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Molecular genetic dissection of inflammatory linear verrucous epidermal naevus leads to successful targeted therapy

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Title

Molecular genetic dissection of inflammatory linear verrucous epidermal naevus leads to successful targeted therapy

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Abbreviations:

Inflammatory linear verrucous epidermal naevus (ILVEN), Caspase Recruitment Domain Family Member 14 (CARD14), Pityriasis rubra pilaris (PRP), Interleukin (IL).

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To the editor,

Inflammatory linear verrucous epidermal naevus (ILVEN) is a rare skin condition. Classically it presents at birth or within the first year of life, frequently progressing during early childhood. Diagnostic criteria are erythematous verrucous hyperkeratosis in a fine and whorled Blaschko-linear pattern, intense pruritus, early age of onset, histological features, and resistance to treatment(Morag and Metzker, 1985). The cause of ILVEN has been unknown, however a single case of mosaicism in gene *GJA1* has recently been reported (Umegaki-Arao et al., 2017). We sought to investigate the genetics of ILVEN with a view to new therapeutic angles.

Fifteen children with ILVEN and six normal controls (from surgery where excess normal skin was available) were recruited with written informed consent and Research Ethics Committee approval from Great Ormond Street Hospital Research and Development office. The patients' parents/guardians consented to the publication of the patient images. DNA and RNA were extracted from skin biopsies of affected tissue, and DNA from blood by standard methods, and affected skin keratinocytes cultured and immortalised where possible (Lenti-HPV-16 E6/E7 Virus). Deep whole exome sequencing (WES) of blood and affected skin was performed on patient samples and data analysed using an optimised bioinformatic pathway for the detection of low-level somatic variants as previously published (Al-Olabi et al., 2018). Pathogenic *GJA1* variants were not found in any patient. Clinical and histological features of patients 1 and 2 are demonstrated in **Fig.1** and **2A-B**.

Heterozygous missense variants in gene Caspase Recruitment Domain Family Member 14 (*CARD14)* were detected in 2/15 patients (**Fig.2C-D**). In both patients the allelic load was compatible with that of a mosaic variant – in patient 1 the variant was present at 20% in both blood and DNA extracted directly from a whole punch biopsy of affected skin (c.356T>A, p. (M119K)), and in patient 2 at 1% from DNA extracted directly from the epidermis of affected skin and undetectable in blood (4/313 reads in skin, c.277A>G, p.(K93E)). We had intended that WES of the epidermis in patient 2 might have increased the mutant allele load, however this was not the case, and the 1% load may have been due to mainly cornified epidermis being sequenced. Both variants were, however, convincing on WES raw data, and both were clearly confirmed by Sanger sequencing (**Fig.2E-F**). The missense variant in patient 1 affects the same codon as one previously published in a non-mosaic state causing pityriasis rubra pilaris (Lwin et al., 2018), supporting its likely pathogenicity *in vivo*, and also supported by *in silico* predictions (SIFT Tolerated, Polyphen2 Benign, Mutation Taster Disease Causing, PROVEAN Neutral, CONDEL Neutral, CADD score 22.6) .The variant in patient 2 is predicted overall likely pathogenic *in silico* (SIFT Tolerated, Polyphen2 Probably Damaging, Mutation Taster Disease Causing, PROVEAN Neutral, CONDEL Deleterious, CADD score 24.1), and as it was to our knowledge previously unreported, we went on to characterise its functional effects. Cultured patient keratinocytes from patient 2 were used to model the variants in the most biologically-similar manner. Additionally, the patient 2 variant was modelled in a keratinocyte cell line (SVK_{14}) that was transfected (Lipofectamine 2000) with CARD14 wild-type and mutant (c.277A>G) pcDNA3.1-HA constructs (**Fig.2O)**. Culture of keratinocytes from patient 1 unfortunately failed,

and it was not deemed ethical to take further biopsies from a child for this purpose only.

Quantitative real-time polymerase chain reaction (qRT-PCR) demonstrated a significant increase in IL12A and IL23A in cultured patient keratinocytes and SVK_{14} cells transfected with the mutant *CARD14* construct_compared to identically-handled keratinocytes from grouped normal controls **(Fig.2G)** and SVK_{14} cells transfected with the wild-type *CARD14* construct **(Fig.2H)**. This was further validated at protein level by IL12/IL23 p40 ELISA (**Fig.2M-N**) (Invitrogen, United States). In addition, WST-1 assay (Sigma-Aldrich, USA) demonstrated a significant increase in proliferation rate in patient keratinocytes and SVK_{14} cells transfected with the mutant *CARD14* construct (**Fig.2I-J**). A significant increase in NF-κB p65 subunit activity was demonstrated by ELISA in nuclear extracts from SVK_{14} cells transfected with the mutant *CARD14* construct (**Fig.2L**) but not in patient keratinocyte nuclear extracts (**Fig.2K**) (Abcam, United Kingdom), potentially due to the less physiological model of overexpression in the cell line model.

Inherited (non-mosaic) heterozygous mutations in *CARD14* were recently described as rare causes of psoriasis(Jordan et al., 2012) and pityriasis rubra pilaris (PRP)(Fuchs-Telem et al., 2012). Variants affecting certain domains of CARD14 were initially described as leading to activation of NF-κB in the skin(Fuchs-Telem et al., 2012). However, differences between wild type and variant *CARD14* effects on NF-κB are modest (Li et al., 2015), and not all pathogenic variants increase activation of NF-κB (Bertin et al., 2001). This includes some of those located in the CARD domain

(aa 15-107) (Israel and Mellet, 2018) such as that in patient 2. Treatment of patients with germline *CARD14* variants with Ustekinumab has been highly successful (Eytan et al., 2014, Lwin et al., 2018), however direct measurement of the effect of *CARD14* variants on IL12 and IL23 expression has not previously been performed. Our findings suggest that IL12 and IL23 could be increased by *CARD14* variants in a non-NF-κB dependent manner.

Patient 1 had been resistant to multiple therapies (cyclosporine, acitretin, oral prednisolone), and she had faltering growth (height and weight below the 0.4th centile by three years of age; birth weight 50th- 75th centile). With hospital drug and therapeutics committee approval we started treatment at the age of six with Ustekinumab (0.75mg/kg/ dose at 0 and 1 months, 3 monthly thereafter, as per psoriasis protocol). She has had a dramatic and sustained improvement in her skin, now 20 months into treatment, but has required an increase to 8-weekly dosing to maintain effect between doses. She also exhibited catch-up growth, with height and weight improving from the <0.4th to 2nd-9th percentile within three months **(Fig.1D-F)**, and no adverse effects. Patient 2 is younger and less symptomatic **(Fig.1G-J)** and has not required treatment.

Historically there has been debate about clinical and histopathological similarities of ILVEN to Congenital Hemidysplasia, Ichthyosiform Erythroderma and Limb Defects (CHILD) syndrome, and to psoriasis(Happle, 1991, Ito et al., 1991, Moss and Burn, 1990, Welch et al., 1993). We consider that these debates are likely the result of

genetic heterogeneity in ILVEN, and that the term ILVEN is a clinical description rather than a single histopathological or genetic entity.

We identify here that heterozygous missense variants in *CARD14* are a recurrent cause of this phenotype, leading to successful targeted medical therapy in one patient. Indications for treatment should be made on an individual patient basis. Genetic counselling should be considered in ILVEN, as in these cases it could be passed on as PRP or psoriasis. These findings underline the power of molecular genetic characterisation of rare diseases alongside clinical and histopathological phenotyping.

Data availability

No datasets were generated or analysed during the current study.

Conflict of interest

The authors state no conflict of interest.

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Figure Legends

Figure 1. Clinical features of *CARD14*-mosaic ILVEN and dramatic response to targeted therapy in one patient. Patient 1 pre-treatment (A-C) and 3 months post commencing Ustekinumab (D-F), showing dramatic reduction in erythema and hyperkeratosis. Patient 2 pre-treatment showing predominantly left-sided Blaschko-linear inflammatory, hyperkeratotic skin lesions at one year (G, I) and 4 years (H, J). The patients' parents/guardians consented to the publication of the patient images.

Figure 2.

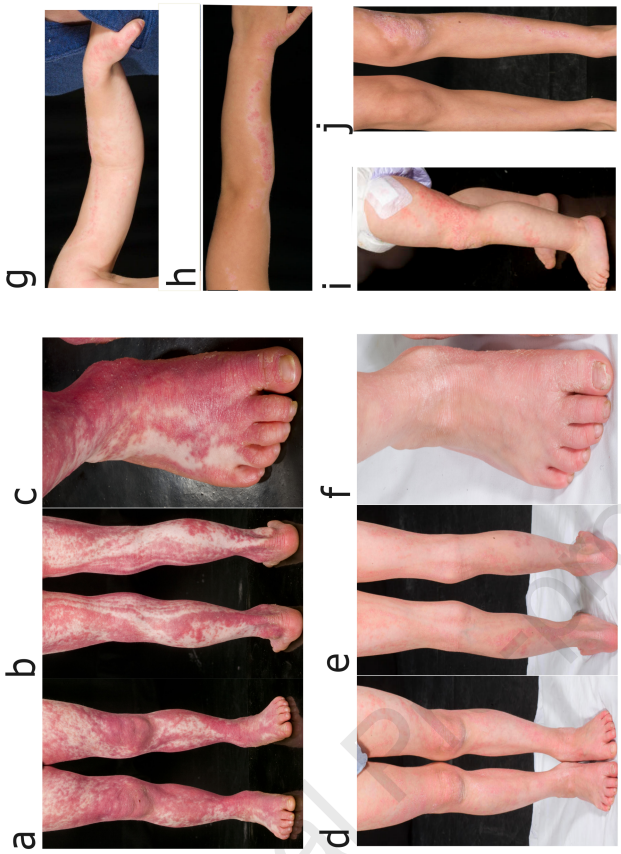
Histological features and mosaic genetic variants in *CARD14*-ILVEN. Patient 1 – (A, C, E), patient 2 - (B, D, F, G, H, I, J, K,L,M,N).

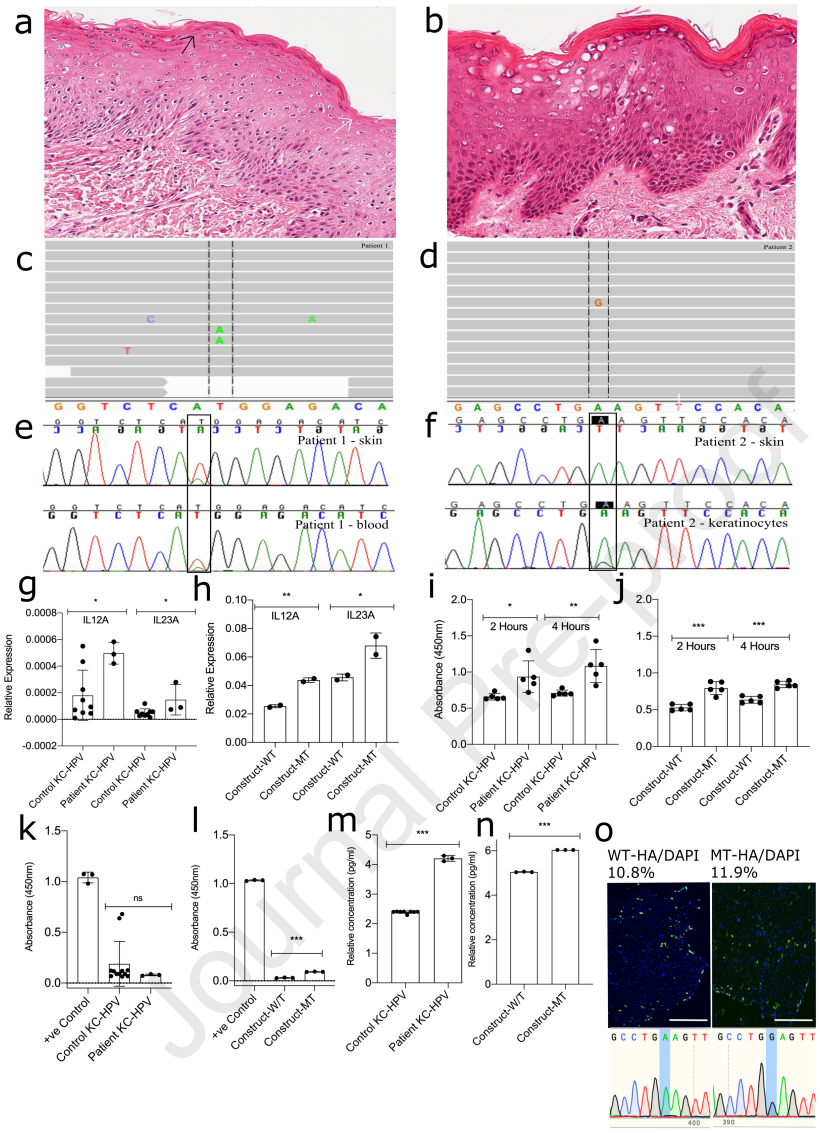
(A, B) Histology demonstrating alternating orthokeratosis (white arrow) and parakeratosis (black arrow) in patient 1, with generalised disruption of cornification in patient 2. Histological variability between ILVEN samples (from clinical diagnosis) was found to be very broad. (C, D) Whole exome sequencing visualised in the Integrative Genomics Viewer (Broad Institute) demonstrates mosaic *CARD14* missense variants c. 356T>A, p. (M119K) (patient 1, C), and c.277A>G, p.(K93E) (patient 2, D). (E-F) Sanger sequencing chromatograms confirm the variants.

Cultured patient keratinocytes and SVK_{14} cells transfected with a mutant *CARD14* construct express increased IL12 and IL23 at mRNA and protein level, proliferate faster than controls and show variable activity of NF-κB p65. (G,H) qRT-PCR

demonstrating a significant increase in IL12A and IL23A in cultured keratinocytes from affected skin from patient 2 and in SVK_{14} cells transfected with the mutant *CARD14* construct in comparison to control patient keratinocytes (n=3) and SVK_{14} cells transfected with the wild-type *CARD14* construct, respectively .Mean relative gene expression of five replicates per patient sample and duplicates per SVK_{14} sample was calculated with standard deviation. (I, J) WST-1 proliferation assay showing a proliferation increase in keratinocytes cultured from patient 2 and in SVK_{14} cells transfected with the mutant *CARD14* construct compared to control patient keratinocytes (n=3) and SVK_{14} cells transfected with the wild-type *CARD14* construct respectively, measured at 450 nm after two and four hours. The keratinocytes were cultured for 8 days prior to proliferation measurement. Mean absorbance of five replicates is shown with standard deviation. (K) Nuclear extracts from patient 2 keratinocytes do not show a difference in NF-κB p65 activity when compared to control patient keratinocytes (n=6). (L) Nuclear extracts from SVK_{14} cells transfected with the mutant *CARD14* construct show an increase in NF-κB p65 activity when compared to SVK_{14} cells transfected with the wild-type *CARD14* construct. Mean absorbance of triplicates for patient/control keratinocytes and positive control is shown with standard deviation. (M,N) Patient 2 keratinocytes and SVK_{14} cells transfected with the mutant *CARD14* construct have significantly increased levels of IL12 and IL23 secreted in the supernatant compared to control keratinocyte cell lines (n=4) and SVK_{14} cells transfected with the wild-type *CARD14* construct, respectively . Mean absorbance of triplicates is shown with standard deviation. All p-values were calculated by Students t-test using Prism v7.0 (Graphpad). Asterisks indicate p-value of <0.05. (O) Immunofluorescent anti-HA

staining of SVK_{14} cells transfected with *CARD14* wild-type and mutant pcDNA3.1-HA constructs with Sanger sequencing validation (scale bar = 400um).





	Patient 1	Patient 2
Age of onset	11 months	1 year
Lesion type	Blaschko-linear erythematous, hyperkeratotic, pruritic	Blaschko-linear erythematous, hyperkeratotic
Lesion distribution	Generalised	Appeared on left thumb at 4-6 weeks of age
Lesion extent	Facial, truncal and all limbs	Facial, truncal, all limbs
Unilateral / Bilateral	Bilateral	Initially unilateral on the left side, progressed to bilateral
Palmoplantar involvement (Y/N) and which type	Diffuse palmoplantar keratoderma	Linear palmoplantar keratoderma in continuity with arm lesions

Table S1: Detailed clinical features of patients 1 and 2.