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ORIGINAL ARTICLE

Primary antibody deficiencies in Turkey: molecular and clinical aspects

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Abstract

Primary antibody deficiencies (PAD) are the most common subtype of primary immunodeficiencies, characterized by increased susceptibility to infections and autoimmunity, allergy, or malignancy predisposition. PAD syndromes comprise of immune system genes highlighted the key role of B cell activation, proliferation, migration, somatic hypermutation, or isotype switching have a wide spectrum from agammaglobulinemia to selective Ig deficiency. In this study, we describe the molecular and the clinical aspects of fifty-two PAD patients. The most common symptoms of our cohort were upper and lower respiratory infections, bronchiectasis, diarrhea, and recurrent fever. Almost all patients (98%) had at least one of the symptoms like autoimmunity, lymphoproliferation, allergy, or gastrointestinal disease. A custom-made next-generation sequencing (NGS) panel, which contains 24 genes, was designed to identify well-known disease-causing variants in our cohort. We identified eight variants (15.4%) among 52 PAD patients. The variants mapped to *BTK* ($n=4$), *CD40L* ($n=1$), *ICOS* ($n=1$), *IGHM* ($n=1$), and *TCF3* ($n=1$) genes. Three novel variants were described in the *BTK (p.G414W)*, *ICOS (p.G60*)*, and *IGHM* (p.S19*) genes. We performed Sanger sequencing to validate pathogenic variants and check for allelic segregation in the family. Targeted NGS panel sequencing can be beneficial as a suitable diagnostic modality for diagnosing well-known monogenic PAD diseases (only 2–10% of PADs); however, screening only the coding regions of the genome may not be adequately powered to solve the pathogenesis of PAD in all cases. Deciphering the regulatory regions of the genome and better understanding the epigenetic modifications will elucidate the molecular basis of complex PADs.

Keywords Primary antibody deficiencies · Common variable immune deficiency · Targeted next-generation sequencing

Introduction

Primary antibody deficiencies (PAD) are the most frequent subtype of primary immunodeficiency (PID), with a prevalence of 1 in 600 people [1, 2]. PAD is characterized by an absence or marked decrease of immunoglobulins. The main clinical phenotypes include agammaglobulinemia, common variable immune deficiencies (CVID), immunoglobulin (Ig) isotype deficiencies, hyper-IgM phenotype (HIgM), and specific antibody deficiency. Deregulation in B cell development, activation and proliferation or abnormal class-switch recombination and in somatic hypermutation, and apoptosis have been identified in the pathogenesis of PAD [3]. PAD patients have increased susceptibility to infections, autoimmunity, interstitial lung disease, enteropathy, lymphoproliferation, cancer, and allergic diseases. Subcutaneous or intravenous immunoglobulin replacement therapy is the primary therapeutic strategy for restoring decreased immunoglobulins. Hematopoietic stem cell transplantation can be an option for subjects with B cell disorders accompanied with an impaired T cell compartment [4].

Agammaglobulinemia, the most severe form among the PADs, mostly manifests in early childhood. Agammaglobulinemia is characterized by the absence of all major serum immunoglobulin classes due to severe reduction of B cells (< 2%) in peripheral blood. X-linked agammaglobulinemia (XLA) is the most common form of agammaglobulinemia, with an estimated incidence of around 1:100,000 in live births [5]. XLA is caused by deleterious mutations in

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the *Bruton's tyrosine kinase* (*BTK*) gene. *BTK* mutations account for 85% of all agammaglobulinemic individuals in Western Europe [6]. Autosomal recessive forms of agammaglobulinemia arise due to defects in B cell development or survival in the context of mutations affecting the *IGHM*, *CD79A*, *CD79B*, *IGLL1*, *BLNK*, *PIK3CD*, *TOP2B*, or *PIK3R1* genes [5, 7]. In recent years, autosomal dominant forms of agammaglobulinemia were described, with mutations in the *TCF3* and *LRRC8A* genes [5].

Common variable immune deficiency (CVID) comprises the most frequent form of PAD, which is characterized by hypogammaglobulinemia and reduced immunoglobulin (Ig) levels. The majority of CVID patients have normal number of circulating B cells, suggesting that functional defects affect the later stages of B-cell development. Such mutations may affect B cell signaling and activation, isotype switching machinery, and the somatic hypermutation or cytokine signaling. Likewise, defective T cell-mediated activation of B cells due to impaired signaling through CD40-CD40L engagement plays a major role in PAD [8]. In addition to recurrent infections in CVID, additional clinical symptoms like gastrointestinal disorders, allergy, lymphadenopathy, and/or autoimmune diseases. Autoimmunity is a common event and presents in 20–30% of CVID patients [9]. Clinical and genetic phenotypes of CVID are heterogeneous [10, 11]. Although more than thirty genes have been linked to CVID, the genetic basis remains unclear in most cases, with only 2–10% of the patients having known monogenic disorders. Positive family history and parental consanguinity rates were changed according to countries; with a range20–80% of all CVID cases [12, 13].

International Union of Immunological Societies (IUIS) Expert Committee reported more than 400 immune system-related genes in inborn immunodeficiencies, making genetic characterization more complicated [14]. A molecular diagnosis is often advantageous for the patient and the family; a physician can provide genetic counseling, determine prognosis, observe for disease-specific-complications, and occasionally prescribe specific targeted therapies. Next-generation sequencing (NGS) technologies present a valuable option with timesaving and multi-gene testing for diseases with multiple candidate genes like PIDs. Targeted NGS panels may be used as a first screening step to detect common and/or novel disease-causing variants for genes associated with well-recognized PAD syndromes [15, 16].

Herein, we report on the clinical and molecular characteristics of a group of Turkish PAD patients whose genetic etiologies were tested by a custom-designed disease-specific amplicon panel.

Methods

Patients

We studied 52 PAD patients (31 male/21 female), whose diagnoses were CVID ($n = 34$), agammaglobulinemia ($n = 15$), and hyper-IgM syndrome ($n = 3$), based on the European Society for Immunodeficiency Diseases (ESID) diagnostic classification scheme (https://esid.org/Education/Diagnostic-Criteria-PID). The subjects' median age was 14.4 years (min 2 years; max 62 years), and the median age of the reported symptom onset was 4 years. Median leucocyte counts were 8404 cells/μl (range 1700–28,100), and median lymphocyte counts were 2600 cells/μl (range 700–7200) at diagnosis. The parental consanguinity rate was 57.7% in our cohort. Detailed clinical and immunological findings of the study group are described in Table 1. The ethical board of Istanbul Medical Faculty (2015/492) approved this study and written informed consent obtained from all parents or legal guardians.

Targeted panel screening and data analysis

An amplicon-based targeted NGS panel was developed for the PAD. The custom-designed SmartChip TE technology (WaferGen, USA) was used to amplify the coding regions, promoters, and UTR sites of 24 genes (*BTK*, *IGHM*, *IGLL1*, *CD79α*, *CD79β*, *BLNK*, *PIK3R1*, *TCF3*, *ICOS*, *CD19*, *CD81*, *MS4A1*, *CD21*, *TNFRSF13B*, *LRBA*, *TNFRSF13C*, *TWEAK*, *NFKB2*, *CD40LG*, *CD40*, *AICDA*, *UNG*, *TRNT1*, and *TTC37*) according to International Union of Immunological Societies Expert Committee guidelines [17] with the total target length of 105.138 bps. Our custom designed panel consisted of 555 amplicons with a median length of 338 bp (range 103–451 bp).

According to the manufacturer's instructions, genetic DNA was extracted using the QIAamp DNA Blood Kit (Qiagen). Enrichment of target regions was performed by Seq-Ready™ TE MultiSample NanoDispenser according to manufacturers' protocol. Quantification of libraries was done by Qubit dsDNA HS assay kit (Invitrogen, USA) using Qubit 4 Fluorometer (Invitrogen, USA). Samples were sequenced with Illumina MiSeq Reagent V2 kit on Illumina MiSeq (Illumina, USA) sequencer.

Coding regions, 5′- and 3′-UTR parts, and flanking regions of 24 PAD-related genes were enriched for the sequencing. Although primer designs of 555 amplicons aimed at covering more than 100% of all the coding parts, the median coverage of the targeted regions was 92.38% (min 84.5–max 100), and median transcript coverage was 93.25% (min 72.3–max 100).

Table1 Clinical characteristics and immunological findings in our PAD patients

Patients	*Diseases*	*Cons. Mar*	*Current age (years)*	*Gender*	*Leu. count*	*Lymph. Count*	*B Lymph %*	*IGG mg/dl*	*IGM mg/dl*	*IGA mg/dl*	*IGE I.U*	*Age at onset (years)*	*Admission symptoms*	*Infection*	*Autoimmunity*	*Lymphoproliferation*	*GIS*	*Allergy*
PAD01	CVID	Yes	12	M	8500	1300	4	584 ↓	99,6	0,1 ↓	0,1 ↓	5	Bronchiectasis, otitis media	*Streptococcus pneumoniae*	No	No	No	No
PAD02	CVID	Yes	7	M	5700	2200	41	190 ↓	18 ↓	14 ↓	57 ↓	4	Lymphopenia	N.D	No	No	No	No
PAD03	CVID	No	18	M	8000	2000	5	1918	76	369 ↑	0 ↓	15	URTI, recurrent aphtha	N.D	No	Lymphadenopathy	No	No
PAD04	AGM	No	16	M	11,000	2820	0	129 ↓	70	0,44 ↓	2 ↓		Recurrent fever	N.D	N.D	N.D	N.D	N.D
PAD05	AGM	No	10	M	NA	2100	0	102 ↓	15 ↓	0 ↓	1 ↓	1,5	Severe infection	*Otitis media, ecthyma gangrenosum, sepsis*	No	No	No	No
PAD06	AGM	No	16	M	6400	4600	0	126 ↓	12 ↓	22 ↓	NA	1	Recurrent infection	*Pneumonia, otitis media, chronic diarrhea*	Scleroderma	No	Chronic non atrophic gastric	No
PAD07	CVID	Yes	18	M	8300	1700	14	73 ↓	12 ↓	4 ↓	<2 ↓	10	Bronchiectasis	*Streptococcus pneumoniae*	No	No	No	No
PAD08	CVID	Yes	9	F	10,000	4300	15	345 ↓	48	7 ↓	<2 ↓	5	Eosinophilia, URTI	*Streptococcus pneumoniae*	No	No	No	No
PAD09	CVID	Yes	8	F	13,310	4600	20	180 ↓	68	81	8 ↓	5	Bronchiectasis, urtı	*Streptococcus pneumoniae*	FMF, Celiac Disease	GIS lymphoma	Severe gastrointestinal involvement	No
PAD10	AGM	Yes	3	F	7300	5000	0	56 ↓	7 ↓	27	3,2 ↓	1,5	Sepsis	*Ecthyma Gangrenosum*	No	No	Diarrhea	Chronic sinusitis
PAD11	AGM	Yes	15	M	3700	1300	2	1015	128	116	977 ↑	1	Bronchiectasis, otitis media	*Streptococcus pneumoniae*	Atopic dermatitis	Lymphoid hyperplasia	No	No
PAD12	AGM	No	25	M	5600	1600	0	634 ↓	<1↓	<1 ↓	1 ↓	8 mo	Recurrent fever, URTI	N.D	Anti-tg and anti-tpo positivity	N.D	N.D	N.D
PAD13	CVID	Yes	11	F	5600	1600	6	923	67	61	<↓	8	Pneumonia,otitis	CMV	N.D	N.D	N.D	N.D
PAD14	CVID	No	35	F	1700	1100	4,5	141 ↓	20 ↓	18 ↓	<↓	5	Recurrent pneumonia	*Streptococcus pneumoniae*	Anti-tg and anti-tpo positivity	N.D	Recurrent diarrhea	N.D
PAD15	CVID	Yes	15	M	22,700	7100	8	75 ↓	17 ↓	6 ↓	2 ↓	13	Acute gastroenteritis	*Candida albicans*	No	No	Enteropathy	No
PAD16	CVID	No	3	M	8300	5100	24	206 ↓	29,3 ↓	9,1 ↓	2 ↓	2	URTI	*Pseudomonas aeruginosa*	No	No	No	Eczema
PAD17	CVID	Yes	11	M	20,300	3000	17	769 ↓	127	157	372	6	Bronchiectasis	*Streptococcus pneumonia*	No	No	No	No
PAD18	CVID	Yes	4	F	17,000	7000	63	826	106	29 ↓	36.7	2	Pneumonia	*Streptococcus pneumoniae, influenza type 1*	Autoimmune hemolytic anemia	Splenomegaly, hepatomegaly	No	No

Table1 (continued)

Patients	*Diseases*	*Cons. Mar*	*Current age (years)*	*Gender*	*Leu. count*	*Lymph. Count*	*B Lymph %*	*IGG mg/dl*	*IGM mg/dl*	*IGA mg/dl*	*IGE I.U*	*Age at onset (years)*	*Admission symptoms*	*Infection*	*Autoimmunity*	*Lymphoproliferation*	*GIS*	*Allergy*
PAD19	CVID	Yes	11	M	9100	2400	9	508	178	0,1 ↓	16,5	8	LRTI		ITP, autoimmune hemolytic anemia, rheumatoid arthritis	Splenomegaly, hepatomegaly, lymphadenopathy	No	No
PAD20	CVID	No	17	F	11,500	3000	11	152 ↓	27 ↓	19 ↓	16	6	LRTI	*Haemophilus influenzae serotype B*	No	No	Nodular lymphoid hyperplasia	No
PAD21	CVID	Yes	6	F	6600	2000	24	186 ↓	63	25 ↓	12	3	LRTI	*Haemophilus influenzae serotype B/ Streptococcus pneumoniae*	FMF, rheumatoid arthritis	No	No	No
PAD22	CVID	Yes	16	F	7100	3300	11	65 ↓	28 ↓	43 ↓	< 25	3	Recurrent LRTI	*Streptococcus pneumoniae*	N.D	N.D	Recurrent diarrhea	N.D
PAD23	CVID	No	62	F	5600	1500	21	33 ↓	< ↓	< ↓	< ↓	N.D	Pneumonia, LRTI	*Streptococcus pneumoniae*	Vitiligo	Lymphadenomegaly	N.D	N.D
PAD24	CVID	Yes	11	M	14,000	2800	9	389 ↓	82	12 ↓	2 ↓	2	Recurrent bronchiolitis, URTI	N.D	N.D	N.D	N.D	N.D
PAD25	HIGM	Yes	26	F	6500	900	6	528 ↓	214 ↑	192	1132 ↑	2	Recurrent fever, otitis, sinusitis	*Streptococcus pneumoniae*, herpes labialis,	N.D	Splenomegaly	Recurrent diarrhea	Grass, tree, cat, dog
PAD26	AGM	No	39	F	4200	1600	0	200 ↓	6 ↓	6 ↓	2 ↓	1	arthralgia, SLE	*Streptococcus pneumoniae*, herpes labialis	SLE	Splenomegaly	Recurrent abdominal pain	N.D
PAD27	AGM	No	10	M	8100	3900	0	347 ↓	56	66	1,2 ↓	2	Otitis media, rhinosinusitis	*Streptococcus pneumoniae*	No	No	Acute infectious diarrhea	Allergy
PAD28	CVID	Yes	13	M	14,660	3000	7	777 ↓	93	112	< 10 ↓	7	Bronchiectasis	*Streptococcus pneumoniae*	No	No	No	No
PAD29	CVID	No	5	M	10,900	7200	7	9 ↓	51	54	< 2 ↓	3	LRTI, sequela calcific foci	*M. tuberculosis, Streptococcus pneumoniae*	No	No	Acute infectious diarrhea	No
PAD30	AGM	No	18	M	9200	3350	1	73 ↓	8 ↓	4 ↓	< 17 ↓	1	Bronchiectasis	*Streptococcus pneumoniae, zoster*	No	No	No	No
PAD31	AGM	No	5	M	6000	1250	2	123 ↓	25 ↓	38	< 1 ↓	1	Otitis media	*Streptococcus pneumonia*	ITP	Splenomegaly	No	No
PAD32	CVID	No	10	F	6540	3190	11	580	170	69	18 ↓	3	Obstructive disease		No	Mesenteric lymphadenopathy	No	No
PAD33	AGM	Yes	7	M	17,200	1700	1	132 ↓	6 ↓	15 ↓	23	2	Epilepsy, recurrent pneumonia	*Streptococcus pneumoniae*	No	No	No	Rhinitis, asthma

Table1 (continued)

Patients	*Diseases*	*Cons. Mar*	*Current age (years)*	*Gender*	*Leu. count*	*Lymph. Count*	*B Lymph %*	*IGG mg/dl*	*IGM mg/dl*	*IGA mg/dl*	*IGE I.U*	*Age at onset (years)*	*Admission symptoms*	*Infection*	*Autoimmunity*	*Lymphoproliferation*	*GIS*	*Allergy*
PAD34	CVID	Yes	20	F	13,600	2100	14	369 ↓	109	17 ↓	< 17 ↓	2	Bronchiectasis, otitis media	*Streptococcus pneumoniae*	No	No	No	Rhinitis
PAD35	AGM	Yes	9	F	3270	1100	2	1161	70	414 ↑	24	1	Obstructive disease, otitis media	*Streptococcus pneumoniae*	No	No	Acute infectious diarrhea,enteropathy	Rhinitis
PAD36	CVID	Yes	10	M	5300	2800	5	1085	118	101	163	8	Granulomatous disease	*Streptococcus pneumoniae*	No	No	No	Rhinitis
PAD37	CVID	Yes	20	M	7700	3320	19	522 ↓	9 ↓	8 ↓	< 1 ↓		Pyogenic infections	*N.D*	No	No	No	No
PAD38	CVID	No	19	M	7100	2500	18	591 ↓	61	22 ↓	39	2	Recurrent pneumonia, otitis, sinusitis,	*Streptococcus pneumoniae*	ITP, ANA positivity	N.D	N.D	N.D
PAD39	CVID	Yes	10	M	5730	1310	7	260 ↓	58	18 ↓	< 3 ↓	9	LRTI	*Streptococcus pneumoniae*	No	No	No	Rhinitis
PAD40	AGM	No	11	F	4300	1600	0	464 ↓	3 ↓	10 ↓	< 17 ↓	1	Bronchiectasis	*Streptococcus pneumoniae*	No	No	No	Rhinitis
PAD41	HIGM	Yes	17	M	6300	NA	37	320 ↓	1444 ↑	5 ↓	NA	7	URTI	*Pseudomonas aeruginosa*	No	Splenomegaly, hepatomegaly, lymphadenopathy	No	Eczema
PAD42	CVID	No	18	F	2700	1000	9	433 ↓	16 ↓	46 ↓	2 ↓	2	LRTI	*Haemophilus influenzae serotype B/ Streptococcus pneumoniae*	No	No	No	No
PAD43	CVID	Yes	12	M	5700	2000	10	295 ↓	65	15 ↓	16 ↓	5	Meningitis	*Meningococcal meningitis*	No	No	No	No
PAD45	CVID	Yes	18	F	4700	700	12	405 ↓	15 ↓	16 ↓	17 ↓	3	LRTI	*M. tuberculosis*, *Haemophilus influenzae serotype B*	Autoimmune neutropenia	Splenomegaly	Enteropathy	Asthma, rhinitis
PAD46	CVID	Yes	16	M	4600	1900	7	200 ↓	48	26 ↓	2 ↓	8	Sinusitis	*Streptococcus pneumonia*	No	Splenomegaly	No	Rhinitis
PAD47	CVID	No	15	M	3900	1300	4	336 ↓	25 ↓	6 ↓	17 ↓	2	Acute gastroenteritis	*Haemophilus influenza serotype B/ Streptococcus pneumoniae*	No	Splenomegaly	Enteropathy	No
PAD48	CVID	Yes	20	F	3000	700	6	153 ↓	21 ↓	0 ↓	17 ↓	13	Fever, bronchiectasis	*Haemophilus influenzae serotype B/ Streptococcus pneumoniae*	ITP, Autoimmune hemolytic anemia	Splenomegaly	No	No
PAD49	CVID	No	16	M	7400	4500	8	488 ↓	132	0,1 ↓	12 ↓	2	Fever	*Streptococcus pneumoniae*	No	splenomegaly	No	No

Table1 (continued)

Patients	*Diseases*	*Cons. Mar*	*Current age (years)*	*Gender*	*Leu. count*	*Lymph. Count*	*B Lymph %*	*IGG mg/dl*	*IGM mg/dl*	*IGA mg/dl*	*IGE I.U*	*Age at onset (years)*	*Admission symptoms*	*Infection*	*Autoimmunity*	*Lymphoproliferation*	*GIS*	*Allergy*
PAD50	AGM	Yes	11	M	28,100	1200	0	191 ↓	49 ↓	95 ↓		2	Recurrent fever	*Streptococcus pneumoniae*, herpes labialis	N.D	N.D	N.D	N.D
PAD51	AGM	Yes	15	M	6100	3450	2	842	60	116	6 ↓	2	Recurrent bronchiolitis	Pneumonia, recurrent herpes labialis	N.D	Splenomegaly	N.D	N.D
PAD52	HIGM	No	11	F	4900	1500	16	<145 ↓	355 ↑	6 ↓	<17 ↓	2	Recurrent fever	*Streptococcus pneumoniae*	N.D	N.D	N.D	N.D
PAD53	CVID	No	3	F	3610	1960	3,6	129 ↓	16,8 ↓	5,8 ↓	2 ↓	2	Bronchiectasis, diarrhea	*Streptococcus pneumoniae*	No	No	Chronic diarrhea	No

AGM agammaglobulinemia, *CVID* common variable immunodeficiency, *HIGM* hyper-IgM phenotype, *F* female, *M* male, *mo* month, *Pos* positive, *Neg* negative, ↓ indicates decreased Ig levels according to age-matched healthy controls (ref). *N.D.* not determined, *LRTI* lower respiratory tract infection, *URTI* upper respiratory tract infection, *SLE* systemic lupus erythematosus, *FMF* familial Mediterranean fever, *ITP* idiopathic thrombocytopenic purpura, *GIS* gastrointestinal disease, *CMV* cytomegalovirus, *GIS* gastrointestinal system, *LPD* lymphoproliferative disorders, *ALGY* allergy

Alignment to reference genome (hg19) and variant calling were performed by SEQ 2.8.2. software (Genomize, Turkey). Data analysis and variant interpretation pipeline were previously described by our group [18]. PolyPhen [19], Mutation Taster [20], CADD [21], and SIFT [22] were used for in silico prediction of novel variations. Variants were classified according to ACMG guidelines [23].

Following NGS-based gene panel screening, all genomic variations were validated, and familial segregations were performed by Sanger sequencing and analyzed with CLC software (CLC Genomics Workbench, Qiagen, Netherlands).

Results

Clinical findings

The most common clinical symptoms were upper and lower respiratory infections (32%), bronchiectasis (27%), and recurrent fever (15%) in our cohort. Almost all subjects presented with recurrent infections at disease onset, with the most common infections being pneumonia (71%), otitis media (17%), or sinusitis (9%). The majority of the patients suffered from pneumonia at the time of diagnosis or later in the follow-up (54%), with *H. influenza* detected in microbiologic tests. Two patients manifested tuberculosis, and one suffered bacterial meningitis. Ecthyma gangrenosum infection occurred in two patients. Two patients experienced *Pseudomonas aeruginosa* infections.

Twenty-two cases (42.3%) were diagnosed with one or more autoimmune diseases, including autoimmune hemolytic anemia, idiopathic thrombocytopenia (ITP), rheumatoid arthritis, systemic lupus erythematosus (SLE), autoimmune neutropenia, scleroderma, familial Mediterranean fever (FMF), celiac disease, atopic dermatitis, vitiligo, and anti-Tg/anti-TPO positivity.

Sixteen patients (30.7%) had a history of lymphoproliferative disorders like splenomegaly, hepatomegaly, and lymphadenopathy. One patient developed lymphoid hyperplasia, and one patient was deceased due to B-cell lymphoma. Gastrointestinal system diseases detected in fifteen patients (28.8%) are mostly seen as recurrent diarrhea and enteropathy. Twenty-five percent of our cohort suffered from variable allergic conditions, mostly asthma, eczema, and rhinitis.

Variants in PAD patients

This study identified disease-causing variants in eight (15.4%) out of 52 PAD patients. The majority among these variants is found in the *BTK* gene ($n=4$), followed by *CD40L*, *ICOS*, *IGHM*, and *TCF3*, each in a single subject (Table 2). To verify our custom-designed panel's reliability, we included a patient sample with a known *CD19* splicing

site mutation as a validation sample, and the variant was successfully redetected. All the variants were verified, and parents' carrier status was checked by Sanger sequencing. In our agammaglobulinemia cohort, the genetic etiology was clarified in six (*BTK*, *IGHM*, and *TCF3* gene variants) out of fifteen patients.

The most common genetic variations were found in *BTK* gene. A novel missense *BTK* variant (c.1240G > T;p.G414W) was found in PAD30, who suffered bronchiectasis, recurrent pneumonia, and zoster. The patient had no B lymphocytes and a near-complete lack of Ig production (Fig. 1A). This novel variant was located on the kinase domain of the BTK protein, which is highly conserved (Fig. 1B). A 16-year-old male patient (PAD6) suffered from severe infections, scleroderma, and chronic non-atrophic gastric; he was suspected with agammaglobulinemia and was found to have a missense (c.1732 T > C;p.S578P) variant in the *BTK* gene. Sanger sequencing confirmed the BTK variant's segregation and the de novo status of the variant in the proband only, but not in his mother (Fig. 1A). Two siblings with a similar clinical presentation like recurrent upper respiratory tract infection (URTI) and severe respiratory distress were clinically diagnosed as XLA. One of these siblings (PAD12) was screened in our panel, and a known nonsense variant (c.718G > T;p.Glu240Ter, rs128621191) was detected in the *BTK* gene. Surprisingly, the other affected brother was not a carrier of this pathogenic *BTK* variant and was found hemizygous for the wild-type allele in Sanger analysis (Fig. 1A). Therefore, this affected brother with no *BTK* mutation was investigated by whole-exome sequencing, but no pathogenic variant was detected (data not shown). Furthermore, we detected a known nonsense variant (c.1234C > T;p.Q412*) in a 10-year-old boy (PAD5) who had a history of recurrent severe infections. The patient's mother was heterozygous for the mutation (Fig. 1A).

We identified two different genetic mutations among cases with agammaglobulinemia; a patient had a homozygous *IGHM* mutation, and another case carried a heterozygous variant in the *TCF3* gene, which was previously linked to autosomal dominant agammaglobulinemia. PAD10, a 3-year-old female who had a history of hospital admissions due to severe infections, including sepsis, diarrhea, chronic sinusitis, and an Ecthyma gangrenosum, harbored a nonsense variant (c.56C > A;p.Ser19Ter) in the *IGHM* gene (PAD10) (Fig. 2B).

PAD27, a 10-year-old male (PAD27), carried a known missense variant (c.1388C > T;p.S514L, rs372168347) in the *TCF3* gene (Fig. 2C); the patient had a remarkable infectious history that included otitis media, rhinosinusitis, and acute infectious diarrhea. The immunologic investigation in PAD27 showed lack of B cells and hypogammaglobulinemia Thirty-four patients were diagnosed with common variable immune deficiency (CVID), and we identified a causative mutation in only a single case.

The patient was a 16-year-old female (PAD22) who harbored a disease-causing (c.171_172insG; p.Gln60AlafsTer8) variant in the *ICOS* gene (Fig. 2A).

Among our PAD cohort, a single case (PAD29) had an immunologic profile consistent with a hyper-IgM syndrome and was diagnosed with X-linked *CD40L* deficiency. PAD29 was a 3-year-old boy with reduced B cell counts and hypogammaglobulinemia. He had a history of severe lower respiratory tract infection (LRTI) with tuberculosis, pneumonia, and calcified lung foci. Data analysis showed that he has a previously identified missense variant (c.755G > A;p.G252D) in the *CD40L* gene (Fig. 2D). Sanger sequencing confirmed the segregation of the *CD40L* variant with the phenotype and the maternally inherited status of the variant in the proband.

Discussion

Primary antibody deficiency (PAD) is the most prevalent form of PIDs with serum antibody profiles classified as agammaglobulinemia/hypogammaglobulinemia, immunoglobulin (Ig) isotype deficiencies, hyper-IgM phenotype (HIgM), and common variable immunodeficiency (CVID). The clinical presentation ranges from no symptoms to severe and fatal complications that may present as recurrent infections, autoimmunity, lymphoproliferation, and malignancy [24]. In this study, fifty-two PAD patients (43 children and 9 adults) were evaluated. Our cohort's main features included upper and lower respiratory tract infections, autoimmunity, allergies, and lymphoproliferative disorders. The calculated difference between the median age at diagnosis and the age at onset suggests an average of 10 years of diagnostic delay. Although the rates of autoimmunity and GIS disease in our cohort were similar to other adult CVID studies, malignancy was rare, and the mortality rate was relatively low [25, 26]. We attribute this difference to our group's age, with the majority in our cohort being children may not have lived long enough to experience malignancy or lethal complications yet.

The pathogenic gene variants identified in PAD patients pointed to distinct themes: (*i*) defect in early B cell development, (*ii*) class switch recombination (CSR), and (*iii*) terminal B cell differentiation genes.

However, B cell development, CSR, and B cell differentiation genes were identified in the pathogenesis of primary antibody deficiency disorders, and most of the patients' genetic etiology remains unknown [3, 27].

In this study, we developed a targeted NGS panel for PAD patients, which includes well-known PAD genes, and identified the disease-causing variants in *BTK*, *CD40L*,

Table 2 Identification of disease-causing variants in the PAD patients

Sample ID	*Gene*	*Zygosity*	*Nucleotide change*	*Amino acid change*	*Mutation type*	*ACMG guidelines*	*CADD score*	*Reported*
PAD5	*BTK*	Hemizygous	c.1234C > T	p.Q412*	Stop gain	Pathogenic	38	Chen XF,2016 BTKbase
PAD6	*BTK*	Hemizygous	c.1732 T > C	p.S578P	Missense	VUS	28,4	Lopez-Herrera et al. (2008)
PAD12	*BTK*	Hemizygous	c.718G > T	p.E240*	Stop gain	Pathogenic	38	rs128621191
PAD30	*BTK*	Hemizygous	c.1240G > T	p.G414W	Missense	VUS	31	Novel
PAD29	*CD40LG*	Hemizygous	c.755G > A	p.G252D	Missense	VUS	24,1	N Rezaei (2013)
PAD22	*ICOS*	Homozygous	c.171_172insG	p.G60*	Frameshift	Pathogenic	32	Sefer et al. (2021)
PAD10	*IGHM*	Homozygous	c.56C > A	p.S19*	Stop gain	Pathogenic	25,2	Abolhassani et al. (2016)
PAD40	*TCF3*	Heterozygous	c.1388C > T	p.S514L	Missense	VUS	21,7	rs372168347

VUS variant of unknown significance

ICOS, *IGHM*, and *TCF3* genes with a 15.4% success rate. Our custom-made panel has a better success rate (40%) in agammaglobulinemia patients. Disease causing variants were confirmed in 6 out of 15 agammaglobulinemia patients. Agammaglobulinemia consists of mostly monogenic disorders with a well-known genetic background [24]. X-linked agammaglobulinemia is the most seen form of agammaglobulinemia with Bruton's tyrosine kinase (*BTK*) gene variants. *BTK* is located on the X chromosome, and more than 90% of agammaglobulinemia patients are XLA [28]. This study had seven male agammaglobulinemia patients born to non-consanguineous parents and clinically diagnosed with XLA. Contrary to the clinical predictions, only four harbored *BTK* mutations (57%). Here, we identified a novel missense *BTK* variant. Also, three *BTK* variants previously reported in XLA cases were found in other XLA patients [29]. In addition, we found a dominantly inherited *TCF3* allele in a male agammaglobulinemia patient who was clinically diagnosed with XLA. *TCF3* (also known as *E2A*) gene is a transcription factor that is essential for B cell development; dominant-negative variants in this gene were previously linked to agammaglobulinemia [30]. These results showed that autosomal dominant inherited genes could contribute to the pathogenesis of agammaglobulinemia patients with unrelated parents.

A female patient with agammaglobulinemia was confirmed with a nonsense variant in *IGHM* gene, which was previously described before in one individual with *IGHM* deficiency [31]. *IGHM* (mu heavy chain) gene encodes the heavy chain of immunoglobulins and pre-B cell receptor (BCR), and defects of *IGHM* lead to an absence of B cells and all type of immunoglobulins. This patient has severe complications and was treated in the intensive care unit. *IGHM* deficiency has more severe phenotype compared to XLA, which is characterized by a complete absence of B cells and have an early onset of disease. For the CVID group, we have identified a frameshift variant in *ICOS* gene in one patient resulting in hypogammaglobulinemia and normal B cell count. Detailed clinical complications and immunophenotyping of this patient were discussed in our other study [32]. Inducible T cell costimulatory (*ICOS*) gene was the first discovered disease-causing gene for monogenic CVIDs in 2003 [33]. In our cohort, we have one patient with *ICOS* deficiency in 34 CVID patients (2.9%). Recent studies showed that pediatric CVID patients, especially who have autoimmune disorders, mostly mutated with autoimmune-associated genes like *LRBA* and *AIRE* [34]. Although 42% of our patients have at least one autoimmune disorder, we did not detect any pathogenic variant in autoimmune-related genes. CVID shows genetic and clinic heterogeneity, and only 2–10% of patients have a monogenic inheritance [35]. Our results confirmed that screening known genes is not enough for understanding the pathogenesis of CVID. Finally, among our three hyper-IgM patients, we found a *CD40L* variant in one patient. CD40 ligand expressed in T cell is needed for B-T cell interaction and class switching from IgM to other immunoglobulins on B cells. Hematopoietic stem cell transplantation (HSCT) represents the only curative option for *CD40L* deficiency [36]. Here we reported a *CD40L* variant previously identified in a patient with pneumonia, neutropenia, and hyperimmunoglobulin M phenotype [37]. Our patient has similar findings, and HSCT is planned for his treatment. Genetic diagnosis gives a great opportunity to confirm the diagnosis, understanding clinical outcome, patient management, and genetic counseling. The discovery rate of pathogenic variants using targeted gene panels varies about 25–60% in the literature [15, 16, 38, 39]. We have previously developed an amplicon-based targeted NGS panel for SCID (severe combined immunodeficiency) patients with a 58% success rate [18].

In this study, our panel's diagnostic success was 15.4%, which might be insufficient to use this panel for diagnostic

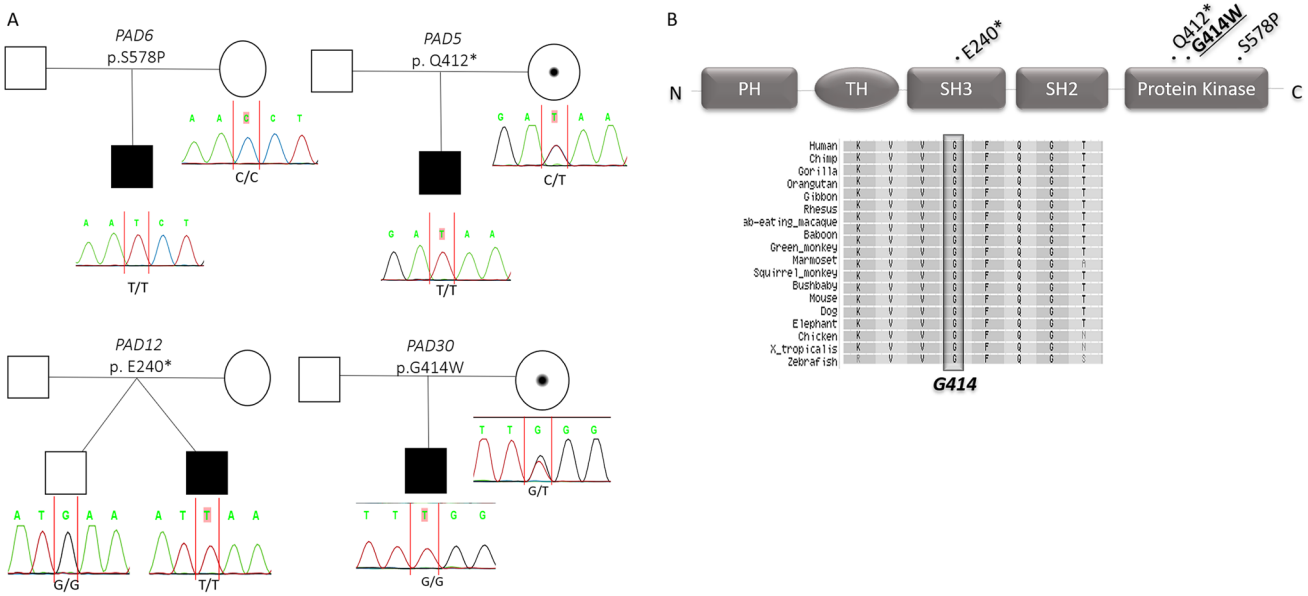


Fig. 1 *BTK* gene variants in the agammaglobulinemia patients. **A** Sanger segregation of the variants in the affected child and the family. Full-filled figures present homozygous patients. Half-filled figures present heterozygous carriers. Square, male; circle, female. **B** Location of all *BTK* variants in the protein and the conservation of the p.G414 residue, denoted by the bold (USCS annotation). PH: pleckstrin homology, TH, Tec homology; SH3, Src homology 3; SH2, Src homology 2

purposes: small cohort size and the limited number of genes affect the success rate. However, addition of whole exome sequencing studies increases the success rate of genetic diagnosis up to 70%; it is still far to understand the genetic background of PADs completely [40]. Advanced omics technologies like whole-genome sequencing (WGS), methylation array, or genome-wide association studies (GWAS) will be essential to identify new disease-associated targets [41]. Targeted NGS technologies represent an accurate, timely, and cost-effective approach for the evaluation of complex PIDs. Molecular diagnosis with wide range-targeted panels can be used as a first-line diagnostic tool for screening of well-known PID genes. Nevertheless, due to the heterogeneous nature of PIDs, sequencing only the most common genes cannot explain all cases. Whole exome sequencing (WES) or whole-genome sequencing (WGS) can help enlighten the molecular background of PIDs.

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Declarations

Conflict of interest The authors declare no competing interests.

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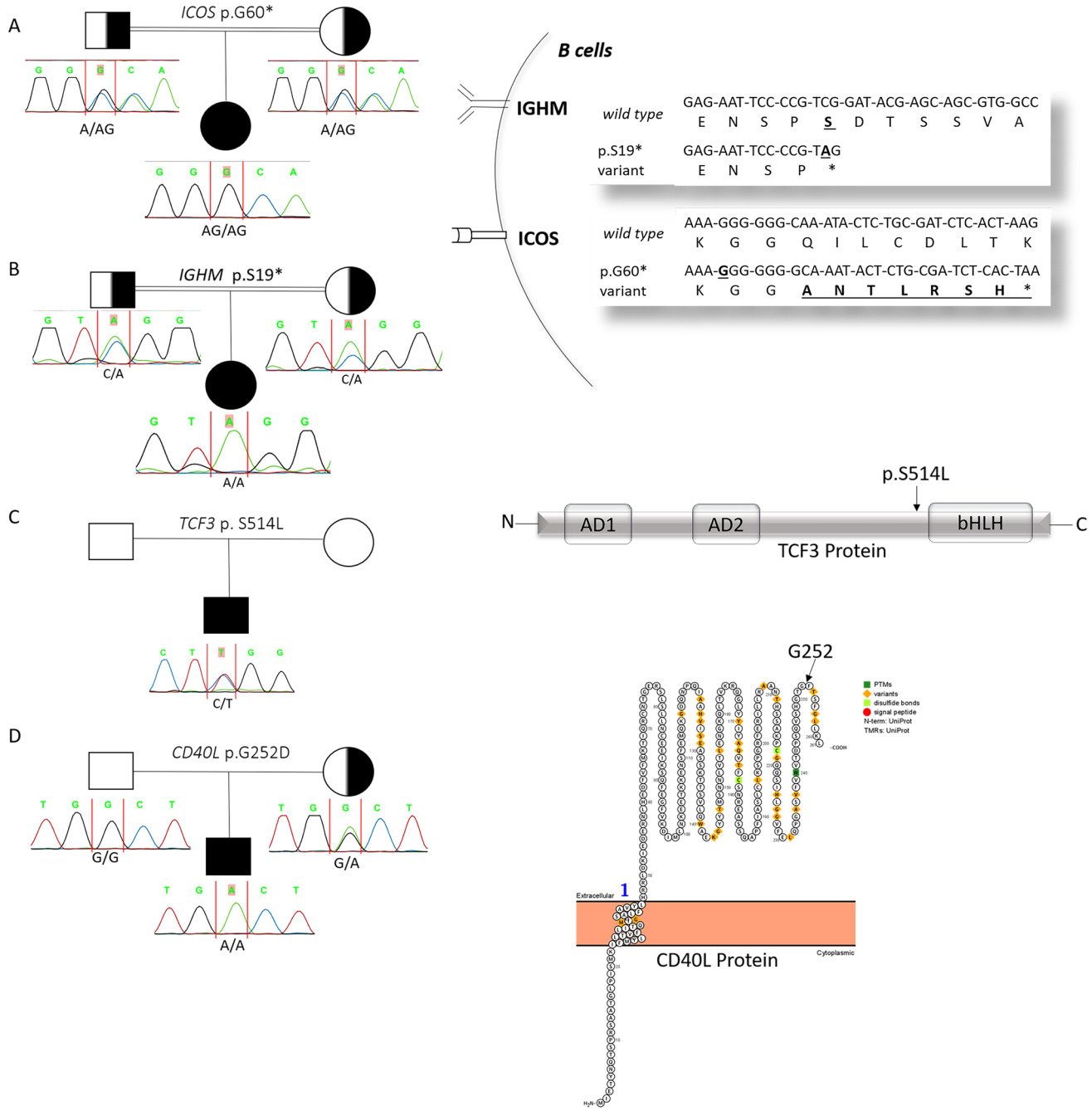


Fig. 2 *ICOS*, *IGHM*, *TCF*, and *CD40LG* variants in the patients: Variant detection in family segregation study was performed with Sanger sequencing. Full-filled figures present homozygous patients. Half-shaded filled figures present heterozygous carriers. Square, male; circle, female. **A** and **B** Sanger segregation of *IGHM* and *ICOS* gene variants in the affected patient and their families and the illustration of the structure of the ICOS and IGHM protein. Nucleotide sequences of wild-type and mutated ICOS and IGHM protein were shown. **C** Sanger segregation of *TCF3* gene variant in the affected child and the variant were shown in the protein. Arrows indicate the location of the variant. AD1, activation domain 1; AD2, activation domain 2; bHLH, basic helix loop helix domain. **D** *CD40LG* gene variant in the affected child and his family. Protein structure was illustrated with Protter, and p.G252 residue was shown by arrow

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