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A new report of autoinflammation and PLCG2-associated antibody deficiency and immune dysregulation (APLAID) with a homozygous pattern from Iran

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Highlights

- Autoinflammation and PLCG2-associated antibody deficiency and immune dysregulation (APLAID) is an autosomal dominant autoinflammatory disease characterized by episodic skin, musculoskeletal, ophthalmic and gastrointestinal tract symptoms. Here we report an 11-year-old girl with a history of repeated episodes of fever, myalgia, arthralgia, abdominal pain, and urticarial rash in the trunk and limbs. Whole exome sequencing was carried out to identify the genetic cause of our patient. We identified a homozygote missense variant (c.579C>G, p. His193Gln) in exon 7 of PLCG2 gene suggesting to be pathogenic in homozygous state for APLAID and the probability of acting in an Autosomal Recessive pattern. Our bioinformatics analyze showed this novel mutation has detrimental effect on 3D structure of PLCG2 protein and it is well conserved among other similar species.

Abstract

Autoinflammation and PLCG2-associated antibody deficiency and immune dysregulation (APLAID) is an autosomal dominant autoinflammatory disease characterized by episodic skin, musculoskeletal, ophthalmic and gastrointestinal tract symptoms. Here we report an 11-year-old girl with a history of repeated episodes of fever, myalgia, arthralgia, abdominal pain, and urticarial rash in the trunk and limbs. Chest and pelvic X-Ray, sacroiliac joints MRI, brain MRI and abdominal CT scan were normal. Anti-nuclear antibody, Rheumatoid factor, cryoglobulin, ANCA/PR3, p-ANCA/MPO, anti-smooth muscle antibody and anti-mitochondrial antibody were negative. Serology for cytomegalovirus, Epstein-Barr, hepatitis B, hepatitis C, and HIV viruses was negative. Serum immunoglobulins were in the normal range. Genetic analysis for familial Mediterranean fever syndrome was negative. Whole exome sequencing was carried out to identify the genetic cause of our patient. We identified a homozygous missense variant (c.579C>G, p. His193Gln) in exon 7 of the *PLCG2* gene. Bioinformatic analysis and clinical symptoms suggests this variant to be pathogenic in the homozygous state for APLAID and thus probably acting in an autosomal recessive manner. Our bioinformatic analysis also showed this novel mutation to have detrimental effects on the 3D structure of the PLCG2 protein, which is well conserved among many other similar species.

Keywords: Autoinflammation; APLAID; Genetic variant; Next-generation sequencing

1. Introduction:

Traditionally, inflammation is categorized as a nonspecific defense mechanism of the human body. This classification can be true for acute inflammation which involves vital organs, but it is incorrect for chronic inflammation involving many parts of the body and is a component of the immune response [1,2]. In 1999, Daniel Kastner and colleagues defined a group of hereditable monogenic conditions characterized by recurrent episodes of sterile inflammation including fever, joint pain, skin lesions and features of disease-specific pattern of organ inflammation, without evidence of autoantibodies or antigen-specific T cells, caused by aberrant regulation of innate immune cells, mostly neutrophils and macrophages, and proposed the term of autoinflammatory diseases (AIDs) for them [1-3]. This concept revealed that autoinflammatory disorders are phenotypically diverse and without genetic analysis, many of these disorders might have gone unrecognized [4].Later, with the arrival of powerful genetic tools, scientists demonstrated that autoinflammatory diseases can be monogenic with Mendelian inheritance, genetically complex or even non-genetic [4, 5]. Autoinflammatory diseases have many shared characteristics with autoimmune diseases, like involving multiple organs of the body and excessive activity of the immune system. However, autoinflammatory diseases arise from disorders of the innate immune system, while dysregulation of both innate and adaptive immunity have a role in the pathology of autoimmune diseases [6-8]. Autoinflammatory diseases are typically present in early childhood and their correct diagnosis, which can help to discover new targeted treatments, relies on the physicians' awareness especially in monogenic types [9]. Due to the rarity of these diseases in the population and the lack of large multi-case families, advanced techniques are required to diagnose them precisely [10, 11].

Using Whole exome sequencing, we have investigated an Iranian- Azeri girl with various defects in multiple organs in the hope that our findings give assistance to reach a better understanding for the pathogenicity of this disease.

2. Materials and Methods

2.1 Patient:

An 11-years-old girl was referred to Tabriz Genetic Analysis Centre (TGAC) for evaluation of repeated episodes of fever, myalgia, arthralgia and abdominal pain. Members of the studies family, consisting of the proband, mother, and father, provided informed consent for themselves and their child for enrollment in this program, to identify the molecular cause of the patient's phenotype. Her disease presented 3 years ago with periodic abdominal pain every 1-2 weeks which lasted 24- 48 hours. Attacks sometimes associated with vomiting and/or fever. After 8 months she developed attacks of arthralgia and myalgia every 1-2 weeks. She also reported episodic urticarial rash in the trunk and limbs. After 3 months she developed episodes of temporal and parietal headache every 2-3 days which lasted 24 hours accompanied by nausea and photosensitivity. Her physical examination during the attacks showed mild and generalized tenderness in the abdomen and the left shoulder, wrists along left MTP joints arthritis. Ophthalmologic examination was normal. Treatment with colchicine 1-2 mg/d was ineffective in the control of the attacks. With the diagnosis of juvenile rheumatoid arthritis, she was treated with prednisolone 5-7.5 mg/d and methotrexate 10-15 mg/week and then Etanercept 25 mg/week for one year without success.

Chest X-ray, pelvic X-Ray, sacroiliac joints magnetic resonance imaging (MRI), brain MRI and abdominal CT scan were normal. Upper and lower gastrointestinal endoscopy and biopsy showed mild esophagitis, mild gastritis and mild eosinophilic infiltration in the lamina propria of the colon.

The laboratory data were as follows: White blood cell count, $5.110\times10^3/\mu L$ (Neutrophils: 72%, Lymphocyte: 25%, Eosinophils: 2%, Basophils: 1%); Hemoglobin, 13.6mg/dL; erythrocyte sedimentation rate, 22mm/h; C-reactive protein 8.7mg/L (normal $\leq$10). Urea, creatinine, urine analysis, liver function tests and electrolytes were normal. Muscle enzymes were in normal ranges. Blood cultures at the episodes of fever were negative.

Anti-nuclear antibody, Rheumatoid factor, cryoglobulin, ANCA/PR3, p-ANCA/MPO, anti-smooth muscle antibody and anti-mitochondrial antibody were negative. Serology for cytomegalovirus, Epstein-Barr, hepatitis B, hepatitis C, and HIV viruses was negative.

Serum immunoglobulins including IgG, IgM, IgD, IgA and IgE were in the normal range. Genetic analysis for familial Mediterranean fever syndrome was negative.

According to episodes of abdominal pain, myalgia, arthritis, urticarial skin lesions and headaches lasting 2-3 days and repeated every 1-2 weeks, lack of autoantibodies and lack of response to colchicine, the possibility of periodic fever syndromes, like Tumor Necrosis Factor Receptor Associated Periodic Syndrome (TRAPS), Hyperimmunoglobulin D syndrome (HIDS) and Mevalonic aciduria (MVA) were suspected. But, genetic analyses for *MEFV* and *MVK* genes were negative respectively.

Due to the consanguineous marriage of parents and her uncharacterized systemic inflammatory disease, performing a comprehensive genetic analysis to identify the illness and its possible causative mutations seemed to be plausible. The family pedigree is presented in Figure 1. To achieve the goal, whole exome sequencing was performed in proband with an average on target coverage of 100X using DNA obtained from peripheral blood by a standard salting-out extraction method.

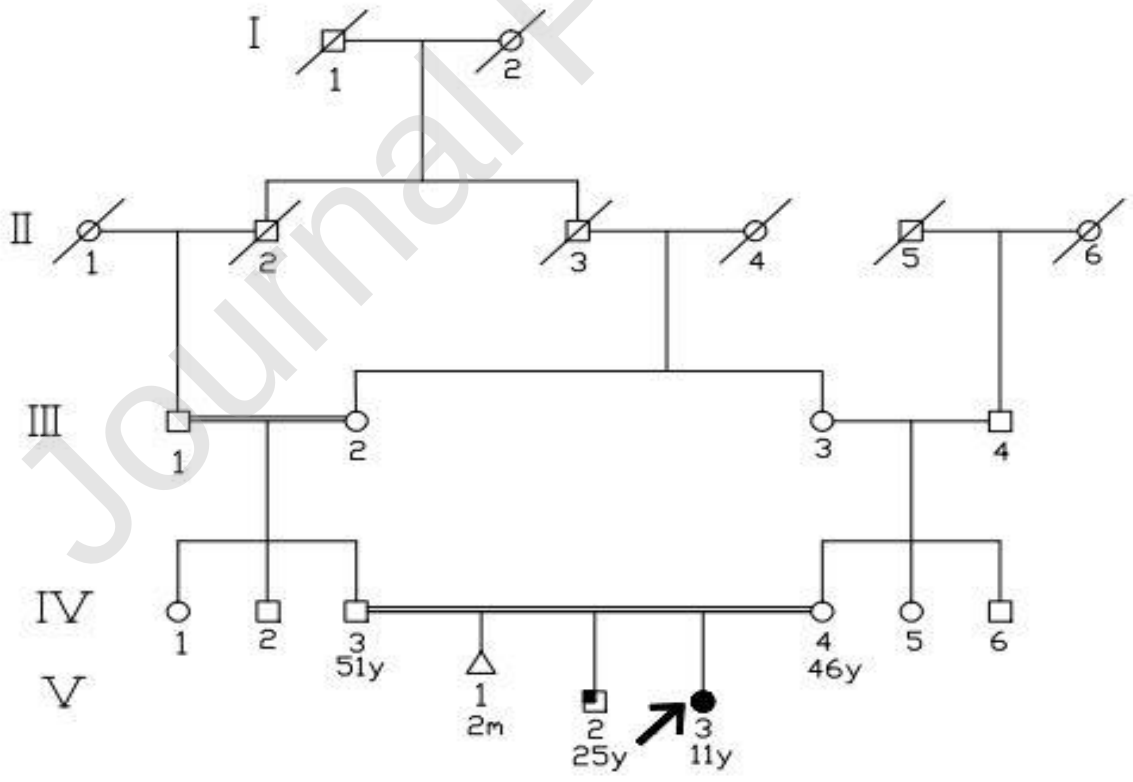

Fig 1. Pedigree of family tree. The filled black symbol indicates unknown physical impairments. All of the white symbols are healthy. The marked square symbol (V:2) has a physical-mental problem. Deceased individual is marked by a slash. The arrow is the proband of the family. Numbers below each symbol indicates the number and age (y: years, m: months). The source of DNA is from blood.

2.2 Whole-Exome Sequencing

The coding and flanking intronic regions were enriched using the in-solution technology from Agilent (SureSelectXT Human All Exon V6) and were sequenced using the Illumina HiSeq 4000 system at CeGaT GmbH, Germany. Fastq files obtained from CeGaT were uploaded to SEQ, a cloud-based variant analysis platform by Genomize Inc., and were processed using analysis version 16.1 (BWA-Freebayes-PCR dedup).

For pathogenicity assessment of 435,796 single nucleotide polymorphisms and indels, we applied a multistep process (Figure 2) starting from analyzing all 9,826 exonic and splice region variants. We filtered out variants with population allele frequencies of more than 5 percent in all normal population to 4,390 rare variants. Public databases including the 1000 Genome Project (1KGP) (2500 samples; http://www.1000genomes.org), the Exome Variant Server (ESP) (6500 WES samples; http://esv.gs.washington.edu/ESV/) and the Exome Aggregation Consortium (ExAC) database (61,468 multiethnic individuals; (http://exac.broadinstitute.org/) were used for normal population allele frequencies along with our own in-house database of Iranian-Azeri allele and variant frequencies compiled using SEQ (total of 232 Iranian-Azeri WES samples). In the next step, we excluded all synonymous variants, keeping all synonymous variants altering the splice site features. Owing to the phenotypic symptoms present in the patient, we decided to focus primarily on the *in silico* gene panel for auto-inflammation and vasculitis based on genes list in Supplementary data 1[12]. This panel includes 113 genes for vasculitis and inflammation panel version 1 and 166 genes for the later version covering aortopathies, diseases associated with intestinal inflammation, Autoimmune lymphoproliferative syndrome (ALPS) and related disorders, Autoinflammatory disease, Complement and regulatory protein deficiencies, Vasculopathic Ehlers-Danlos syndrome, Haemophagocytic lymphohistiocytosis (HLH), Hereditary amyloidosis, Paediatric stroke, SLE and Aicardi-Goutieres syndrome and Vasculitis/vasculopathy diseases. After applying the filter for our patient, 24 variants were selected for further analysis. Simultaneously, we checked all metabolic and immunodeficiency disorders in the patient by analyzing relative gene panels

including 638 genes for epilepsy, metabolic and brain development disorders and 326 genes for blood and immune disorders (Supplementary data 2).

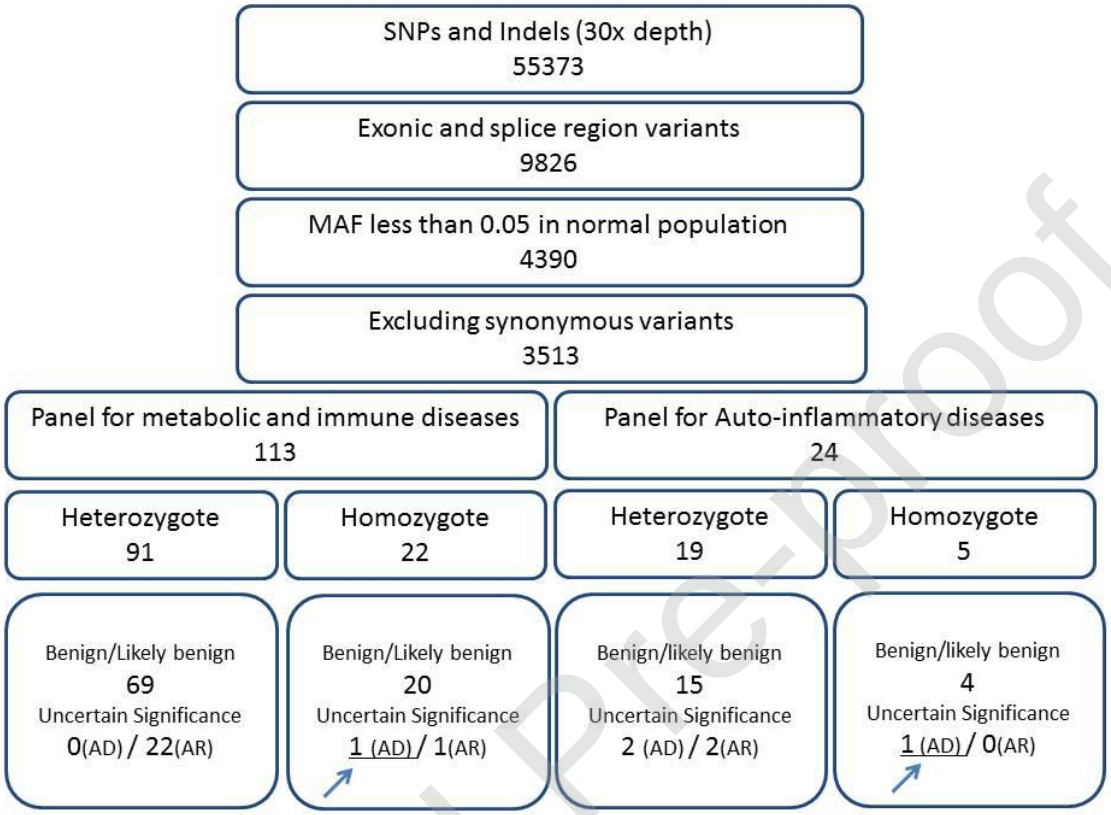

Fig 2. Scheme of workflow for whole exome sequencing data analysis. The numbers below each chart indicate the number of checked variants. The AD and AR represent the Autosomal Dominant and Autosomal Recessive inheritances, respectively.

2.3 Sanger sequencing validation and co-segregation analysis

Validating WES results and co-segregation analysis of resulted mutation in proband and parents, PCR product of *PLCG2* gene exon 7 were amplified using following primers F: TCAAAAGCCATGACACTGAA/ R: CTCATTTATGTTTCCTTGACT and sequenced by ABI 3500.

2.4 Bioinformatics analysis

SWISS-MODEL were used to analyze the effect of identified mutation on 3D structure of protein[13] and NCBI's Conserved Domain Database (CDD) were used to identify involved domains of the protein [14]. To analyze conservation of PLCG2, DNA and protein sequence of Human and other similar species were extracted from Ensemble 98 database and inserted to Clustal Omega multiple sequence alignment program[15] and alignment results is depicted using Jalview Version 2 program [16]. Also, polyphen-2 [17] and Sorting Intolerant from Tolerant (SIFT)[18] software were used to predict functional effect of mutations.

3. Results

3.1 WES analysis of a patient

In total, 137 homozygote and heterozygote variants obtained after applying these mentioned gene panels, were selected for further pathogenicity assessment. The identified variants were individually classified into pathogenicity groups (Class 1: benign; Class 2: likely benign; Class 3: unknown significance; Class 4: likely pathogenic; Class 5; pathogenic), according to the American College of Medical Genetics (ACMG) guideline for Interpretation of Sequence Variants [19]. No pathogenic or likely pathogenic variant were detected according to these criteria. We determined 24 homozygote and 84 heterozygote variants classified as “Benign” and “Likely Benign”. 29 variants with uncertain significance were selected for the next step. Heterozygote variants causing diseases with autosomal recessive inheritance were omitted from further analysis. Finally, one homozygote missense variant (c.579C>G, p.His193Gln) in Exon 7 of *PLCG2* (ENST00000359376.3), one heterozygote variant (c.320+2T>G) in intron 5 splice donor site of *DNASE1* (ENST00000407479.1) and another heterozygote missense variant (c.3568C>T, p.His1190Tyr) in exon 22 of *NOTCH1* (ENST00000277541.6) remained as candidates. The pathogenic variants in *NOTCH1* are known to cause Adams-Oliver syndrome 5 (MIM: 190198) with autosomal dominant inheritance. Since the clinical symptoms of Adams-Oliver syndrome 5 are completely different from our patient, this variant was also omitted from the rest of the analysis. As previously described by Zhou *et al.*, 2012, missense mutations in *PLCG2* can result in a dominantly inherited autoinflammatory disease with immunodeficiency [11]. Mutations in *DNASE1* possibly result in susceptibility to systemic

lupus erythematosus (MIM: 152700) in an autosomal dominant manner. No pathogenic, likely pathogenic and VUS variant with X linked inheritance were detected in this study.

3.2 Validation by Sanger sequencing

Direct Sanger sequencing was carried out for the proband and her parents to validate the variant. This approved the co-segregation of this variant in the family and rejection of the possibility of being a *de novo* variant of that. It was revealed that both parents were heterozygous for this variant but none of them have shown any clinical symptoms (Figure 3).

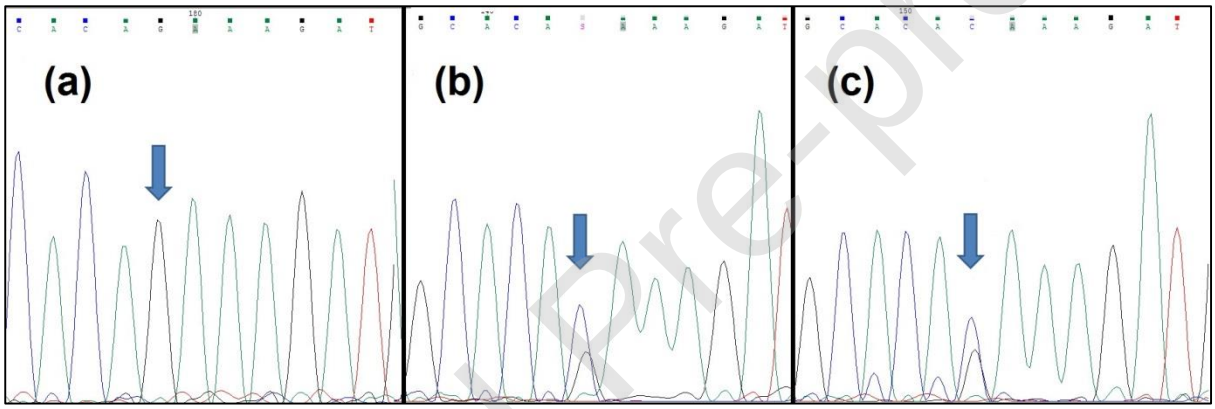

Fig 3. Sanger sequencing validation of mutation discovered by NGS in exon 7 of *PLCG2* gene (c.579C>G, p.His193Gln). The chromatograms successively show the sequences of this mutation relative to proband (a), her father (b) and her mother (c). The position of nucleotide substitution is indicated by an arrow.

3.3 Bioinformatics analysis

As represented in figure 4, identified substitution has been resulted in structural change in PLCG2 protein. Based on amino acid sequence of PLCG2, This protein is composed of multiple domains including: Pleckstrin homology-like, EF hand, Catalytic domain of phosphoinositide-specific phospholipase C-like phosphodiesterases, Src homology 2 (SH2),

Src Homology 3 (SH3) and C2 domain which The p.His1190Tyr substitution is located in EF hand domain of PLCG2 protein (Figure 5).

Alignment results indicates that protein and DNA sequence of PLCG2 are well conserved among human and other close species and all subjects had similar sequence to wild type in humans (Figure 6 and Figure 7).

Also, in silico analysis of the missense mutation found in this case predicted a benign affect according to PolyPhen and tolerated according to SIFT.

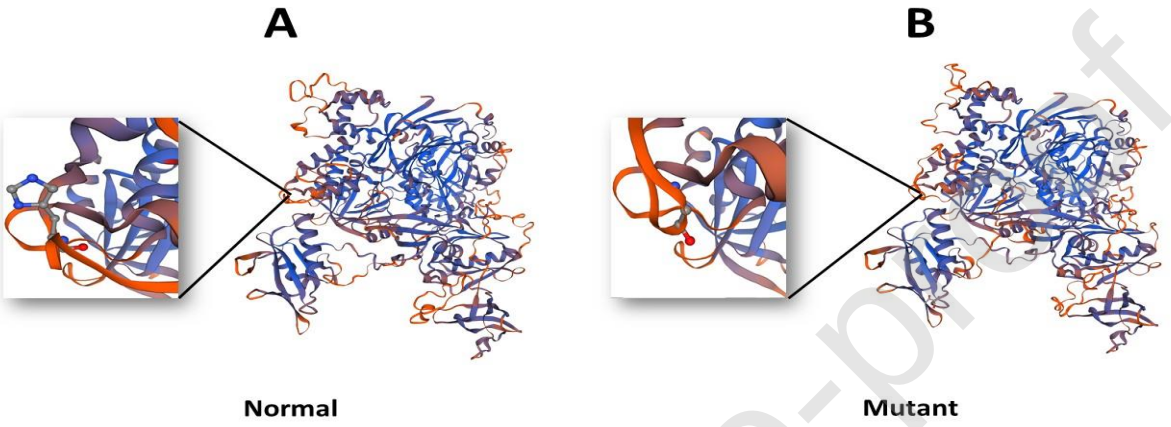

Fig 4. 3-D structure of the PLCG2 protein; A) normal structure and B) mutant structure (p. His1190Tyr)

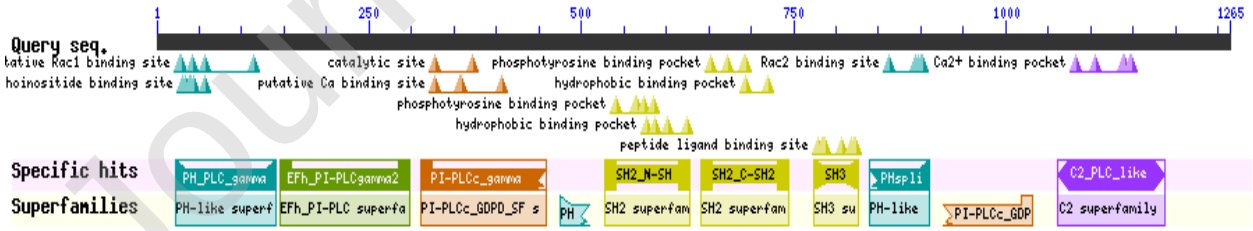

Fig 5. PLCG2 protein domains

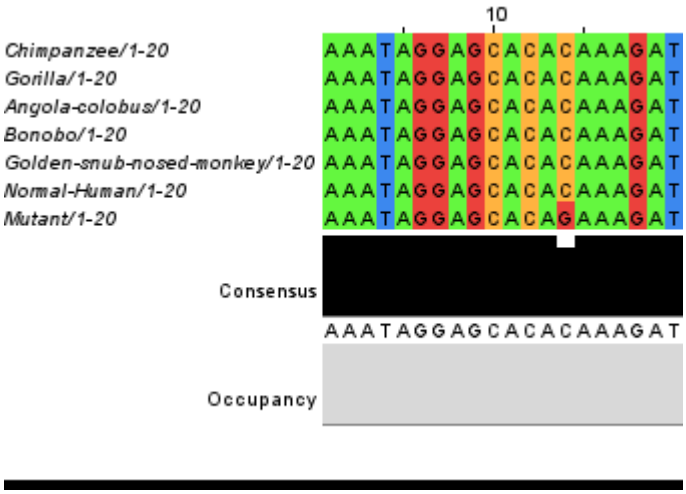

Fig 6. PLCG2 DNA sequence alignment

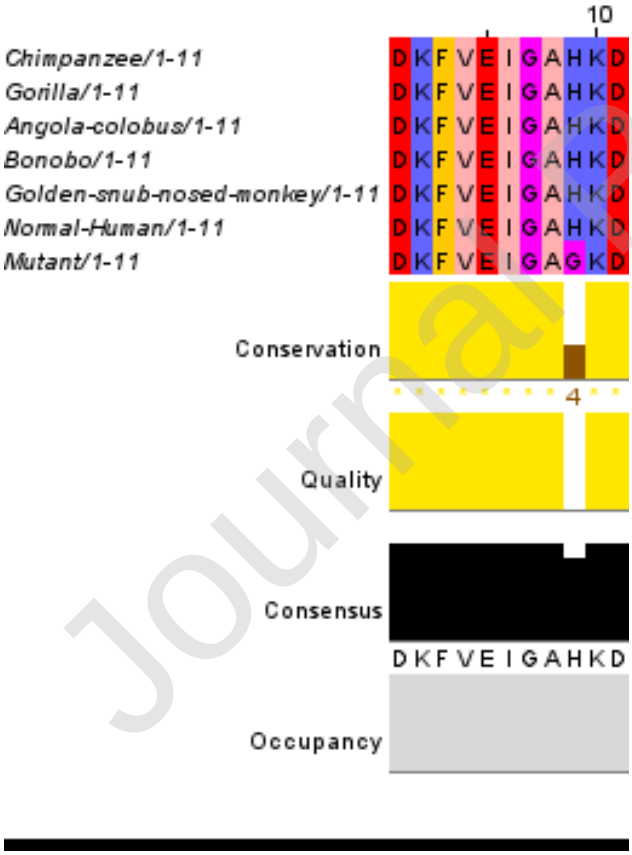

Fig 7. PLCG2 protein sequence alignment

4. Discussion and conclusions

APLAID is a rare autoinflammatory disorder, with a difficult diagnosis process in children. It was first reported by Zhou and his colleagues in 2012 in a father and his daughter, both suffered from early-onset recurrent skin inflammation and granulomata, nonspecific interstitial pneumonitis with respiratory bronchiolitis (NSIP), arthralgia, eye inflammation, enterocolitis, cellulitis, and mild immunodeficiency [11]. Similar to our patient's symptoms, cold urticaria was not observed in APLAID patients while it is a common symptom in PLAID (PLCG2-associated antibody deficiency and immune dysregulation) patients.

PLCG2 encodes phospholipase Cg2 (PLCg2), an enzyme responsible for ligand-mediated signaling in cells of the hematopoietic system with a critical regulatory role in various immune and inflammatory pathways [20]. Previous studies by Ombrello et al demonstrated that genomic heterozygous intragenic deletions in the *PLCG2* gene can cause a dominant variant of the PLAID disease [21]. However, it was identified by Zhou *et al*, that a heterozygous missense mutation (c.2120C>A, p.Ser707Tyr) in exon 20 of *PLCG2* gene causes APLAID [11].

Here, we report a missense mutation (c.579C>G, p.His193Gln) in exon 7 of *PLCG2* gene which was not reported at the time of our study; but recently a study has considered it as a benign variant for familial cold autoinflammatory syndrome 3 [16]. Nevertheless, clinical evidence and expert physicians confirmed the co-segregation of proband symptoms with APLAID syndrome. This is while WES analysis did not show any known pathogenic or other exonic variants (population allele frequencies less than 5) in proband. Besides, parents of the proband are heterozygous without any manifestation of the disease despite the dominant inheritance of this syndrome.

Our bioinformatics analysis indicated that the structure of PLCG2 mutant protein is significantly different compared to normal protein which may crucially affect the protein function (figure 4). Also, based on sequence alignment results, PLCG2 is well conserved among close species to human, so it has fundamental role in cell and any disruption may result in malfunction protein. Moreover, identified amino acid substitution is located in EF hand domain of PLCG2 protein which has important role in calcium fluctuation of the cell [22]. Based on important role of Ca^{2+} ion in inflammation and its related disorders[23, 24], this

substitution may affect the normal Ca^{2+} ion fluctuation in cell and further studies about role of Ca^{2+} ion and this syndrome is required. In the current identified missense mutation, a nonpolar amino acid (Glycine) is replaced with a polar amino acid (Histidine) which may significantly affect the protein structure [25, 26]. Interestingly predicted effect of our mutation using PolyPhen and SIFT, are in discordance with previous mentioned bioinformatics analysis, demonstrates additional functional studies about pathology of our novel mutation is still required.

In summary, based on physicians' diagnosis and genetic analysis of the family members and bioinformatic study on recognized mutation depicting detrimental effect of this mutation on PLCG2 protein, we state the probability of pathogenicity of missense variant (c.579C>G, p. His193Gln) in exon 7 of *PLCG2* gene for APLAID in the homozygote state. Also, we suggest the probability that APLAID can act in an Autosomal Recessive manner in this family and maybe we can extend this claim to the pathogenicity of disease as a whole. Nevertheless, this hypothesis will need further research in other families accompanied by molecular research.

5. Acknowledgments

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