Activating *CARD14* mutations are associated with generalised pustular psoriasis but rarely account for familial recurrence in psoriasis vulgaris

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Abbreviations: APP, acral pustular psoriasis; *CARD14*, Caspase Recruitment Family Member 14; CC, coiled coil; GPP, generalised pustular psoriasis; PRP, pityriasis rubra pilaris; PV, psoriasis vulgaris.

ABSTRACT

Caspase Recruitment Family Member 14 (*CARD14,* also known as *CARMA2*) is a scaffold protein that mediates NF-κB signal transduction in skin keratinocytes. Gain-of-function *CARD14* mutations have been documented in familial forms of psoriasis vulgaris (PV) and pityriasis rubra pilaris (PRP). More recent investigations have also implicated *CARD14* in the pathogenesis of pustular psoriasis. Follow-up studies, however, have been limited, so that it is not clear to what extent *CARD14* alleles account for the above conditions. Here, we sought to address this question by carrying out a systematic *CARD14* analysis in an extended patient cohort (n=416). We observed no disease alleles in subjects with familial PV (n=159), erythrodermic psoriasis (n=23), acral pustular psoriasis (n=105) or sporadic pityriasis rubra pilaris (n=29). Conversely, our analysis of 100 individuals with generalised pustular psoriasis (GPP) identified a low-frequency variant (p.Asp176His) which causes constitutive CARD14 oligomerization and shows significant association with GPP in Asian populations (P=8.4x10^{-5}; OR=6.4). These data indicate that the analysis of *CARD14* mutations could help to stratify pustular psoriasis cohorts but would be mostly uninformative in the context of psoriasis and sporadic pityriasis rubra pilaris.

INTRODUCTION

Caspase Recruitment Family Member 14 (*CARD14*, also known as *CARMA2*) is a conserved scaffold protein that mediates TRAF2-dependent activation of NF-κB signalling (Scudiero *et al.*, 2011). The gene is most prominently expressed in skin, as transcript levels are high in keratinocytes and moderately abundant in dermal endothelial cells (Fuchs-Telem *et al.*, 2012; Harden *et al.*, 2014). Conversely, *CARD14* mRNA is virtually undetectable in T-lymphocytes and monocytes, suggesting that the gene is specifically required for the maintenance of skin immune homeostasis (Fuchs-Telem *et al.*, 2012; Harden *et al.*, 2014). In keeping with this notion, gain-of-function *CARD14* mutations have been linked to a number of inflammatory dermatoses.

Disease alleles resulting in enhanced NF-κB signalling were first described in two multi-generation pedigrees where psoriasis vulgaris (PV) segregated as an autosomal dominant trait (Jordan *et al.*, 2012b). Three further activating mutations were subsequently reported in individuals with familial pityriasis rubra pilaris (PRP), a papulosquamous condition phenotypically related to psoriasis (Fuchs-Telem *et al.*, 2012). Finally, the analysis of a small Japanese resource identified a tentative association between a *CARD14* variant (p.Asp176His) and generalised pustular psoriasis (GPP), a rare neutrophilic dermatosis that often presents with systemic upset and concurrent PV (Sugiura *et al.*, 2014).

Of note, the follow-up to the findings originally obtained in familial PV has been very limited, with a single survey of seven North-African pedigrees published in the literature (Ammar *et al.*, 2013). Likewise, studies of *CARD14* in PRP have mostly been restricted to cohorts recruited in the Middle East (Eytan *et al.*, 2014) and the association between p.Asp176His and generalised pustular psoriasis has yet to be validated, as it was only supported by marginal statistical significance. Thus, large gaps remain in our understanding of the phenotypes that are caused by *CARD14* mutations and it is not clear whether the genetic analysis of this locus might aid disease stratification.

Here, we have sought to address this issue by investigating an extended and ethnically diverse patient resource. We found that *CARD14* alleles are unlikely to account for a significant proportion

of familial PV or sporadic PRP cases. Conversely we demonstrated that the p.Asp176His variant has a substantial impact on protein function and is strongly associated with generalised pustular psoriasis in the Chinese and Japanese populations.

RESULTS

CARD14 mutations do not play a significant role in the pathogenesis of familial PV, sporadic pityriasis rubra pilaris or acral pustular psoriasis

Since all *CARD14* mutations described to date cluster to exons 3 and 4 (Eytan *et al.*, 2014; Fuchs-Telem *et al.*, 2012; Jordan *et al.*, 2012b; Sugiura *et al.*, 2014), we initially focused our genetic screening on this region. We sequenced the two exons in a total of 416 patients, affected by acral pustular psoriasis (APP; n=100), erythrodermic psoriasis (n=23), familial PV (n=159), generalised pustular psoriasis (n=105) or pityriasis rubra pilaris (n=29) (Table 1, Supplementary Table 1). Importantly, our calculations showed that all datasets had adequate power (>80%) to detect mutations accounting for >5% of disease cases (Figure 1). In fact, the power of the familial PV, APP and GPP samples exceeded 95% (Figure 1). This is in keeping with previous analyses of the APP and GPP cohorts, which allowed us to identify a number of low-frequency mutations (Setta-Kaffetzi *et al.*, 2013; Setta-Kaffetzi *et al.*, 2014).

Despite the evidence for adequate power, we could not detect any deleterious alleles among patients affected by acral pustular psoriasis, familial PV or erythrodermic psoriasis. In fact, the only coding change observed in these datasets was a p.Ser200Asn substitution, which was identified in 2 cases of acral pustular psoriasis and four patients affected by familial PV (Table 2). This variant was however classified as benign by multiple pathogenicity prediction algorithms. Moreover, its frequency in APP (1.0%) and familial PV subjects (1.25%) did not exceed that observed in European Controls (1.3%, according to the data generated by the Exome Aggregation Consortium).

The screening of the PRP sample revealed a single change with pathogenic potential (p.Gln136Lys; Table 2). However, this was observed in a patient who underwent parallel investigations of the *GJB4*

gene to explore a differential diagnosis of erythrokeratoderma. Given that a damaging p.Cys169Trp change was identified in *GJB4*, it was not possible to reach a firm conclusion as to which variant was causing the disease phenotype.

Importantly, sequencing of the entire *CARD14* coding region in a representative patient subset (n=82; Supplementary Table 2) did not identify any additional changes with deleterious potential. Thus, our findings indicate that *CARD14* disease alleles are unlikely to account for a significant proportion of familial PV, sporadic pityriasis rubra pilaris or acral pustular psoriasis cases.

The CARD14 p.Asp176His variant is associated with generalised pustular psoriasis in the Chinese and Japanese populations

The analysis of the generalised pustular psoriasis resource identified three unrelated subjects of Chinese descent, who carried a deleterious p.Asp176His substitution (Figure 2a; Table 2; Supplementary Table 3). Of note, one of the patients had inherited the variant from a maternal aunt, who was also affected by GPP. While the change occurs at low frequency in the Chinese population, we found that it was more common in GPP cases compared to controls (6.2% vs. 1.0%, *P*=0.03; Table 3). As these observations mirror those previously reported in a small Japanese dataset (Sugiura *et al.*, 2014), we undertook a meta-analysis of the two studies. To maximise statistical power, we increased by fourfold the size of the Japanese control resource by obtaining frequency data for an additional 322 individuals (104 subjects sequenced by the 1000 Genomes Project and 218 screened in-house). This validated the association with the p.Asp176His substitution (*P*=0.008) and showed that the Japanese and Chinese datasets were genetically homogeneous (I^2:0%). The subsequent meta-analysis of the two studies also demonstrated that the p.Asp176His change confers a very substantial increase in disease risk (OR: 6.4; 95%CI: 2.5-16.1; $P=8.4x10^{-5}$).

Since p.Asp176His occurs on the background of a single haplotype in the Japanese population (Sugiura *et al.*, 2014), we typed four tagging SNPs in our mutation bearing patients, in order to establish whether they carried the same ancestral chromosome. We found that three subjects were

homozygous and one likely heterozygous for the same intragenic haplotype that was described in the Japanese individuals carrying the p.Asp176His variant (Supplementary Table 4). Thus, our data strongly support the notion that the spread of the mutation across East Asia is the result of a founder effect.

Sequencing of the entire *CARD14* coding region in a representative patient subset (n=15) did not uncover any further disease alleles, indicating that the association with GPP is specific to the p.Asp176His change.

The p.Asp176His variant is a deleterious allele associated with constitutive CARD14 oligomerization

To investigate the functional consequences of the p.Asp176His substitution, we first used bioinformatics to assess its potential effect on the coiled-coil (CC) domain encoded by exons 3 and 4. We found that the change from a negatively charged aspartic acid to a basic histidine significantly reduces the likelihood of CC formation (Figure 2b). This effect is comparable to the predicted impact of p.Leu156Pro and p.Glu138Ala (Figure 2b), two disease alleles previously associated with pityriasis rubra pilaris and psoriasis (Fuchs-Telem *et al.*, 2012; Jordan *et al.*, 2012b).

Of note, the coiled-coil domain of *CARD11* (a well characterised *CARD14* paralogue) keeps the protein in an inactive conformation which in the absence of inflammatory stimuli, precludes auto-oligomerization and downstream signal transduction (Lamason *et al.*, 2010). In fact, gain-of-function mutations within the coiled coil of *CARD11* cause spontaneous protein aggregation and constitutive activation of NF-κB signalling, leading to the onset of diffuse large B-cell lymphoma (Lenz *et al.*, 2008).

To investigate the possibility that the p.Asp176His substitution may also promote protein oligomerization, we transfected HEK293 cells with wild-type or mutant FLAG-CARD14 and monitored recombinant protein levels with an anti-FLAG antibody. While western blotting of whole-cell extracts showed comparable transfection efficiencies for the two constructs (Figure 2c), the analysis of soluble proteins demonstrated the p.Asp176His change was associated with a significant decrease in

the levels of free CARD14 (Figure 2d and 2e). This suggested that the mutant protein was forming insoluble oligomers. To validate this hypothesis, we used the anti-FLAG antibody to analyse the insoluble fraction of the cell extracts. As predicted, we found that CARD14 aggregates were significantly more abundant in the cells that had been transfected with the p.Asp176His cDNA (Figure 2f and 2g). Interestingly a similar effect was noted for the constructs harbouring the p.Leu156Pro and p.Glu138Ala disease alleles (Figure 2d, 2e, 2f and 2g). Thus, our findings indicate that the pathogenic potential of the p.Asp176His change is comparable to that of previously validated mutations.

DISCUSSION

In the last few years, *CARD14* mutations have been associated with a range of inflammatory skin disorders, including familial psoriasis and clinically related conditions such as pityriasis rubra pilaris and generalised pustular psoriasis (Fuchs-Telem *et al.*, 2012; Jordan *et al.*, 2012b; Sugiura *et al.*, 2014). While these findings have highlighted *CARD14* as a key regulator of skin immune homeostasis, their applicability to clinical practice has been limited. Very little information is available on mutation frequencies in different conditions or ethnic groups, so that it is unclear whether *CARD14* screening would aid the diagnosis or stratification of inflammatory dermatoses. Here we sought to address this issue by analysing a sizeable and well characterised patient resource, recruited in Europe and East Asia.

We first examined a familial PV cohort including > 150 unrelated cases. Despite the significant power of this dataset, we could not identify novel disease alleles or detect any of the mutations reported in previous studies (Jordan *et al.*, 2012a; Jordan *et al.*, 2012b). While it could be argued that our definition of the phenotype allowed the inclusion of many affected sib-pairs who were unlikely to suffer from monogenic psoriasis, our resource also comprised 51 families with four or more affected individuals (Supplementary Table 1). Thus, our results indicate that Mendelian forms

of psoriasis are likely to be genetically heterogeneous, with *CARD14* mutations accounting for a small minority of cases.

The subsequent analysis of the pityriasis rubra pilaris cohort generated similarly negative results, validating the emerging notion that *CARD14* mutations do not contribute to sporadic forms of the disease (Eytan *et al.*, 2014). While the idea that *CARD14* plays a role in the pathogenesis of PV and PRP is supported by observations of gene over-expression in patient skin and by the results of genome-wide association studies (Eytan *et al.*, 2014; Tsoi *et al.*, 2012), our findings indicate that screening this locus for deleterious mutations is unlikely to help the stratification of patient resources.

The analysis of the GPP dataset demonstrated a significant association with a non-conservative amino acid substitution (p.Asp176His) that is only found in Asian populations. The functional characterization of this nucleotide change indicated that it is likely to affect coiled coil formation, leading to loss of CARD14 auto-inhibition and spontaneous protein oligomerization. While we did not formally demonstrate that these abnormalities result in enhanced transcription of NF-κB target genes, this outcome is supported by several lines of evidence. First of all, it has been shown that CARD11 oligomerization levels strongly correlate with increased NF-κB activation (Lenz *et al.*, 2008). Secondly, the effect of p.Asp176His on CC formation is comparable to that of p.Leu156Pro and p.Glu138Ala, which have been extensively characterised and linked to abnormal NF-κB signalling *in-vitro* and *ex*-vivo (Fuchs-Telem *et al.*, 2012; Harden *et al.*, 2014; Jordan *et al.*, 2012b). Finally, a systematic analysis of rare *CARD14* variants has shown that over-expression of p.Asp176His mutant constructs results in increased NF-κB reporter activity (Jordan *et al.*, 2012a). Thus, our findings validate p.Asp176His as a deleterious gain-of-function allele.

Of interest, Mossner et al have very recently observed the p.Asp176His mutation in two acral pustular psoriasis cases recruited in Estonia (Mossner *et al.*, 2015). As the p.Asp176His substitution was not detected in any of the European datasets sequenced by the 1000 Genomes Project, it is not surprising that the variant did not appear in our APP cohort. In fact, its presence among Estonian

patients is likely to reflect the distinctive population history of the Baltic countries. In this context, the analysis of p.Asp176His in Asian datasets will be required to confirm the association with APP and provide further evidence for a genetic overlap between generalised and localised pustular psoriasis (Setta-Kaffetzi *et al.*, 2013).

While the evidence presented here strongly supports a pathogenic role for p.Asp176His, no association with GPP was observed in a recent analysis of a Han Chinese resource (Qin *et al.*, 2014). It is interesting however, that none of the cases that were examined had been screened for mutations in *IL36RN*, the major genetic determinant of GPP (Onoufriadis *et al.*, 2011). Conversely, both our study and the analysis carried out by Sugiura et al (Sugiura *et al.*, 2014) were restricted to *IL36RN* negative cases. Thus, our results support the notion that the sequential screening of disease genes will enhance the power to detect pathogenic mutations and facilitate the stratification of GPP cohorts.

MATERIALS & METHODS

Subjects

This study was undertaken in accordance with the principles of the Declaration of Helsinki and with the approval of the ethics committees of all participating institutions. Patients affected by psoriasis vulgaris, erythrodermic psoriasis, generalised pustular psoriasis, acral pustular psoriasis (APP, including both Acrodermatitis Continua of Hallopeau and Palmar Plantar Pustulosis) were ascertained as described elsewhere (Berki *et al.*, 2014; Hussain *et al.*, 2014). Pityriasis rubra pilaris was diagnosed on the basis of established criteria (Judge *et al.*, 2004). All patients with pustular psoriasis had been previously screened for *IL36RN* and *AP1S3* mutations, so as to exclude any subjects carrying disease alleles at known loci (Onoufriadis et al., 2011; Setta-Kaffetzi et al., 2014). Individuals who were affected by psoriasis vulgaris and had at least one first-degree relative with the same disease were considered cases of familial PV (Supplementary Table 1). The demographics of the various patient cohorts are summarised in Table 1.

Allele frequency data for 208 unrelated Chinese controls were initially collated from the 1,000 Genome Project (CHB and CHS datasets) (Abecasis *et al.*, 2012). To match the genetic make-up of cases, 8 individuals with *IL36RN* mutations were subsequently excluded from the association analysis. Allele frequency data for 422 independent Japanese controls were obtained from the 1,000 Genome Project (JPT dataset, n=104), the work published by Sugiura et al (n=100) (Sugiura *et al.*, 2014) and from the analysis of 218 healthy individuals, recruited at the National Cerebral and Cardiovascular Center in Osaka (Japan), and genotyped in house by Sanger Sequencing. None of the above individuals carried *IL36RN* mutations. Allele frequency data for the European population were obtained by mining the data generated from the Exome Aggregation Consortium (http://exac.broadinstitute.org/).

All study participants granted their written informed consent.

Sanger sequencing and pathogenicity predictions

Primers were designed to amplify all *CARD14* coding exons and exon-intron junctions, as well as the genomic regions spanning haplotype tagging SNPs (Supplementary Table 5). Sequenced PCR products were loaded on an ABI3730*xl* DNA Analyzer (Applied Biosystems, Waltham, MA) and nucleotide changes were detected using Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI). The pathogenic potential of non-synonymous sequence variants occurring with a minor allele frequency <2% was assessed with the following programs: SIFT (Kumar *et al.*, 2009), PolyPhen-2 (Adzhubei *et al.*, 2013), PROVEAN (Choi *et al.*, 2012), MutationTaster (Schwarz *et al.*, 2010), CADD (Kircher *et al.*, 2014), Align GVGD (Mathe et al, 2006); SROOGLE (which was also used to compute Senepathy scores) (Schwartz *et al.*, 2009) and MaxEntScan (Yeo and Burge, 2004). The impact of damaging nucleotide changes on coiled coil domains was further investigated with the NCOILS 1.0 software (Lupas *et al.*, 1991).

Site-directed mutagenesis, cell culture and transfection

The previously described CARMA2-sh construct (Scudiero *et al.*, 2011) was used as a template for site-directed mutagenesis, since it encodes the CARD14 isoform with the highest expression in skin (Jordan *et al.*, 2012b). Reactions were prepared using the QuikChange Lightning Site Directed mutagenesis Kit (Agilent, Santa Clara, CA) and the primers listed in Supplementary Table 6. The integrity of all constructs was verified by Sanger sequencing of the *CARD14/CARMA2* coding region, FLAG tag, CMV promoter and BGH poly-adenylation signal. HEK293 cells were grown in DMEM medium, supplemented with 1% penicillin/streptomycin and 10% Fetal Calf Serum (all from Life Technologies, Carlsbad, CA). Cells were seeded on 12-well plates at a concentration of $2.5x10^5$/ml and transfected with the indicated constructs, using Lipofectamine 2000 (Life Technologies). All cultures were harvested 48h after transfection.

Western blotting and densitometry

Whole-cell protein extracts were prepared by treating cells with a denaturing lysis buffer (5% SDS, 200Mm Tris-HCl pH 6.8, 1mM EDTA, 1.5% β-Mercaptoethanol, 8M Urea, 1X Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland)). For the analysis of the soluble and insoluble fractions, cells were initially incubated with a non-denaturing buffer (50mM Tris-HCl pH 7.4, 50mM NaCl, 5mM EDTA, 10% Glycerol, 1% NP40, 1X Complete Protease Inhibitor Cocktail)and centrifuged for 15min at 13,000rpm, at 4C. The supernatant was then stored as a soluble fraction, while the pellets containing the insoluble protein aggregates were lysed with the denaturing lysis buffer. Following poly-acrylamide gel electrophoresis and transfer to nitrocellulose membranes, blots were probed with 1:2,500 mouse monoclonal anti-FLAG (Sigma-Aldrich, St Louis, MO) and 1:1,000 rabbit polyclonal anti β-actin (Cell Signalling Technology, Beverly, MA) antibodies. Autoradiography films were scanned and densitometric analysis was undertaken with the Image J software (Schneider *et al.*, 2012).

Statistical analyses

Power calculations were implemented with the binomial probability calculator available at stattrek.com/online-calculator/binomial.aspx. The frequency of the p.Asp176His substitution was compared in cases vs. controls with Fisher's exact test. The meta-analysis of two association studies was undertaken by using the RevMan 5.2 software (Cochrane Collaboration, 2012) to calculate a weighted pooled odds ratio and Z score. The densitometry data were analysed with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA) by one-way ANOVA followed by a Dunnett's post-test. *P* values < 0.05 were considered statistically significant.

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Table 1: Patient resource summary statistics

Disease	N. of Cases	Sex	Ethnicity
Acral Pustular Psoriasis[a,]	100	34M, 66F	North-European
Erythrodermic Psoriasis	23	18M, 5F	North-European
Familial Psoriasis Vulgaris	159	78M, 81F	North-European
Generalised Pustular Psoriasis	105	34M, 71F	North-European (n =12) Chinese (n =24) Malay (n =52) Indian (n =17)
Pityriasis rubra pilaris	29	16M, 13F	North-European (n=24) Afro-Caribbean (n=2) Unknown (n=3)
TOTAL	416	180M, 236F	North-European (n =318) Asian (n=93) Afro-Caribbean (n=2) Unknown (n=3)

[a]Including Palmar Plantar Pustulosis (n=92) and Acrodermatitis Continua of Hallopeau (n=8); F: Females; M: Males;

Table 2A: Rare coding variants detected in the study resource

Change (rs ID)	Patient phenotype	Pathogenicity prediction						
		SIFT	**Polyphen-2**	**PROVEAN**	**Mutation Taster**	**Align GVGD**	**CADD score[a]**	**CONSENSUS**
p.Met119Arg (novel)	PRP (n =1)	Tolerated	Benign	Neutral	Disease Causing	Pathogenic	Damaging	NEUTRAL
p.Gln136Lys (novel)	PRP (n =1)	Damaging	Probably Damaging	Neutral	Disease Causing	Pathogenic	Damaging	DAMAGING
p.Glu168Lys (novel)	GPP (n =1)	Tolerated	Benign	Neutral	Polymorphism	Pathogenic	Neutral	NEUTRAL
p.Asp176His (rs144475004)	**GPP (n =3)**	**Damaging**	**Probably damaging**	**Neutral**	**Disease Causing**	**Pathogenic**	**Damaging**	**DAMAGING**
p.Ser200Asn (rs114688446)	PV (n =4) APP (n=2)	Tolerated	Benign	Neutral	Polymorphism	Likely Benign	Neutral	NEUTRAL
p.Ala216Thr (novel)	GPP (n =1)	Tolerated	Benign	Neutral	Polymorphism	Likely Pathogenic	Neutral	NEUTRAL

Table 2B: Rare splicing variant detected in the study resource

Change (rs ID)	Patient phenotype	Pathogenicity prediction					
		Max entropy	**SROOGLE**	**Senepathy**	**MutationTaster**	**CADD score[a]**	**CONSENSUS**
c.2569+4T>C (rs146678380)	APP (n=1)	Neutral	Neutral	Neutral	Disease Causing	Neutral	NEUTRAL

[a] Although the CADD algorithm does not return qualitative pathogenicity predictions, scores >15.0 are generally considered as evidence of pathogenicity;
[b] Variants were classified as pathogenic if they were predicted to be deleterious by at least four algorithms

Table 3: Genetic analysis of the p.Asp176His variant

	Allele counts (%)		
Ethnicity	**Cases**	**Controls**	***P* value**
Japanese	4/42 (9.5%)	14/844 (1.7%)	0.008
Chinese [a]	3/48 (6.2%)	4/400 (1.0%)	0.030
Meta-analysis			**8.4×10^{-5}**

[a] As the association analysis was carried out on unrelated cases, the aunt of patient T014369 was excluded from the dataset

Figure 1: A binomial calculation calculator was used to estimate the power to detect *CARD14* mutations accounting for variable proportions of disease cases. This demonstrated that sequencing exons 3 and 4 in the various patient cohorts would have >80% power to uncover mutations found in > 5% of disease cases.

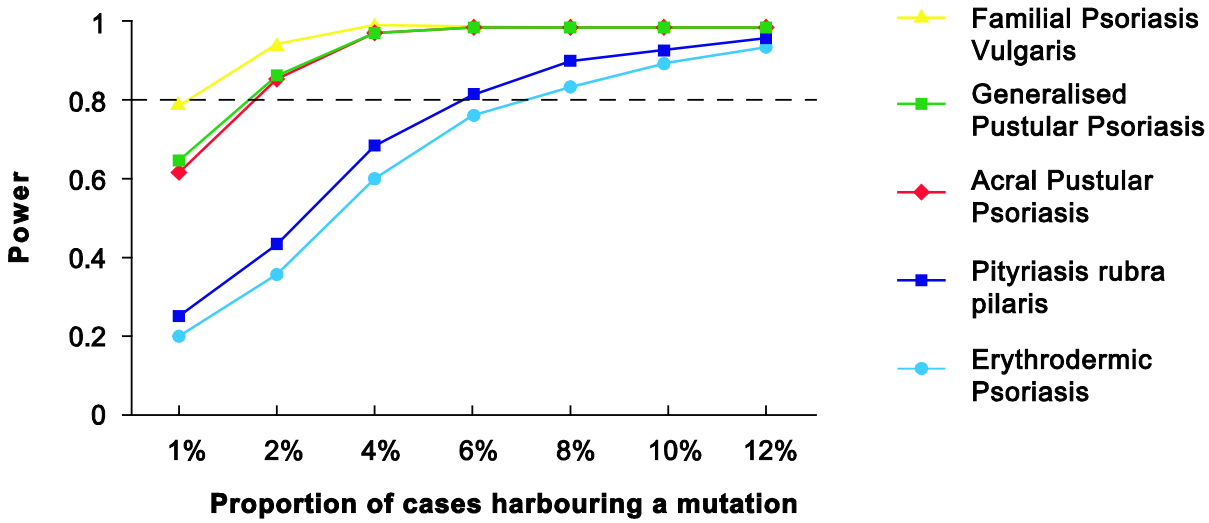
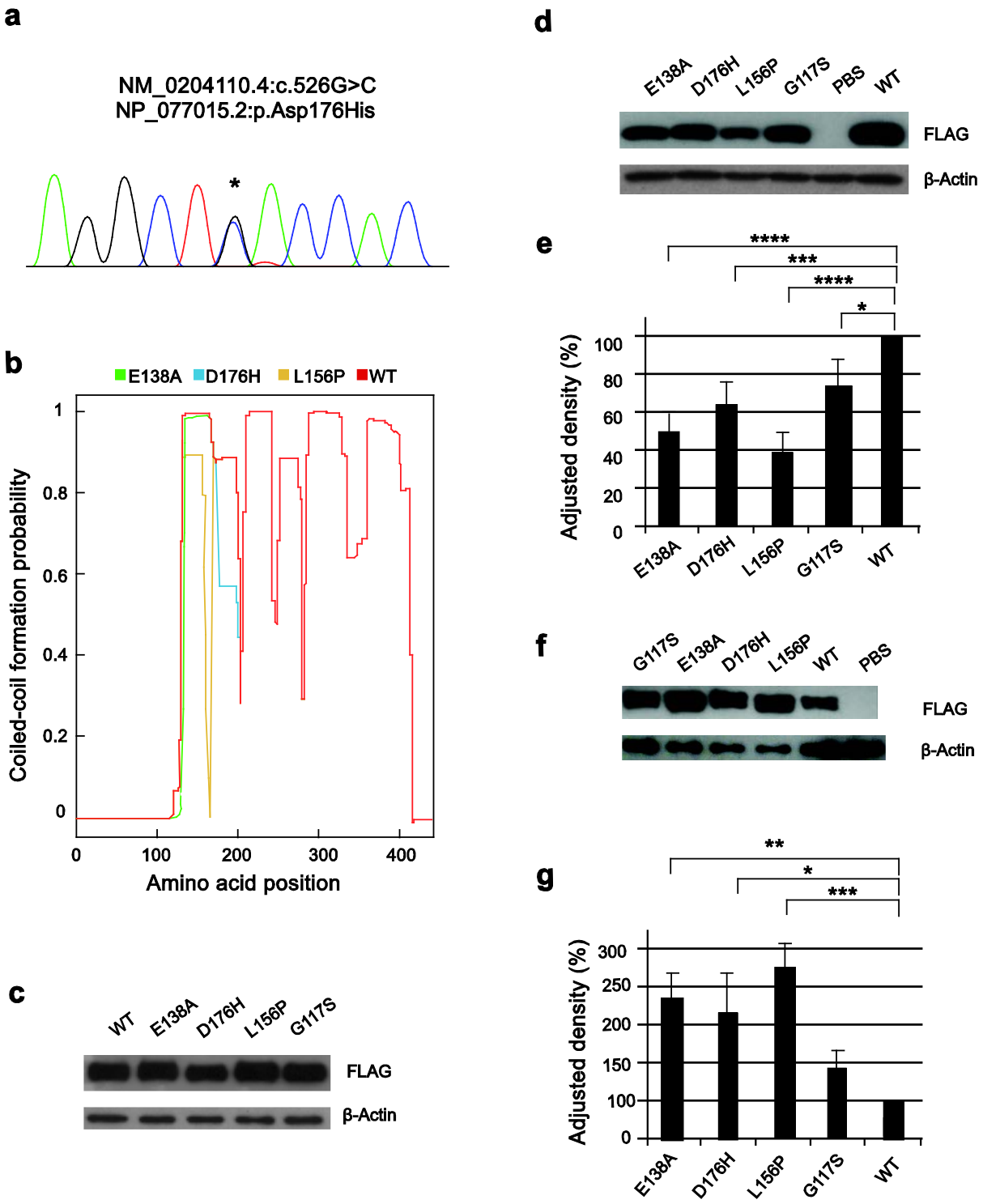


Figure 2: The p.Asp176His substitution causes spontaneous CARD14 oligomerization. A) Chromatogram showing the p.Asp176His (c.526G>C) change, which is predicted to disrupt coiled-coil formation (B). C) Western blotting of total cell lysates shows comparable amounts of wild-type and mutant CARD14. D,E) Western blotting and densitometric analysis of soluble proteins shows reduced accumulation of CC mutants (p.Glu138Ala, p.Pro156Leu, p.Asp176His) compared to wild-type CARD14 and the non-CC mutant p.Gly117Ser. F,G) Western blotting and densitometric analysis of insoluble proteins shows enhanced aggregation of CC mutants compared to wild-type CARD14 and p.Gly117Ser. All densitometry data are expressed as mean ±SD of at least two independent experiments. Comparable results were obtained when the levels of soluble (or insoluble) protein were normalised to total CARD14, rather than β-actin. **P*<0.05; ****P*<0.001; *****P*<0.0001. WT: wild-type.


a
NM_0204110.4:c.526G>C
NP_077015.2:p.Asp176His
*
b
E138A
D176H
L156P
WT
Coiled-coil formation probability
Amino acid position
c
WT
E138A
D176H
L156P
G117S
FLAG
β-Actin
d
E138A
D176H
L156P
G117S
PBS
WT
FLAG
β-Actin
e
Adjusted density (%)
****
***
****
*
E138A
D176H
L156P
G117S
WT
f
G117S
E138A
D176H
L156P
WT
PBS
FLAG
β-Actin
g
Adjusted density (%)
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*
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E138A
D176H
L156P
G117S
WT