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RESEARCH

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Novel homozygous CARD11 variants in two patients with combined immunodeficiency and atopic skin disease

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Abstract

Background Caspase recruitment domain family, member 11 (CARD11) is an important protein which plays a fundamental role in the activation of NF-κβ pathway in lymphocytes. CARD11 deficiency can be inherited in either autosomal dominant or autosomal recessive forms and present with different phenotypes including combined immunodeficiency, atopic dermatitis, and other variable manifestations. The present report describes clinical phenotypes and immunological defects of two unrelated patients with missense homozygous variants in CARD11 presenting with combined immunodeficiency (CID) and atopic skin disease resembling that reported in dominant negative CARD11 deficiency. The patients underwent next generation sequencing, immunophenotyping of T and B subsets by flow cytometry, T cell stimulation, and evaluation of CARD11 expression.

Results Both patients had features suggesting CID including repeated pneumoniae with ICU admissions, chronic diarrhea, and itchy atopic skin disease. Patient-1 has homozygous missense variant in the C terminal domain (c.2839G > A, p.Glu947Lys), and patient-2 has homozygous variant in the inhibitory domain (c.1073C > G, p.Pro568Arg). Both have profound defects in Tregs with normal recent thymic emigrants, memory, and naïve CD4+T cells. However, in response to stimulation, T cells failed to upregulate the expression of CD25. CARD11 expression by flow cytometry was decreased rather than abolished as previously described in patients with autosomal recessive CARD11 deficiency. B cells showed marked deficiency of switched memory and increase in transitional B cells.

Conclusion Missense variants causing CARD11 deficiency may affect the protein function rather than the expression and can result in a phenotype combining the atopic skin disease and the features of CID.

Keywords CARD11, Combined immunodeficiency, CMB-complex, Loss of function

Introduction

Caspase recruitment domain family, member 11 (CARD11) is an important, 1154-amino acid protein which plays a crucial role in connecting antigen recognition with downstream activation of the NF-κβ pathway in lymphocytes [1]. CARD11 associates with B-cell leukemia/lymphoma 10 (BCL10) and Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) to form a complex called CBM. The CBM complex is basically involved in signal transduction of TCR and BCR leading to the activation of the IκB Kinase (IKK)/

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NF-κβ, c-Jun N-terminal kinase (JNK), and mechanistic target of rapamycin complex (mTORC1) pathways in lymphocytes [2–5]. The CARD11 protein consists of an N-terminal caspase recruitment domain, LATCH, coiled-coil domains, inhibitory domain (ID) and a C-terminal membrane-associated guanylate kinase domain comprised of PDZ, SH3 and GUK domains [2]. Pathogenic variants in CARD11 have been associated with a wide spectrum of phenotypes [3]. Germline heterozygous gain-of-functions (GOF) pathogenic variants in CARD11 manifest as lymphoproliferative disorder named BENTA (B cell Expansion with NF-κB and T cell Anergy) [4, 6, 7], whereas somatic GOF CARD11 variants are commonly detected in non-Hodgkin Lymphoma [2]. Biallelic loss-of-functions (LOF) pathogenic variants of CARD11 result in profound combined T and B-cell immune deficiency, while dominant negative heterozygous LOF were reported in patients with variable manifestations similar to those described in STAT3-deficiency, DOCK8 deficiency, cutaneous viral infections, inflammatory bowel disease, and common variable immune deficiency (CVID) [2, 8, 9]. Several reviews have described the importance of CARD11 and the CBM complex in lymphocytes' function and development, highlighting the effect of their deficiencies on the immune system [10, 11]. However, few described the clinical phenotypes of AR LOF of CARD11 [7]. To date, only eight cases have been reported with AR CARD11 deficiency [2, 10, 12–14]. Affected individuals present early in life with manifestations of combined immunodeficiency including P.jirovecii pneumonia, CMV persistent infection, enteropathy, and Omenn-like features. Pathogenic variants reported to result in LOF CARD11 were mostly nonsense variants in 6 patients with premature stop codon and truncated protein while one patient had complete exon 21 deletion [2, 10, 13]. Recently, a patient with inflammatory skin condition was reported to have AR CARD11 deficiency due to a missense variant [14]. Herein, we describe 2 patients with novel missense variants in CARD11. Both patients presented with CID phenotype associated with atopic skin disease, and T and B cells defects.

Patients and methods

Study participants and consents

All investigations were conducted after obtaining informed written consents from the patients' parents. The study was approved by the institutional review board, according to the Declaration of Helsinki. Two patients with manifestations of CID were included in the study.

Serum immunoglobulin levels quantitation

Serum immunoglobulins (IgM, IgG, IgA) were quantitated by nephelometry (Omlipo, Goldsite), while ELISA was used to evaluate serum IgE (General Biologicals Corporation, ref. 4S00062).

Flow cytometry and lymphocytes immunophenotyping

Immunophenotyping of lymphocytes was performed by FCM (BD FACS CANTO-II) on EDTA-peripheral blood samples collected from patients and controls as previously described by Meshaal et al. [15]. The monoclonal antibodies utilized were: anti-CD3(PE-TR), anti-CD4 FITC, anti-CD8 PE, anti-CD19 PE-Cy5.1 and anti-CD56 PE-Cy7. Naïve and memory T cells were assessed using anti-CD45RA FITC, anti-CD45RO PE, anti-CD4 PE-Cy7. Anti-CD31 PE was used as a marker of recent thymic emigrants (RTE). Anti-CD25 PE and anti-Foxp3 Alexa Fluor®647 antibodies were used for Tregs quantification following permeabilization and fixation by Beckman Coulter IM2389 IntraPrep permeabilization Reagent according to manufacturer. B cells subsets were assessed by anti-CD27 PE and anti-IgD FITC, anti-CD10 Alexa Fluor®647, anti-CD24 Alexa Fluor®647, and anti-CD38 Alexa Fluor®647. All monoclonal antibodies were purchased from Beckman Coulter (Kendall, FL) and BD Biosciences.

CD25 Expression in response to in vitro stimulation

Five ml of peripheral blood was withdrawn on Na-heparin from both patients as well as age and sex-matched controls. Peripheral blood mononuclear cells were separated by density gradient centrifugation using Ficoll hypaque. PBMCs (1×10^6/ml) were incubated in 1 ml of phosphate-buffered saline containing 2% fetal calf serum, either without stimulation or with stimulation by 5 μM Ca+2 ionomycin (ab120116) at 37 °C for 24 h. The expression of CD25 was assessed on CD4+ T cells before and after stimulation.

CARD11 expression

EDTA peripheral blood was collected from both the patients as well as age matched controls to assess CARD11 expression by FCM. 50 μl EDTA-blood was permeabilized and fixed by Beckman Coulter IM2389 IntraPrep permeabilization Reagent according to manufacturer, followed by staining with rabbit polyclonal anti-CARD11 antibody (Cell signaling, Cat.4440s) then incubation for 2 h. Cells were then washed and goat Alexa-flour488-conjugated anti-Rabbit IgG (Abcam, Ab150077) was added and incubated for 30 min. Excess unbound antibodies were washed. Cells only incubated with anti-rabbit IgG were used as an isotypic control. At least 30,000 events were acquired on the FCM for analysis.

Genetic testing

DNA was extracted from peripheral blood using QIAamp DNA Blood Mini Kit (Qiagen). Targeted next-generation sequencing (NGS) was done using a designed panel of 55 genes involved in CID and B cell/Antibody deficiencies. The panel was designed based on the international union of immunological societies' classification of inborn errors of immunity [16] (Additional file 1). Since CID constitutes a group of rare genetic disorders, only variants having an allele frequency of < 1% were analyzed. The panel was designed to cover all the exons, exon–intron junctions, as well as the UTRs. Detected variants were confirmed with Sanger sequencing. To predict the functional effects of amino acid substitutions we used: Polymorphism Phenotyping version 2 software tool (PolyPhen-2), sorting intolerant from tolerant (SIFT) software, Combined Annotation Dependent Depletion (CADD), Functional Analysis through hidden Markov Model (V2.3) (FATHMM-XL). The reported variants' clinical significance and the genotype–phenotype correlation were evaluated by clinically relevant variant (ClinVar) database, VARSOME and protein variation effect analyzer (PROVEAN). Variants' frequencies were checked in population databases (the Genome Aggregation database (gnomAD), ExAC, dbSNP and 1000G). The effect on splicing was assessed by Genomnis Human splicing Finder (HSF-Pro).

Results

Clinical and immunological features

Patient-1

Is a female patient born to first-degree consanguineous parents, who are cousins from both sides. This family had a history of two previous sibling deaths: one female sib died at age of 18 days following Neonatal intensive Care Unit (NICU) admission due to severe respiratory distress; and one male sib died at age of 14 months with pneumonia and had a history of exfoliative dermatitis, draining ears and elevated serum IgE. Additionally, 4 maternal uncles died at ages between 4 and 18 months due to infections. She was admitted to NICU at the age of 16 days with respiratory distress that required mechanical ventilation. Since the age of 2 months, she suffered generalized itchy exfoliative erythematous rash complicated with cellulitis in the anterior abdominal wall, persistent diarrhea, failure to thrive, recurrent oral thrush, and diaper rash. She received a blood transfusion once at the age of 2 months due to marked anemia. She received regular IVIG since the age of 8 months with clinical improvement. At the age of 2 years, the patient developed very itchy erythematous dermatitis which improved significantly after the IVIG. At the age of 27 months, she had warts affecting the area behind the ears and the buttocks. The patient had low levels of serum immunoglobulins IgG and IgM, while the levels of IgA and IgE were normal. She has one living 10-year-old brother who complains of eczema with marked lichenification of the palms and soles; as well as the uncle (father's twin) with a similar complaint as the brother. Both, the father, and mother have no complaints. RAG sequencing was done which revealed no pathogenic variants. Also, DOCK8 expression was assessed with normal protein level.

Patient-2

Is a male patient born to first-degree consanguineous parents, he presented to the PID clinic at the age of 8.5 years with pneumonia. He has two healthy siblings: a 12-year-old female and a 15-year-old male. Since the age of 18 months, the patient has been hospitalized several times because of repeated pneumoniae; including pneumonia complicated with pleural and pericardial effusion at the age of 6 years. Additionally, he has a history of recurrent skin boils and superficial abscesses. Currently, he has atopic skin disease with significant lichenification of the palms and soles. The patient has normal serum immunoglobulins. DOCK8 expression was assessed and was normal.

Lymphocytes subsets

Detailed T and B lymphocytes subsets percent and absolute counts are shown in Table 1.

T cells subsets and CD25 expression

Both patients had markedly decreased Tregs compared to the age and sex matched controls and to the reference for age. Patient 2 has high memory T-helper cells (CD4+CD45RO+) percent and absolute count. Both patients had normal RTEs and naïve T-helper cells. In response to in vitro stimulation, T cells from both patients failed to upregulate the expression of the activation marker CD25 compared to the age and sex matched controls. Gated on CD4+ cells: CD25 expression in the control was 9% versus 13% following the in vitro stimulation, while for patients (1&2), CD25 expression was 2% before stimulation and 2.6% and 2.8% after the stimulation, respectively.

B cells subsets

Both patients had low CD19+B cells percents, while patient-2 had low B cells absolute count as well. Switched memory B cells (CD27+IgD−) were markedly decreased in both patients with expansion of naïve B cell compartment (CD27-IgD+) in terms of percent and absolute count (Table 1). Transitional B cells (TrB cells) were identified by lack of expression of CD27 with high expression

Table 1 Peripheral blood Lymphocytes immunophenotyping of the patients:

	Pt 1 (Ref range for pt 1: 2.2 yrs) Abs no (×10^3/ml) %		**Pt 2 (Ref range for pt 2: 9.5 yrs) Abs no (×10^3/ml) %**	
WBCs	12.9 (6.00–10.8)		15.5 (5.7–9.9)	
Lymphocytes	5.74 (2.3–6.9)	44.5 (29.6–69.2)	10.2 (2.3–6.9)	**66** (29.6–49.8)
Eosinophils	0.7 (0–0.2)	6 (1–4)	2.33 (0–0.2)	15 (1–4)
CD3+T cells	4.2 (1.40–3.70)	73.3 (56–75)	9.5 (1.20–2.60)	**93** (60–76)
CD3+CD4+	2.94 (0.70–2.2)	51.3 (28–47)	1.42(0.65–1.5)	**14** (31–47)
CD3+CD8+	1.12 (0.49–1.3)	19.6 (16–30)	5.8 (0.37–1.1)	57 (8–35)
CD3−CD56+	0.44 (0.13–0.72)	7.6 (4–17)	0.27 (0.10–0.48)	2.7 (4–17)
Tregs (CD4+CD25+FoxP3)	**0.006** (0.039–0.15)	**0.2** (3–17)	**0.007** (0.02–0.27)	**0.5** (4–14)
Gated on CD4:				
CD31+CD45RA+	2.05 (0.19–2.6)	69.9 (73–100)	0.49 (0.2–1.7)	**33** (41–81)
CD45RO+	0.37 (0.22–0.66)	12.5 (9–26)	**0.92** (0.23–0.63)	**65** (20–46)
CD45RA+	**2.5** (0.43–1.5)	84.8 (53–86)	0.5 (0.32–1)	**35** (46–77)
CD19+	0.63 (0.4–1.7)	**11** (14.1–28.5)	**0.23** (0.3–0.6)	**2.3** (9.7–23.7)
Gated on CD19				
CD27+IgD−	**0** (0.02–0.22)	**0** (1.5–4.1)	**0.0046** (0.04–0.14)	**1.5** (5·2–12·1)
CD27+IgD+	**0.0012** (0.038–0.09)	**0.2** (4.2–6.9)	**0.0023** (0.02–0.1)	**1** (7·5–12·4)
CD27−IgD+	0.58 (0.57–1.33)	**92.3**(83.4–90.1)	**0.196** (2.3–11)	**85** (69·4–80·4)
CD27−IgD−	0.047 (0.01–0.08)	7.5 (1.6–3.6)	0.037 (0.01–0.07)	**16** (3·5–6·6)
CD27−CD10+	**0.29**	**47.2** (4)	**0.04**	**18.9** (4)
CD27−CD24++	0.6	**94** (5.1–10.7)	**0.16**	**70.8** (3·9–7·8)
CD27−CD38++	**0.59**	**93.5** (5.7–13)	**0.18**	**79.9** (5.2–12.7)
Serum Immunoglobulins:				
IgM	**19.7** (37–184) mg/dL		201 (38–251) mg/dL	
IgG	**<150** (295–1156) mg/dL		616 (462–1,682) mg/dL	
IgA	43.2 (27–246) mg/dL		278 (34–274) mg/dL	
IgE	14 (≤ 97) U/L		86 (≤ 696) U/L	

Reference range for B cells from Morbach et al. [26], reference for T cells from [27, 28]

of CD24 and CD38 [17]. CD19+CD38++CD27− and CD19+CD24++CD27− were significantly higher in the patients compared to the controls and the reference range for age (Table 1, Fig. 1).

CARD 11 expression by flow cytometry

Gated on total lymphocytes and CD3+T cells, both patients showed slightly dim expression of CARD11 when compared to the control by flow cytometry. Gated on CD3+T cells Mean fluorescence intensity of CARD11 was 9300, 7900, 7800 in the control, patient 1 and patient 2, respectively (Fig. 2).

Novel CARD11 variants

A novel missense variant in CARD11 (NM_032415.7, Chr7:2954871:C > T, c.2839G > A, p. Glu947Lys) [18] was detected in homozygous form in patient 1. Sanger sequencing was done to confirm the variant and was detected in heterozygous form in both parents, her sib and uncle (Fig. 3). This variant was predicted VUS by Varsome, damaging by FATHMM-MKL, disease causing by MutationTaster, deleterious by CADD (PHRED score 32), neutral by PROVEAN, benign by Polyphen-2 and tolerated by SIFT. The variant is not present in gnomAD, ExAC, dbSNP and 1000G. The variant lies at intron–exon border, 2 bases from the splicing site and predicted to affect the splicing with new acceptor/donor site by HSF-Pro.

Patient 2 has a novel homozygous missense variant in a highly conserved region in the inhibitory domain (ID) (NM_032415.7, c.1073C>G, p.Pro568Arg) [18]. The variant was confirmed by Sanger sequencing and was detected in heterozygous form in both parents and absent in his healthy sister (Fig. 3). This variant was predicted VUS by Varsome, damaging by FATHMM-MKL, disease causing by MutationTaster, deleterious by CADD (PHRED score 25.1), deleterious by PROVEAN, probably damaging by Polyphen-2 and damaging by SIFT. The variant is not present in gnomAD, ExAC, dbSNP and 1000G.

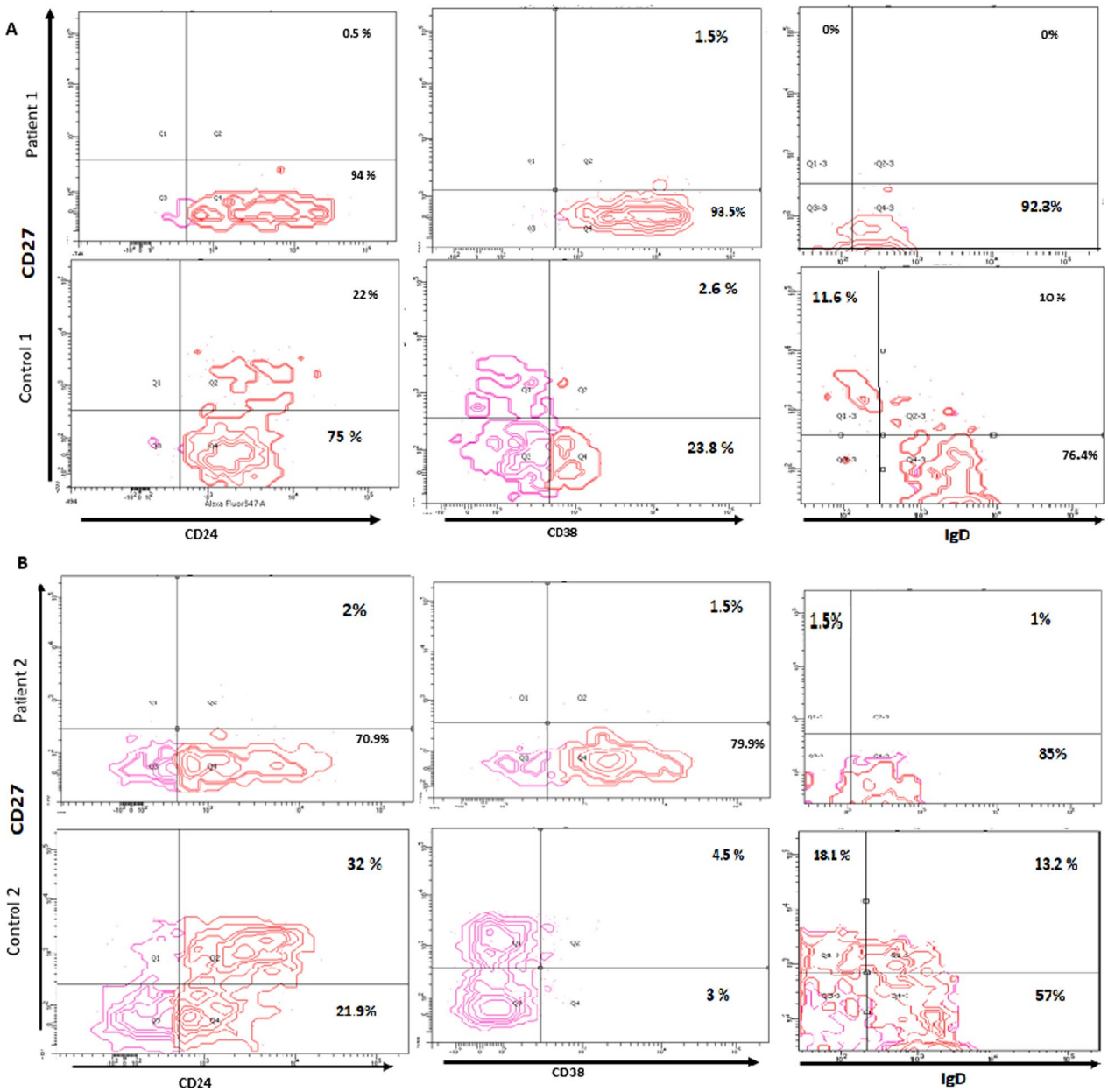

Fig. 1 Immunophenotyping of B cells subsets: **A** Patient-1 versus age matched control: Gating on B cells from the patient shows increased CD27−CD24+, CD27−CD38++, CD27-IgD+ subpopulations and absent switched memory CD27+IgD−B cells. **B** Patient-2 versus age matched control: Gating on B cells from the patient shows increased CD27−CD24+, CD27−CD38++, CD27−IgD+ subpopulations and markedly decreased switched memory CD27+IgD− B cells

Discussion

During lymphocytes signaling, the CARD11 protein interacts with more than 20 different proteins and presents its interaction surfaces in response to signaling inputs [10]. In response to TCR or BCR engagement, CARD11 is converted from the resting closed inactive form into active open scaffold to bind signaling partners [10].

Herein, we present two patients with 2 homozygous missense variants in CARD11, presented clinically with CID features and atopic skin disease. Patient 1 has a missense pathogenic variant in the C-terminal domain at amino acid 947 where Glutamic acid is replaced by Lysine. The C-terminal GUK domain of CARD11 is crucial for T cell activation. It is responsible for membrane association, which results in recruitment of CARD11

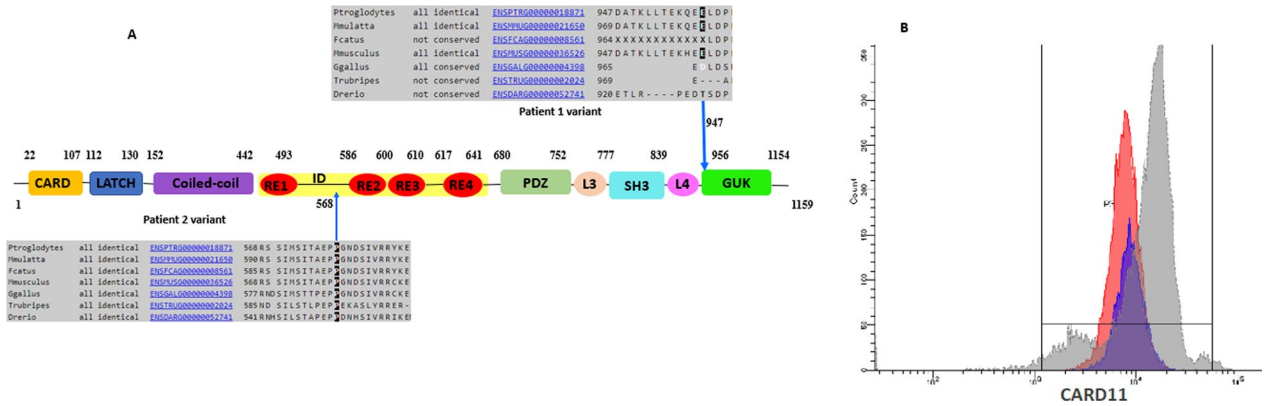

Fig. 2 CARD11 variants detected in the patients: **A** Schematic representation of the CARD11 protein and the locations of the p.Glu947Lys variant affecting the GUK region and detected in patient-1 and the p.Pro568Arg variant affecting the ID and detected in patient-2. The regions were aligned to sequences of model organisms reflecting the conservation status of both regions. **B.** CARD11 expression by flow cytometry with overlay of patient-1 (in purple), patient-2 (in red) versus the control (in gray)

to PKCθ at the immunological synapse [1, 19]. PKCθ then mediates the phosphorylation process of the ID domain which transforms CARD11 into the active form [20]. GUK domain also interacts with the SH3 domain in a manner crucial for CARD11-mediated activation of NF-κB signaling pathway [21]. Mutations disrupting this interaction led to failure of NF-κB activation with impaired T cells activation and B cells development [22]. Pathogenic variants in GUK domains have been previously reported in homozygous form in a patient with CID and as heterozygous dominant negative LOF in a patient with immunodeficiency with atopy [12, 23]. The variant reported in patient 1 (Chr7:2,954,871:C > T, c.2839G > A) is 2 bases from the splicing site and was predicted by HSF-Pro as pathogenic affecting the splicing of exon 21 with new acceptor/donor site. This patient had hypogammaglobulinemia with Omenn-like symptoms in the form of exfoliative erythematous rash, pneumonia, and persistent diarrhea. Recently, a homozygous missense variant in the GUK domain of CARD11 was reported in a patient with inflammatory skin disease resembling skin lesions of Omenn Syndrome [14]. The patient's brother and uncle complained from atopic dermatitis with lichenification with no evidence of CID. Both had the variant in a heterozygous form.

Patient 2 has a homozygous missense variant affecting the amino acid proline at position 568 which is replaced by Arginine. This position is highly conserved and located in the ID of the CARD11 close to the serine residues. These serine residues are phosphorylated as a result of antigen receptor signaling and serve as part of the phosphorylation events allowing CARD11 cofactors to bind and signaling events to ensue [10, 24]. The ID has an internal autoinhibitory function that keeps CARD11 inactive in the absence of receptor triggering and regulates the association of CARD11 with several signaling cofactors [10, 21]. The effect of pathogenic variants affecting the ID of CARD11 was not previously investigated. However, the integration of phosphorylation process of the ID is crucial for the proper function of CARD11 and to allow its binding to cofactors essential for NF-κβ pathway activation [20]. This patient suffered repeated pneumonia which required hospitalization several times. Later the patient developed skin eczema and lichenification. To our knowledge, this is the first report to describe the effect and phenotype of a pathogenic variant in the ID of CARD11.

Both patients had decreased expression of CARD11 but not absent as previously reported in most patients with CARD11 deficiency [2, 7, 12]. However, most of the previously reported patients had early termination of the protein or exon deletion. Nguyen et al. reported normal expression with altered function of CARD11 in their patient who had a missense variant in GUK domain presented with AR LOF of CARD11 [14].

Both patients presented in our study had markedly deficient Tregs with normal RTE and memory and naïve T cells. CARD11 and its cofactors play an important role in Tregs development in the thymus. CARD11- deficient mouse model lack thymic Tregs but have normal development of CD4+and CD8+T cells [20]. Interestingly, thymic Treg generation in CARD11-deficient mice can be rescued by crossing these strains to transgenic mice expressing a constitutively active mutant form of IKKβ [25]. Tregs deficiency was previously reported in patients with AR LOF of CARD11 [7, 12, 13].

In response to in vitro stimulation with Ionomycin, T cells from both patients showed compromised

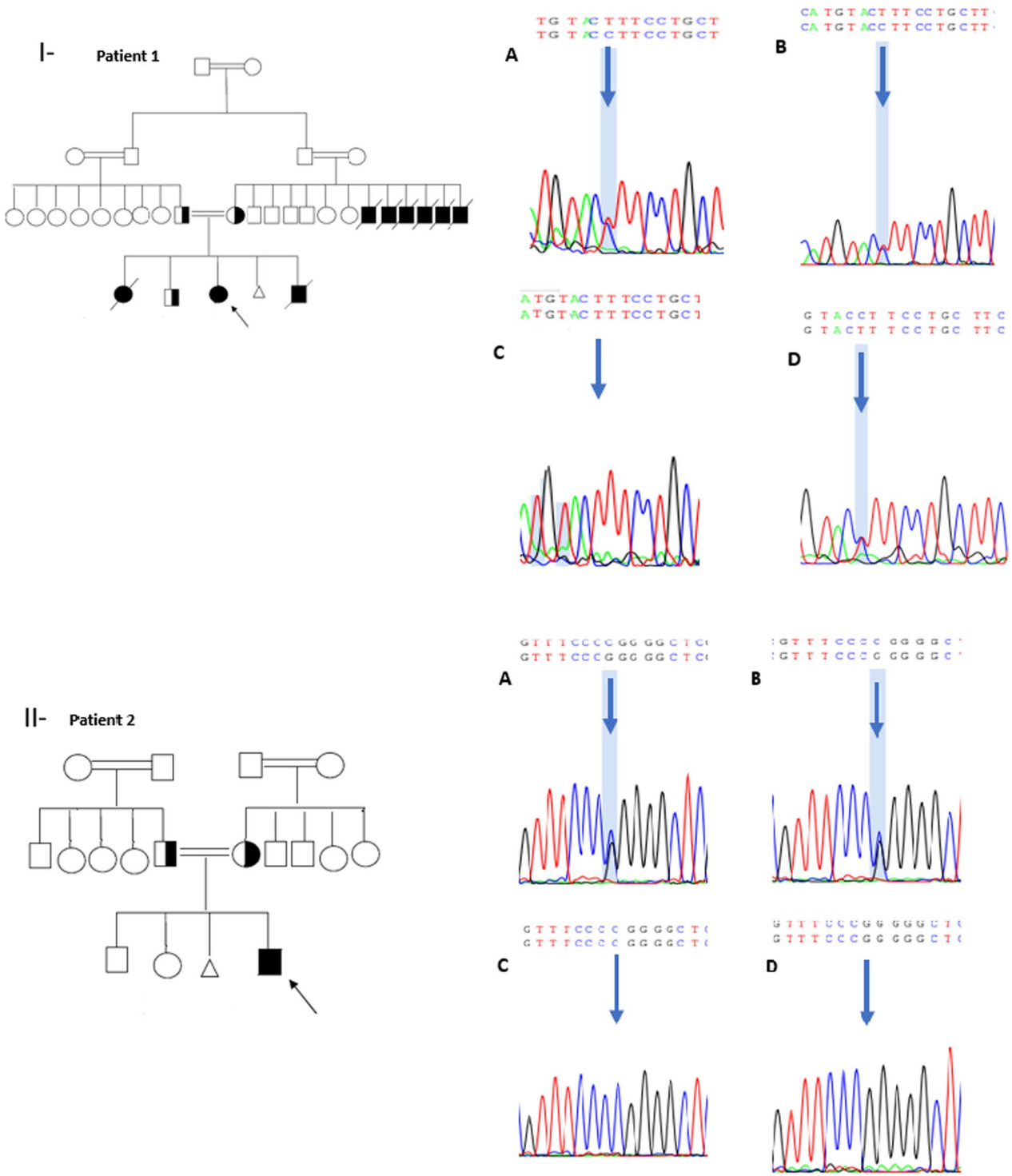

Fig. 3 Families pedigree and Sanger sequencing of the families' members: **I**- Family pedigree of patient-1 and Sanger sequencing of both parents (**A**, **B**) showing the heterozygous state of the variant, the patient has the variant in homozygous state (**C**) and her sibling (**D**) having the variant in heterozygous state. **II-** Family pedigree of patient-2 and Sanger sequencing of both parents (**A**, **B**) showing the heterozygous state of the variant, the patient has the variant in homozygous state (**C**) and his sibling (**D**) is wildtype

upregulation of expression of the activation marker CD25. The defect of T cell response to in vitro stimulation was also previously reported in CARD 11-deficient patients [7]. In both patients, B cells development was impaired with a marked increase in TrB cells percent and count and absent switched memory B cells. Patient 1 had complete absence of switched memory B cells, while they were markedly deficient in patient 2. These defects in B cells development have been previously reported in patients with autosomal recessive LOF of CARD11 [7, 12, 13].

Conclusion

Little is known about the different phenotypes of AR LOF of CARD11 specially those occurring as a result of missense variants. Missense variants causing CARD11 deficiency affect the function of the protein rather than the level of expression. Herein, we report on missense CARD11 variants which result in a phenotype combining the atopic skin disease and the features of CID. CARD11 deficiency should be considered in patients presenting early in life with Omenn Syndrome-like features.

Abbreviations

BCL10	B cell chronic lymphocytic leukemia/lymphoma 10
BCR	B-cell receptor
CARD11	Caspase recruitment domain family member 11
CBM	CARD11-BCL10-MALT1
CID	Combined immunodeficiency
IKKb	Inhibitor of nuclear factor kappa B kinase subunit beta
JNK	C-Jun N-terminal kinase
MALT1	MALT1 paracaspase
mTORC1	Mechanistic target of rapamycin complex 1
TCR	T-cell receptor
TrB	Transitional B
Treg	Regulatory T

Supplementary Information

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Additional file 1. List of the genes included in the panel.

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Author contributions

All authors contributed to the study conception and design. Patients followed by DAE, RA, SL, NG and AE. JB, MS and EC contributed to the study design. Flow cytometric analysis was performed by SM, REH, AE, and AE. Genetic studies were done by SM, RE and RD. The first draft of the manuscript was written by SM, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability

The authors confirm that data supporting the findings of the study are available in the article. Raw data were generated in Cairo University Specialized Children Hospital. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by Cairo University Faculty of Medicine Research Ethics Committee (N-114-2018). Written informed consent was obtained from the parents of the patients. Consent to Publish Informed consents were obtained from patients' guardians. The manuscript does not include any patients' pictures or data that may identify them.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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