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A tale of two sisters: identical *IL36RN* mutations and discordant phenotypes

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SIR- Recessive mutations in the *IL36RN* gene, encoding interleukin 36 receptor antagonist (IL-36Ra), have illuminated the understanding of the pathogenesis of generalized pustular psoriasis (GPP).^{1,2} Consanguineous Tunisian families were central to the discovery of homozygous mutations in *IL36RN* being associated with early onset GPP.² Studies in populations from different genetic backgrounds

highlighted the requirement of other genes in cases that are mutation negative, or
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only carry a mutation in a single allele.³ Sibling pairs with compound heterozygote mutations in European and Asian populations, an intriguing, non-consanguineous, genetic background to study phenotypes associated with *IL36RN*, are rare. Furthermore, the genetic differences in autozygosity seen in consanguineous families are currently being delineated at a population level, and support the exploration and comparison of phenotypes seen in this genetic background with other non-consanguineous genetic backgrounds.⁴ Only 1 compound heterozygous case of Japanese twins is reported as affected siblings. In this case, both twins developed GPP at the age of 2, following treatment with amoxicillin. Siblings with homozygous *IL36RN* mutations have been reported to present within 11 years of each other (Supplementary Table 1a). Here we present a report of two sisters that carry identical compound heterozygous mutations in *IL36RN*, yet first presented 34 years apart, exemplifying the requirement of further genetic or environmental factors.

A 38-year-old, female, European patient had been attending our department with features consistent with severe GPP since 6 months of age. Widespread small pustules were seen on the trunk and limbs, and numerous hospital admissions with severe pustulation and fever were a recurrent feature of childhood (Figure 1a). The patient's history was negative for recognised triggers of GPP such as antibiotic usage, infection and menstruation. Histological examination of the sterile pustules demonstrated a brisk neutrophilic infiltrate, with some spongiosis (Figure 1d). Acrodermatitis continua of Hallopeau involving the fingernails prevented nail growth. Extensive scalp involvement could not be controlled such that the patient resorted to wearing a hat. Pustules were seen on the oral mucosa during severe flares and widespread lymphadenopathy was noted. Methotrexate therapy was commenced at the age of six, and used alone and in combination with etretinate, but

neither regime gave good disease control. Hydroxyurea was ineffective. Oral prednisolone suppressed pustulation, however could not be sustained due to Cushingoid side effects. Deterioration of her skin was noted during her both pregnancies, consistent with a diagnosis of impetigo herpetiformis, warranting treatment with ciclosporin during the 2nd-3rd trimester in her first pregnancy and an elective termination of her second pregnancy. In addition to the physical morbidity the patient had been treated for depression relating to her cutaneous disease.

Recombinant soluble TNF-alpha receptor treatment was chosen at the age of 30, given the severity of her disease (Figures 1b-c). Following treatment with Etanercept at a dose of 25 mg subcutaneously twice weekly, her cutaneous disease activity was brought under control. Methotrexate and prednisolone were weaned successfully and the patient had normal nail growth for the first time. After 11 months of treatment, she demonstrated plaque psoriasis without pustulation. The patient was then treated with Adalimumab 40mg subcutaneously every 2 weeks, with improved control and has continued on this for the last 6 years. There have been no hospital admissions for the last 7 years.

By contrast, the proband's sister who had never previously had skin disease, first developed widespread pustular psoriasis at the age of 34 warranting hospital admission. Her clinical history was negative for antecedent infection, medication use or pregnancy. She was admitted to hospital and managed with topical therapy, prompting resolution of the rash after 12 days. She has subsequently had minimal disease affecting the scalp for a 6-month period. Notably she did not have oral pustules.

Mutation analysis of the *IL36RN* gene was performed given the phenotype, and demonstrated identical mutations (sequencing primers and conditions as

previously described¹). Both sisters carried compound heterozygous mutations in exon 5 of the *IL36RN*: c.338C>T (p.Ser113Leu) and c.368C>T (p.Thr123Met) (Figure 2a). The c.338C>T mutation is a known founder mutation in European populations, whilst c.368C>T has been reported only in Japanese cases⁵, making this a novel finding in Europeans. As the mutation occurred at a CpG dinucleotide sequence, we postulate that the position c.368C of the *IL36RN* gene could be a mutational hot spot across different populations. PCR products were cloned to confirm that the two mutations resided on separate alleles.

In order to determine the biochemical effect of the missense mutation p.Thr123Met on the expression of IL-36Ra, we generated a N-terminal Flag-tagged expression vector for the p.Thr123Met mutant (Met-mutant) protein using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) with the wild type (Wt) IL-36Ra-expression vector as a template.⁶ A p.Thr123Arg-Mut vector (Arg-mutant), containing a mutation known to influence IL-36Ra levels, was used as a positive control. Then we overexpressed the Wt, Met-mutant and Arg-mutant Flag-IL-36Ra vectors in HEK293T cells, collected the cell lysate, and performed SDS-PAGE separation of proteins followed by immunoblotting with mouse monoclonal anti-Flag M2 antibody (diluted 1:1,000; Sigma-Aldrich, St. Louis, MO). Similar to the Arg-mutant of IL-36Ra protein, expression of the Met-mutant was markedly reduced as compared to the Wt, suggesting an instability of the Met-mutant IL-36Ra protein (Figure 2b).⁶

To investigate the functional effect of this instability, we performed reporter gene assays using the *IL8* promoter in HeLa cells as described previously.⁶ The Wt IL-36Ra protein significantly repressed the promoter activity induced by IL-36 γ and IL-1RL2 (Figure 2c), whereas neither the Met-mutant nor the Arg-mutant IL-36Ra

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proteins reduced the activity at all ($p < 0.01$; students t-test), consistent with the mutation resulting in loss of function (Figure 2c).

To improve our understanding of how the mutation may have an effect on tertiary protein structure, we performed molecular modeling of the patient's mutation (Thr123->Met) (Figure 2d). Thr123 is predicted to be situated in loop10 of IL-36Ra, which is a structural element for a core hydrophobic patch, where a large number of amino acid residues are densely embedded. The mutation into methionine at Thr123, which has bulkier side chain than threonine, is likely to cause steric clashing with adjacent amino acid residues. Furthermore the mutation is predicted to disrupt the hydrophobic interaction and hydrogen bond network contributing to the stabilisation of IL-36Ra conformation, resulting in protein misfolding. Taken together with the overexpression and reporter studies, and previous clinical data of changes at this residue, these data support the finding that the c.368C>T mutation in our patient is pathogenic.

We present this case to highlight the strikingly distinct phenotypes seen in 2 *IL36RN* mutation carriers from the same non-consanguineous pedigree. Homozygous carriers of previously identified *IL36RN* mutations across families have been observed to have a range of age of presentations, that suggest further genetic and/or environmental risk factors may influence the age of onset³ (Supplementary Table 1b). Comparison within a family, despite the caveats associated with smaller numbers of patients, control for some of this variation and is informative in our understanding of this disease. We also highlight enoral pustulation that was seen in our patient and has been recognised by Navarini *et al.*⁷ as a useful indicator of *IL36RN* mutations.

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Figure Legends

Fig 1. Clinical features and histopathological findings of the patient. (a) The proband seen at age 6, with generalised pustular lesions seen on the torso and legs, on a background of erythroderma. (b) The proband prior to starting anti-TNF therapy at age 30, with a close up image (c) highlighting the pustulation and superficial desquamation seen. (d) A dense neutrophilic infiltrate seen in the epidermis in a skin biopsy of a pustule taken at age 6.

Fig 2. Mutation analysis of *IL36RN* in both patients, and functional and molecular evidence supporting the pathogenicity of the T123R change.

(a) Sanger sequencing analysis reveals compound heterozygous mutations in the proband's DNA. Mutations are shown at 2 positions within *IL36RN* exon5 ("Patient"), c.338C>T and c.368C>T, which differs from the wild type sequence ("wild type"). Single allele analysis, carried out by cloning the PCR products followed by

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sequencing, reveals the presence of the c.338 C>T mutation “Allele a” and the c.368C>T on the other chromosome, “Allele b”. (b) Expression of the p.Thr123Met mutant IL-36Ra protein is severely impaired as compared to that of the wild type (Wt) IL-36Ra in HEK293T cells. All constructs were verified by direct sequencing. Beta-actin was blotted as a normalization control. (c) *IL8* promoter-reporter gene assays demonstrate loss-of-function of the p.Thr123Met mutant IL-36Ra protein in HeLa cells. The *IL8* promoter activity induced by IL-36 γ and IL-1RL2 was significantly ($p<0.01$; students t-test) downregulated by the wild type (Wt) IL-36Ra, but not by the p.Thr123Met or the p.Thr123Arg mutant IL-36Ra.). All experiments were performed in triplicate and repeated three times. The error bar indicates standard error of the mean of a single representative experiment. (N.S. not significant). (d) Cartoon diagrams of the core hydrophobic patch in the human IL-36Ra protein. The three-dimensional structure of human IL-36Ra was modelled (Swiss model; <http://swissmodel.expasy.org/>)^{8,9} using a reported murine IL-1F5 structure (PDB ID: 1MD6¹⁰) as a template. The predicted structure was generated using Pymol v0.99 (DeLano Scientific LLC, San Carlos, CA). Three β -hairpins of IL-36Ra are shown in purple (residues 1-46), green (residues 47-104), and orange (residues 105-155) ribbon representations. Amino acid residues that contribute to the stability of the conformation of IL-36Ra that interact with Thr123 are highlighted by stick presentations in red and blue. Suggested hydrogen bonds between these residues are shown as dotted lines.

Figure 1

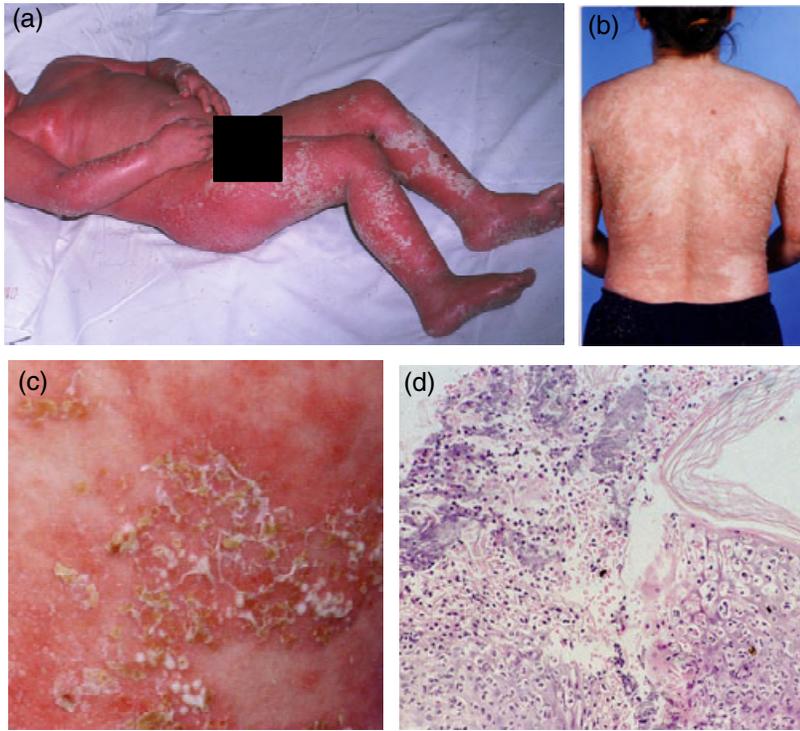


Figure 2

