelet (white blood cells 1.81×10^9 cells/L, neutrophils 0.53×10^9 cells/L, lymphocytes 1.10×10^9 cells/L, platelet 15×10^9 cells/L), high serum ferritin (1528.9 ng/mL), high triglyceride (8.59 mmol/L), and low fibrinogen (0.52 g/L), clinically typical of hemophagocytic lymphohistiocytosis syndrome.

5. Conclusion

Altogether, targeted HTS is an accurate, sensitive, and cost-effective diagnostic tool that can detect a large number of mutations at the same time and often gives a precise molecular diagnosis, particularly for patients with atypical presentations. This method also provides a large number of findings with ambiguous significance.

Whether these variants would contribute to the disease, more functional evidence was needed.

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