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ORIGINAL ARTICLE

A single-center pilot study in Malaysia on the clinical utility of whole-exome sequencing for inborn errors of immunity

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Abstract

Primary immunodeficiency diseases refer to inborn errors of immunity (IEI) that affect the normal development and function of the immune system. The phenotypical and genetic heterogeneity of IEI have made their diagnosis challenging. Hence, whole-exome sequencing (WES) was employed in this pilot study to identify the genetic etiology of 30 pediatric patients clinically diagnosed with IEI. The potential causative variants identified by WES were validated using Sanger sequencing. Genetic diagnosis was attained in 46.7% (14 of 30) of the patients and categorized into autoinflammatory disorders ($n = 3$), diseases of immune dysregulation ($n = 3$), defects in intrinsic and innate immunity ($n = 3$), predominantly antibody deficiencies ($n = 2$), combined immunodeficiencies with associated and syndromic features ($n = 2$) and immunodeficiencies affecting cellular and humoral immunity ($n = 1$). Of the 15 genetic variants identified, two were novel variants. Genetic findings differed from the provisional clinical diagnoses in seven cases (50.0%). This study showed that WES enhances the capacity to diagnose IEI, allowing more patients to receive appropriate therapy and disease management.

KEYWORDS

bioinformatics analysis, genetic diagnosis, genetic variant, inborn errors of immunity, whole-exome sequencing

INTRODUCTION

The term 'inborn errors of immunity' (IEI) was recently adopted to describe primary immunodeficiencies caused by monogenic defects [1]. These conditions are characterized by increased susceptibility to recurrent infections, autoimmune disorders, autoinflammatory diseases, allergy and malignancies [2]. To date, 430 genetic defects have been reported by the International Union of Immunological Societies (IUIS) [2]. The number of IEI-related genes is expected to rise with advances in next-generation sequencing (NGS) technology. Diagnosing IEI using laboratory screening of immunological parameters presents a challenge because of their phenotypical and genetic heterogeneity [3,4]. Hence, a definitive diagnosis of IEI requires genetic testing to identify the disease-causing mutation.

For large-scale sequencing, NGS is more cost-effective than Sanger sequencing [5]. While a targeted gene panel works for cases with a specific syndrome or typical clinical features, whole-genome sequencing (WGS) and whole-exome sequencing (WES) are more suitable for clinically ambiguous cases. The targeted gene panel excludes the exploration of genetic mutations outside the predetermined set of genes. With a wider genome coverage, WGS enables the detection of structural and deep-intronic variants. However, WGS is less practical for diagnostic testing due to its massive data output and high cost. Compared to WGS, data output from WES is more manageable, as it sequences approximately 2% of the genome that harbors approximately 85% of disease-causing mutations [6,7]. Taken together, WES is preferable for genetic diagnosis, given its relatively high diagnostic yield, lower cost and accessible pipelines for efficient data analysis and interpretation.

Earlier studies showed a diagnostic yield of 21–40% when WES was used to diagnose IEI [3,8–12]. Definitive genetic diagnosis is crucial in providing patients with timely appropriate treatment to reduce morbidity and mortality. For instance, severe combined immunodeficiency (SCID) patients detected early may receive hematopoietic stem cell transplant (HSCT) before opportunistic infections develop, hence improving their survival [13]. Furthermore, counseling following genetic testing is necessary so that patients and their families are aware of the disease prognosis and the risk of its development in other family members.

A Malaysian epidemiological study, conducted between 1987 and 2006, reported 52 IEI cases [14]. Despite lacking genetic diagnosis, this report raised awareness among healthcare providers and patients of IEI, once believed to be rare in the community. The estimated prevalence of 0.37 IEI cases per 100 000 population in Malaysia suggests under-reporting when compared to a prevalence of 1.1–7.5 per 100 000 population in other countries [15–20].

These factors argue the need for effective genetic testing to diagnose IEI. Thus, this is the first study, to our knowledge, that aims to use WES to determine genetic diagnosis in patients suspected of IEI in Malaysia.

METHODS

Study population

Thirty patients with clinical suspicion of IEI were recruited by the referral center, Institute for Medical Research, from government hospitals across Malaysia between 2016 and 2018. Patients aged 18 years and below were enrolled if they experienced at least one of these clinical features: (a) signs and symptoms suggestive of immune dysregulation with or without an opportunistic infection and (b) prolonged or recurrent infection requiring long or repeated cycles of anti-microbial drugs. Patients with typical presentation of X-linked agammaglobulinemia (XLA) and chronic granulomatous disease (CGD) were excluded from the study, as these are diagnosed using relevant assays; namely, Bruton's tyrosine kinase (BTK) protein expression and neutrophil oxidative burst activity detection, respectively. Peripheral venous blood with written informed consent was obtained from the patients and their parents. In addition, clinical history and relevant laboratory results were recorded. The study protocol was approved by the Medical Research and Ethics Committee, Ministry of Health Malaysia (KMM/NIHSEC/P16–837) and conducted according to the Declaration of Helsinki.

Laboratory testing

Lymphocyte subsets T, B and natural killer (NK) cell enumeration were performed using the BD Multitest™ four-color T, B and NK cells (TBNK) reagent kit on a BD FACSCanto™ II (Becton Dickinson Biosciences, San Jose, California, USA). Serum immunoglobulin and complement levels of immunoglobulin (Ig)G, IgM, IgA, C3 and C4 were quantified using the SPAPLUS® immunoturbidimeter (The Binding Site, Birmingham, United Kingdom). Total IgE concentration was measured by a fluorescent enzyme immunoassay.

Whole-exome sequencing and causative variant prioritization

Genomic DNA was extracted from peripheral blood mononuclear cells using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Paired-end sequencing of the enriched exomes was done on a HiSeq 4000 sequencer (Illumina, San Diego, California, USA) at ×100 coverage. The workflow for processing sequencing data and variant prioritization strategy have been previously described [21]. The variants were interpreted according to the American College of Medical Genetics and Genomics (ACMG) guidelines [22]. Correlation between clinical features and the potential causative variants in patients was assessed through a comprehensive literature search. Causative variants were validated by bidirectional Sanger sequencing. The disease inheritance was confirmed by sequencing the parents' blood when available. Cases with positive findings were grouped according to their genetic defects and disease-associated features, as in the 2017 IUIS IEI classification [23]. The diagnostic workflow is illustrated in Figure 1.

RESULTS

Cohort demographics

Of the 30 patients, 18 subjects were male, accounting for 60.0% of the study population (Supporting information, Table S1). Most of the patients were Malay ($n = 16$), followed by Chinese ($n = 6$) and Indian ($n = 3$). Four patients were indigenous Malaysians: Bajau ($n = 2$), Iban ($n = 1$) and Kadazan ($n = 1$). One patient in the cohort was a Malaysian Thai. The age of onset ranged from 1 month to 10 years, with a median of 1 year. All patients were from unrelated families with no parental consanguinity except P29, whose parents were third cousins.

Clinical and laboratory findings of 30 patients

Respiratory tract infection was the most common clinical manifestation observed among the cohort (Figure 2a). Lower respiratory tract infections (LRTI) ($n = 32$) had a higher occurrence than upper respiratory tract infections

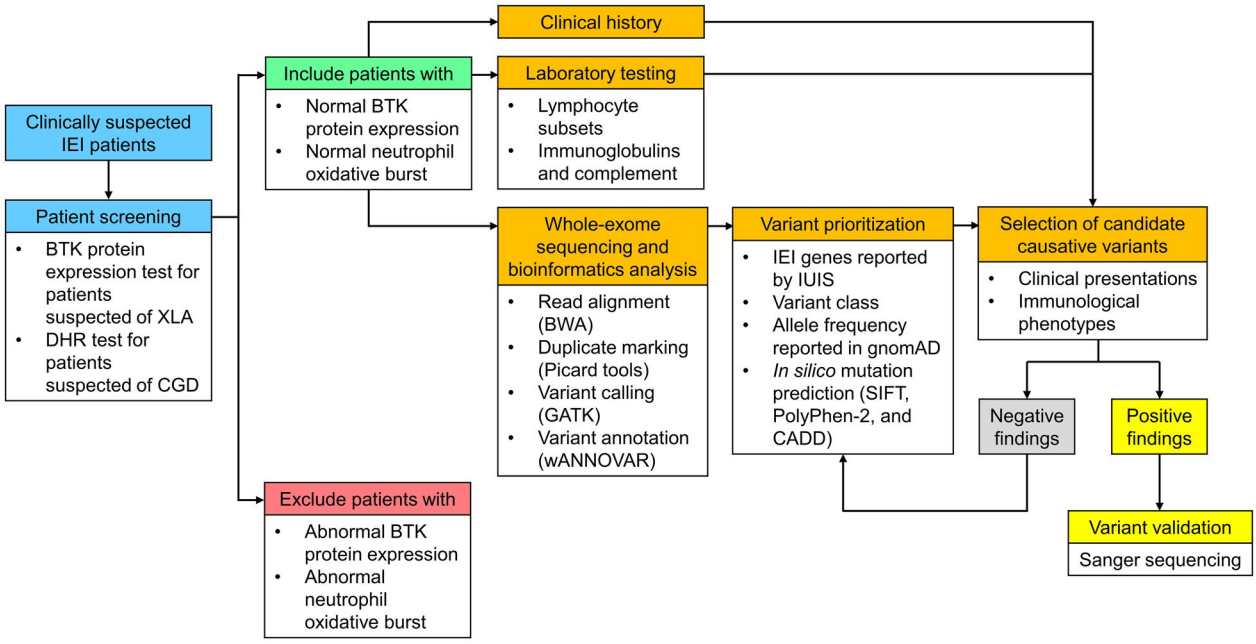

FIGURE 1 Overview of the diagnostic workflow using whole-exome sequencing (WES) of 30 pediatric patients aged 18 years and below. Patients with clinical suspicion of X-linked agammaglobulinemia (XLA) and chronic granulomatous disease (CGD) were excluded based on their abnormal Bruton's tyrosine kinase (BTK) protein expression and abnormal neutrophil oxidative burst, respectively. Patient clinical history was collected along with peripheral venous blood samples and written informed consent. Laboratory testing including lymphocyte subset enumeration and quantitation of serum immunoglobulins and complement were performed. Purified genomic DNA from patients was subjected to WES that utilized the Illumina paired-end sequencing approach. Bioinformatics processing of the sequencing data and variant filtering were performed following the workflows previously described [21]. Lastly, causative variants with functional impact relating to the clinical and immunological features of individual patient were validated using Sanger sequencing. Disease inheritance was confirmed by sequencing the parents' DNA when available. For patients with negative findings, the exome data were reanalyzed based on the updated International Union of Immunological Societies (IUIS) gene list and latest published reports

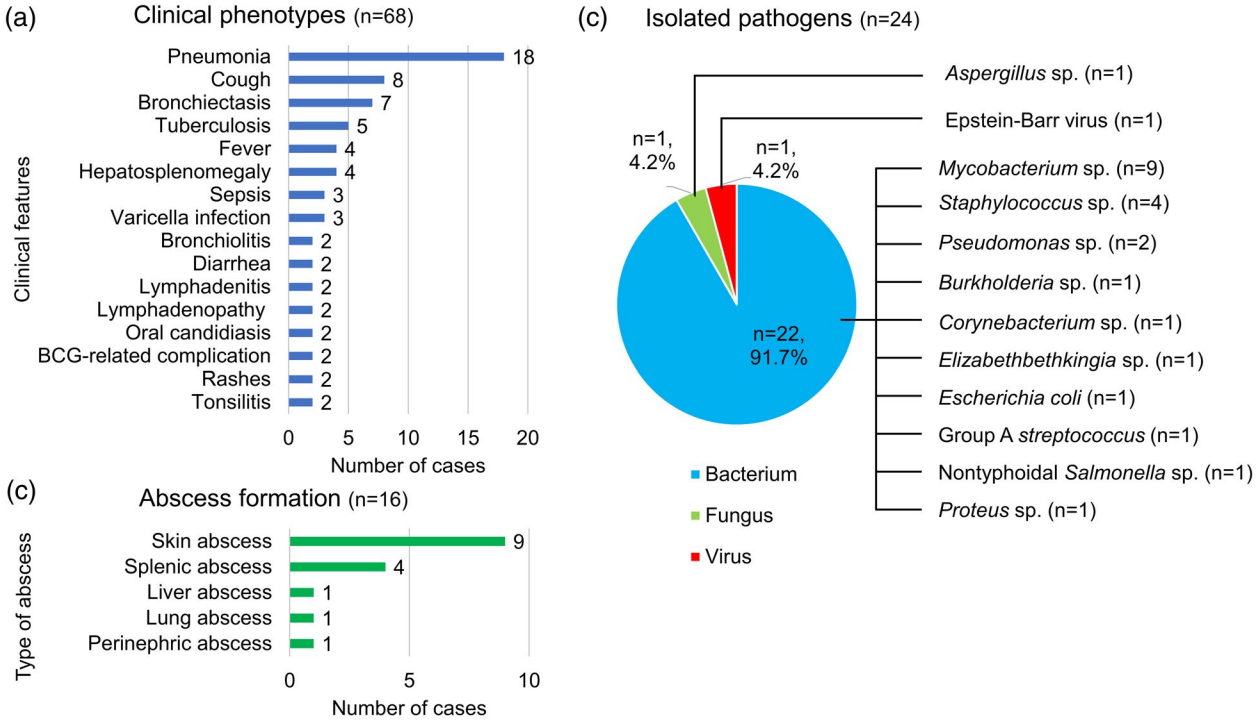

FIGURE 2 Clinical presentations and laboratory findings of 30 patients. (a) Inborn errors of immunity (IEI) patients were more likely to experience respiratory tract infections, particularly lower respiratory tract infections (LRTI), including pneumonia, bronchiectasis, tuberculosis and bronchiolitis. (b) Skin abscesses were more commonly seen than internal abscesses. (c) Most patients contracted infections caused by bacteria while only a minority had fungal and viral infections. Infections induced by *Mycobacterium* sp. and *Staphylococcus* sp. were common among the IEI patients

(URTI) ($n = 10$). Of all the LRTI cases, pneumonia ($n = 18$) was more common than bronchiectasis ($n = 7$), tuberculosis ($n = 5$) and bronchiolitis ($n = 2$), whereas with URTI, cough ($n = 8$) occurred more frequently than tonsillitis ($n = 2$). Apart from respiratory tract infections, fever ($n = 4$) and hepatosplenomegaly ($n = 4$) were noted in the cohort; among the 16 cases with abscess development, skin abscess ($n = 9$) was the most common type (Figure 2b). Culture and sensitivity testing of patients' blood, cerebrospinal fluid and abscess drainage fluid showed that nine patients had mycobacterial infections caused by *Mycobacterium tuberculosis* ($n = 8$) and *Mycobacterium bovis* ($n = 1$), while *Staphylococcus aureus*, commonly found in immunocompromised children, was detected in four patients (Figure 2c). Viral and fungal infections were less frequent, in that one patient was diagnosed with Epstein–Barr virus (EBV) encephalitis and another with invasive pulmonary aspergillosis. Immune profile testing showed three patients with a universal depletion of lymphocyte subsets; nine patients had elevated IgE while one patient displayed low levels of IgG, IgM and IgA. The clinical and immunological phenotypes of all 30 patients are illustrated in Supporting information, Table S1.

Bioinformatic interpretation of 30 exomes

The exomes had an average guanine–cytosine (GC) content of 49.6%. WES generated a range of 47 192 790 to 151 435 510 paired-end reads, with a median of 58 868 128 reads for 30 samples. On average, 99.0% of the sequencing reads were properly aligned to the human reference genome GRCh38. On the whole, 655 728 single nucleotide variants (SNVs) and 16 604 short insertions or deletions (indels) were detected in all the targeted exons and splice sites (Supporting information, Table S2). Of the total SNVs, 51.6% were synonymous and 48.4% were non-synonymous. The non-synonymous SNVs consisted mainly of missense substitutions (98.8%), followed by stopgain (0.9%), startloss (0.2%) and stoploss (0.1%) mutations, while the indels comprised 61.0% non-frameshift and 36.2% frameshift indels. Filtering against known IEI genes reduced the total variants to 10 974 SNVs and 56 indels (Supporting information, Table S2). On average, 0.7% and 0.3% of the variants in an exome were non-synonymous SNVs and indels associated with IEI, respectively. The number of variants was reduced further based on minor allele frequency and variant impact prediction to 15 potentially causative variants in 14 patients.

Genetic diagnosis of 14 patients

We identified causative variants in 14 patients using WES, amounting to a diagnostic yield of 46.7%. The median duration from age of onset to recruitment for WES was 4 years (Figure 3). Autoinflammatory disorders ($n = 3$), diseases of immune dysregulation ($n = 3$) and defects in intrinsic and innate immunity ($n = 3$) were the most common disease categories in our study cohort. Two categories, namely predominantly antibody deficiencies and combined immunodeficiencies with associated and syndromic features, were detected in two patients each. Only one patient was diagnosed with an immunodeficiency affecting cellular and humoral immunity. WES findings differed from the provisional clinical diagnosis in seven of the 14 cases (50.0%). Fifteen causative variants harbored in 13 genes were identified: namely, *SH2D1A*, *PIK3CD*, *NOD2*, *IL17F*, *STAT1*-GOF, *IL12RB1*, *STAT3*-GOF, *NFAT5*, *PNP*, *IL2RG*, *COPA*, *NLRC4*-GOF, *CD79A* and *STAT3*-LOF. Of the identified genetic defects ($n = 15$), missense SNVs ($n = 10$) were the most common mutation, followed by SNVs resulting in premature termination ($n = 2$) (Figure 4a). In addition to exonic SNVs, WES detected two splice site mutations and a frameshift deletion. Most of the variants identified were autosomal dominant disorders (64.3%), while the rest were autosomal recessive (21.4%) and X-linked (14.3%) (Figure 4b). P17 was the only patient identified with a compound heterozygous mutation in *IL12RB1* (Figure 4c). Novel mutations were found in P22 and P26. Familial segregation testing using Sanger sequencing was performed on six patients and their parent(s). Among these six cases, half (P13, P16 and P28) had sporadic mutations while the remainder (P3, P17 and P25) were familial. Unfortunately, five patients (P1, P3, P21, P22 and P26) succumbed to their illnesses in mid-study, giving a mortality rate of 16.7%. The provisional diagnosis, genetic findings and clinical outcomes of 14 patients with genetic mutations identified by WES are summarized in Table 1.

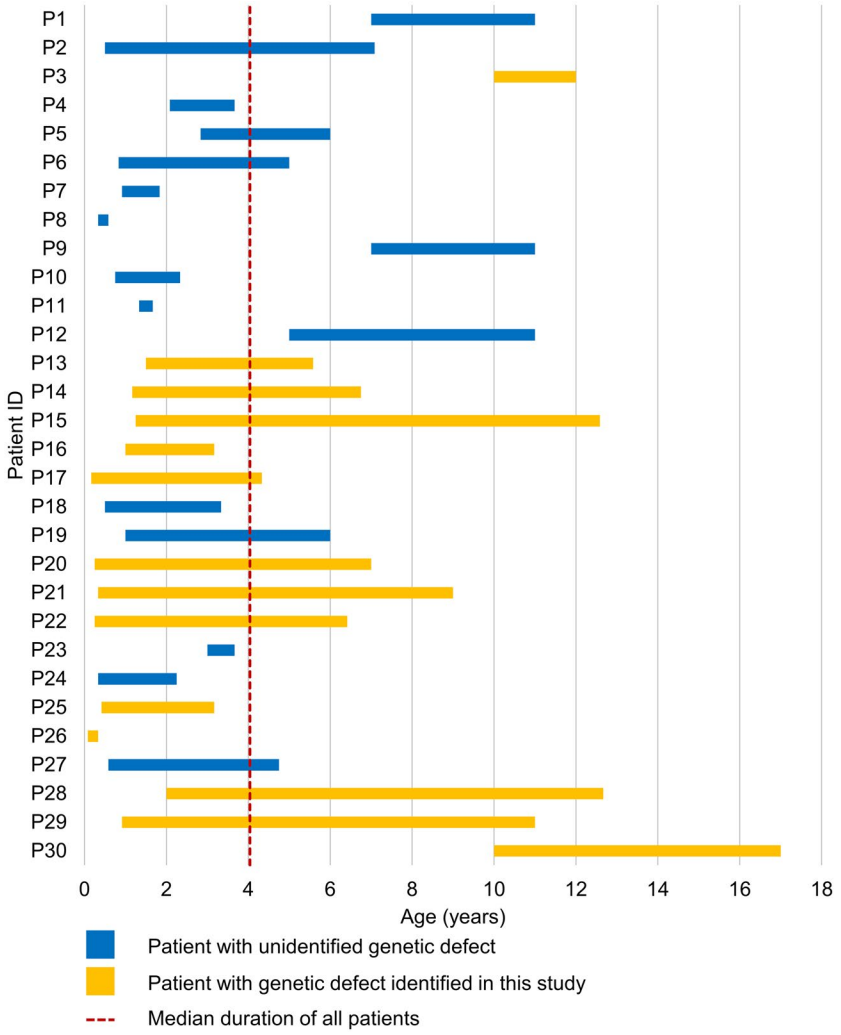

FIGURE 3 Duration from the age of onset to age recruited for whole-exome sequencing (WES). A median duration of 4 years was observed from the onset of symptoms to the recruitment for WES

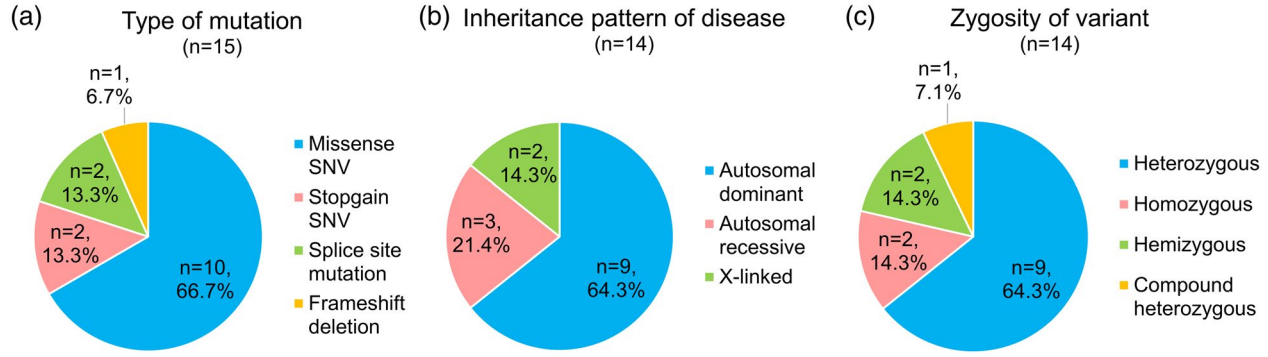

FIGURE 4 Genetic variants uncovered by whole-exome sequencing (WES) in 14 patients. (a) Of the 15 variants identified, 10 were missense single nucleotide variants (SNVs), whereas two were stopgain SNVs. Two splice site mutations and a frameshift deletion were also detected by WES. (b) Most of the variants detected led to autosomal dominant disorders (64.3%). Familial segregation examined by Sanger sequencing of six patients showed three patients had *de-novo* mutations and the other three had familial mutations. (c) One compound heterozygous mutation induced by a missense SNV and a frameshift deletion was detected

DISCUSSION

The clinical variability and genetic heterogeneity make IEI diagnosis a challenge, often leading to a substantial delay in diagnosis. As Noh *et al*. [14] observed, diagnostic delay was the predominant cause for the high mortality and morbidity rate in these patients. This becomes more significant when patients present atypically [26]. Currently, the targeted gene panel to diagnose IEI is widely available. However, as newer IEI-associated genes are discovered gene panels would have to be updated, incurring higher costs. Between WES and WGS, WES is more practical for clinical use because of its lower cost and smaller data output. The larger and more comprehensive genomic data generated by WGS may lead to diagnostic delay due to longer data-processing time. This study demonstrated a diagnostic yield of 46.7%, which is comparable to that obtained using WES in other populations [3,8–12].

We detected two novel variants in this cohort. A *PNP* mutation was confirmed in P22 (combined immunodeficiencies with associated and syndromic features), but by that time the patient had already progressed to severe neuroregression and HSCT was no longer a beneficial option. She eventually succumbed to aspiration pneumonia. The other novel variant in our cohort was P26, who had a *COPA* mutation (autoinflammatory disorder). This patient had an underlying interrupted aortic arch and she died due to nosocomial sepsis before genetic diagnosis was confirmed.

Establishing definitive genetic defects in suspected IEI cases is important for management and treatment of cases. P25, who has an *IL2RG* mutation, is currently being evaluated for HSCT. In P28, the confirmation of a *NLRC4* mutation enabled her intermittent joint pains to be co-managed by a rheumatologist which resulted in better pain control. However, in cases where HSCT or gene therapy is not an option, successful molecular diagnosis is mainly important for family counseling, carrier detection and prognostication. For example, the parents of P17, whose child was initially suspected of Mendelian susceptibility to mycobacterial disease, had difficulty accepting his condition and blamed it on the bacille Calmette–Guérin (BCG) vaccination. This also resulted in poor compliance to antibiotic prophylaxis and frequent default from follow-up. Since the diagnosis of an *IL12RB1* gene defect, treatment and follow-up compliance has improved. His younger brother was BCG-vaccinated only after testing confirmed that he did not have this genetic defect. In another case, P16, the identification of the genetic defect assisted the clinician in anticipating possible disease manifestations. He initially presented with a *STAT1*-LOF feature (BCG-lymphadenitis), but a later candidiasis infection and cytopenia were more consistent with a *STAT1*-GOF etiology. The confirmation of a *STAT1* mutation prompted more aggressive anti-fungal therapy and oral fluconazole was added to his regimen. He is currently free of any serious infection.

Genetic diagnosis remains undetermined in the majority of our cohort. WES is limited in its ability to detect mutations in pseudogenes and highly repetitive sequencing [27]. Interestingly, in a study of 32 patients with unknown genetic defects, WGS identified causative mutations in 53% of the cohort [28]. Therefore, WGS should be considered in those cases where WES fails to identify a genetic defect.

Our diagnostic approach has limitations. While our cohort comprises individuals from various ethnic groups in Malaysia, there is no database reflecting such ethnic diversity. Consequently, our analysis may be affected, as the combined allele frequency used is that of the gnomAD database derived from Caucasian data. Secondly, poor retrieval of parental DNA impeded the examination of inheritance pattern in our cohort. Thirdly, as we were unable to conduct *in-vitro* functional assays to demonstrate the pathogenicity of identified variants, we used *in-silico* tools to predict this in our cohort.

TABLE 1 Admitting clinical diagnosis, genetic diagnosis, treatment plan and disease progression for 14 patients with identified genetic mutations

Patient ID	Gender	Ethnicity	Age at presentation	Admitting clinical diagnosis	Genetic diagnosis (IUIS disease category)	Genetic mutation	Disease inheritance (zygosity)	dbSNP or reported case	SIFT	Polyphen-2	CADD phred	gnomAD	ACMG classification	Treatment plan and/or disease progression
Concordant findings														
P13	F	Malay	1 year 6 months	CVID	CVID (predominantly antibody deficiencies)	*PIK3CD* NM_005026.5 c.3061G>A p.E1021K	AD (Het)	rs397518423	D	D	33	0	Pathogenic	On regular IVIg; No recurrent infection
P15	M	Chinese	1 year 3 months	CMC	CMC (defects in intrinsic and innate immunity)	*IL17F* NM_052872.4 c.365C>T p.P122L	AD (Het)	rs533677359	D	D	24.1	2.12E-05	Benign	No severe infection
P17	M	Malay	2 months	MSMD	MSMD (defects in intrinsic and innate immunity)	*IL12RB1* NM_005535.3 c.523C>T p.R175W; c.599delT p.L200Rfs*3	AR (CH)	rs750667928; rs1169002203	D; NA	D; NA	22.2; NA	8.06E-06; 3.98E-06	Probably benign; probably pathogenic	On antibiotic prophylaxis; parents refused BMT/HSCT
P22	F	Malaysian Thai	3 years	PNP deficiency	PNP deficiency (combined immunodeficiencies with associated and syndromic features)	*PNP* NM_000270.4 c.550C>T p.Q184*	AR (Hom)	Novel	NA	NA	27	NA	Probably pathogenic	Died before diagnosis
P28	F	Malay	2 years	Autoinflammatory disorder	Defects affecting the inflammasome (autoinflammatory disorder)	*NLRC4* NM_021209.4 c.A1970T p.Q657L	AD GOF (Het)	[21]	D	D	24.6	NA	Uncertain significance	Symptomatic management for fever and joint pain
P29	M	Bajau	11 months	Agammaglobulinemia	Agammaglobulinemia (predominantly antibody deficiencies)	*CD79A* NM_001783.4 c.379+1G>A	AR (Hom)	rs1555843601	NA	NA	25.2	4.10E-06	Pathogenic	On regular IVIg
P30	M	Malay	10 years	HIES	HIES (combined immunodeficiencies with associated and syndromic features)	*STAT3* NM_003150.4 c.1934T>A p.L645Q	AD LOF (Het)	[24]	D	D	31	NA	Probably pathogenic	On antibiotic prophylaxis
Discordant findings														

(Continues)

TABLE 1 (Continued)

Patient ID	Gender	Ethnicity	Age at presentation	Admitting clinical diagnosis	Genetic diagnosis (IUIS disease category)	Genetic mutation	Disease inheritance (zygosity)	dbSNP or reported case	SIFT	Polyphen-2	CADD phred	gnomAD	ACMG classification	Treatment plan and/or disease progression
P3	M	Indian	10 years	CVID	XLP (diseases of immune dysregulation)	*SH2D1A* NM_002351.5 c.163C>T p.R55*	XL (Hem)	rs111033623	NA	NA	36	NA	Pathogenic	Died due to sepsis
P14	M	Chinese	1 year 2 months	SCID	Non-inflammasome-related condition (autoinflammatory disorders)	*NOD2* NM_022162.3 c.1834G>A p.A612T	AD (Het)	rs104895438	D	D	25.2	5.98E-04	Uncertain significance	On regular IVIg and planned for BMT/HSCT; Crohn's disease was well-controlled
P16	M	Kadazan	3 months	MSMD	CMC (defects in intrinsic and innate immunity)	*STAT1* NM_007315.4 c.1154C>T p.T385M	AD GOF (Het)	rs587777630	D	D	26.7	NA	Pathogenic	Relatively well
P20	M	Jawa	3 months	ALPS	Regulatory T cell defect (diseases of immune dysregulation)	*STAT3* NM_003150.4 c.1974G>C p.K658N	AD GOF (Het)	rs587777650	D	D	24.9	NA	Pathogenic	No severe infection
P21	F	Malay	4 months	Cell-mediated immunodeficiency	Immune dysregulation with colitis (diseases of immune dysregulation)	*NFAT5* NM_138713.4 c.4498G>A p.E1500K	AD (Het)	rs758828053	D	D	27.9	1.99E-05	Uncertain significance	Died before diagnosis
P25	M	Chinese	5 months	MSMD	SCID (immunodeficiencies affecting cellular and humoral immunity)	*IL2RG* NM_000206.3 c.854+2T>C	XL (Hem)	[25]	NA	NA	33	NA	Pathogenic	Planned for BMT/HSCT
P26	F	Chinese	1 month	SCID	Non-inflammasome-related condition (autoinflammatory disorders)	*COPA* NM_001098398.2 c.223A>C p.I75L	AD (Het)	Novel	D	D	28.2	NA	Uncertain significance	Passed away due to nosocomial sepsis with underlying heart problems (interrupted aortic arch)

F = female; M = male; CVID = common variable immune deficiency; CMC = chronic mucocutaneous candidiasis; MSMD = Mendelian susceptibility to mycobacterial disease; PNP = purine nucleoside phosphorylase; HIES = hyper-immunoglobulin (Ig)E syndrome; SCID = severe combined immunodeficiency; ALPS = autoimmune lymphoproliferative syndrome; IUIS = International Union of Immunological Societies; XLP = X-linked lymphoproliferative disorder; AD = autosomal dominant inheritance; AR = autosomal recessive inheritance; XL = X-linked inheritance; GOF = gain-of-function; LOF = loss-of-function; Het = heterozygous; CH = compound heterozygous; Hom = homozygous; Hem = hemizygous; D = deleterious; NA = not available; ACMG = American College of Medical Genetics and Genomics; IVIg = intravenous immunoglobulin; BMT = bone marrow transplant; HSCT = hematopoietic stem cell transplantation.

CONCLUSION

This is the first study, to our knowledge, to determine the genetic etiology of IEI in Malaysian pediatric patients using WES. A definitive diagnosis was achieved in 46.7% of the cohort, and also revealed a 50% discordance between the provisional clinical diagnosis and this diagnosis. This illustrates the complexity of diagnosis in patients with heterogenous clinical features and argues for WES to be used in the diagnosis of IEI.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Adiratna Mat Ripen and Saharuddin Bin Mohamad designed the study framework and supervised the study. Chai Teng Chear, Mohd Farid Baharin, Rikeish R. Muralitharan, Mei Yee Chiow, Munirah Hishamshah and Hamidah Ghani performed experiments and data analysis. Revathy Nallusamy, Kwai Cheng Chan, Asiah Kassim, Chong Ming Choo, Ke Juin Wong, Siew Moy Fong, Kah Kee Tan, Jeyaseelan P. Nachiappan and Kai Ru Teo assisted in patient recruitment, provided clinical treatments and contributed critical views to the study. Adiratna Mat Ripen, Rikeish R. Muralitharan and Mei Yee Chiow drafted the manuscript. All co-authors critically reviewed and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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