more ideal big data platform would be a research tool not tied to a company core business model and may allow for integration of traditional data sources such as CDC data.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Analysis of *CARD14* Polymorphisms in Pityriasis Rubra Pilaris: Activation of NF-κB

Journal of Investigative Dermatology (2015) **135,** 1905–1908; doi:10.1038/jid.2015.65; published online 2 April 2015

TO THE EDITOR

Pityriasis rubra pilaris (PRP) is a rare inflammatory papulosquamous disorder manifesting with palmoplantar keratoderma and follicular hyperkeratotic papules that tend to coalesce into large, scaly, erythematous plaques often progressing to exfoliative erythroderma (Klein *et al.*, 2010; Petrof *et al.*, 2013). PRP is often misdiagnosed as psoriasis, a more common papulosquamous inflammatory disorder. Nevertheless, the two conditions, in their classic presentations, are clearly distinct, and can be distinguished by clinical findings and histopathologic features (Magro and Crowson, 1997). Clinically, PRP manifests with characteristic "sparing islands" of apparently normal skin, palmoplantar keratoderma, and follicular papules. The disease is frequently self-limiting within a few years' timeframe. Histopathology of PRP is characterized by alternating ortho- and parakeratosis rete ridges oriented in vertical and horizontal arrays ("checkerboard pattern"), acanthosis with broadened bases, follicular plugging, perivascular lymphocytic infiltrate in the dermis, and lack of neutrophils in the epidermis. Currently, there is no specific or uniformly effective treatment for PRP. Most cases of PRP are sporadic without family history, but a familial form with an autosomal dominant inheritance with partial penetrance represents $<6\%$ of all cases. We recently demonstrated that patients with the familial form of PRP harbor gain-of-function mutations in the *CARD14* gene encoding the caspase recruitment domain family, member 14 (CARD14) (Fuchs-Telem *et al.*, 2012). This protein is an activator of NF-κB (Blonska and Lin, 2011), and it has also been implicated in cases of familial psoriasis (Jordan *et al.*, 2012a, b). This study investigates whether *CARD14* mutations might also underlie cases of sporadic PRP.

Patients with PRP were solicited through a website (www.prp-support.org) that serves as a focus of PRP information exchange, frequently visited by patients. A total of 156 patients requesting enrollment were sent an institutional review board (IRB)–approved informed consent, a questionnaire, and a saliva collection kit for DNA isolation. This study was approved by the IRB of Thomas Jefferson University. Of these, 48 patients returned a

Abbreviations: CARD14, caspase recruitment domain family, member 14; cDNA, complementary DNA; PRP, pityriasis rubra pilaris; SNP, single-nucleotide polymorphism

Accepted article preview online 3 March 2015; published online 2 April 2015

Table 1. Clinical features and *CARD14* variants in patients with sporadic PRP[1]

Variant[2]	Age (y)/ sex	Age at onset/ diagnosis (y)	Duration (y)	Type[3]	SNP	Variant, (cDNA protein)	Minor allele frequency (%)	Functional consequences on the protein (bioinformatics prediction programs)				
								Polyphen-2	*SIFT*	*PMut*	*SNAP*	*PROVEAN*
1a	72/F	47/57	20	2	rs114688446	c.599G>A,	A: 0.8	Benign	Tolerated	Neutral	Neutral	Neutral
b	56/M	51/51	2	1		p.S200N						
c	62/M	57/57	3	1								
2	75/M	70/70	2	1	rs142246283	c.683T>G, p.L228R	No data	Probably damaging	Tolerated	Pathological	Neutral	Neutral
3	46/F	21/21	2	1	rs117918077	c.2044C>T, p.R682W	T: 1.2	Probably damaging	Damaging	Pathological	Non-neutral	Deleterious
4	35/F	32/32	1	1	No data	c.2406C>A, p.S802R	No data	Benign	Damaging	Pathological	Neutral	Neutral

Abbreviations: *CARD14, caspase recruitment domain family, member 14*; F, female; M, male; PRP, pityriasis rubra pilaris; SNP, single-nucleotide polymorphism; y, years.
[1]A total of 15 genomic variants were identified in the *CARD14* gene in 48 patients with sporadic PRP (Supplementary Table S1 online). The above variants have minor allele frequency of <1.5%.
[2]Note that the variant 1 was disclosed in three different families (a–c).
[3]Type 1: classic adult type; type 2: atypical presentation with prolonged manifestations.

blood or saliva sample with study documents, including written, informed patient consent. Careful review of the available clinical, photographic, and histopathologic information independently by two clinical dermatologists (HJC and MK), allowed us to establish a definitive diagnosis of PRP in 22 patients using predetermined criteria (N Ross *et al.*, manuscript in preparation). Another seven patients had findings suggestive but not definitive for PRP. Seventeen patients had findings associated with PRP, but there were insufficient data to either confirm or rule out the diagnosis by our stringent, predetermined criteria. Finally, two patients were concluded not to have PRP. None of the patients reported family history of PRP.

Genomic DNA was isolated from saliva samples or in some cases from blood by standard techniques, and the *CARD14* gene was examined by sequencing of the exons and the flanking intronic sequences by PCR utilizing specific previously published primers (Fuchs-Telem *et al.*, 2012). Initial amplification of DNA from all 48 patients focused on exons 3 and 4, previously shown to harbor a cluster of mutations in the familial form of PRP and psoriasis. In addition, the remaining 18 exons and flanking intronic sequences were determined in a subset of 20 patients who had a definitive diagnosis of PRP.

Sequencing of *CARD14* in PRP patients identified a total of 15 sequence variants, many of which were neutral and none of which resulted in premature termination codon for translation (Supplementary Table S1 online). A total of eight missense and two single-nucleotide variants within the splice site junction were evaluated by computer programs predicting the consequences of the mutations at protein levels or on mRNA splicing, as well as by comparison with the single-nucleotide polymorphism (SNP) databases. By this approach, six sequence variants were considered to be inconsequential polymorphisms present in populations at large. The remaining four sequence variants (Table 1), all present in the SNP database in the minor allelic frequency of <1.5%, were considered pathogenic (see Table 1) because (1) bioinformatics prediction programs suggested that the mutation was either damaging, or probably damaging, to the protein function (variants 2, 3, and 4), (2) the mutated amino acid is conserved in CARD14 through evolution (variant 2 from *Mus musculus* and variant 3 from *Danio rerio* to *Homo sapiens*), or (3) they have been previously reported to be present in patients with familial PRP and psoriasis (variant 1) (Fuchs-Telem *et al.*, 2012; Jordan *et al.*, 2012a, b). Variant 4 is located in the C-terminus of CARD14 that is not involved in NF-κB activation (Bertin *et al.*, 2001). Note that variant 1 (c.599G>A; p.S200N) was present in three patients and, therefore, a mutant *CARD14* allele was present in a total of 6 out of 48 patients studied (12.5%). Among these variants, p.L228R and p.S802R are previously unpublished.

To examine the consequences of three variants (nos. 1–3) as putative pathogenic mutations on the activation of NF-κB, *in vitro* assays were performed in a HeLa cell line that constitutively expresses low level of luciferase reporter under a NF-κB-responsive element (Signosis, Sunnyvale, CA) when transfected with a plasmid harboring *CARD14* complementary DNA (cDNA), either wild-type or mutant ones in which the corresponding sequence variants were introduced by QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). This approach was validated in a similar system of HEK293 cells by the analysis of two mutations (p.E138del and p.L156P) previously identified in patients with familial PRP (Fuchs-Telem *et al.*, 2012). As indicated in Figure 1a, both mutations resulted in NF-κB activation. The results with variants encountered in sporadic cases of PRP indicated that only one of the putative variants, variant 2 (c.683T>G; p.L228R), present in a

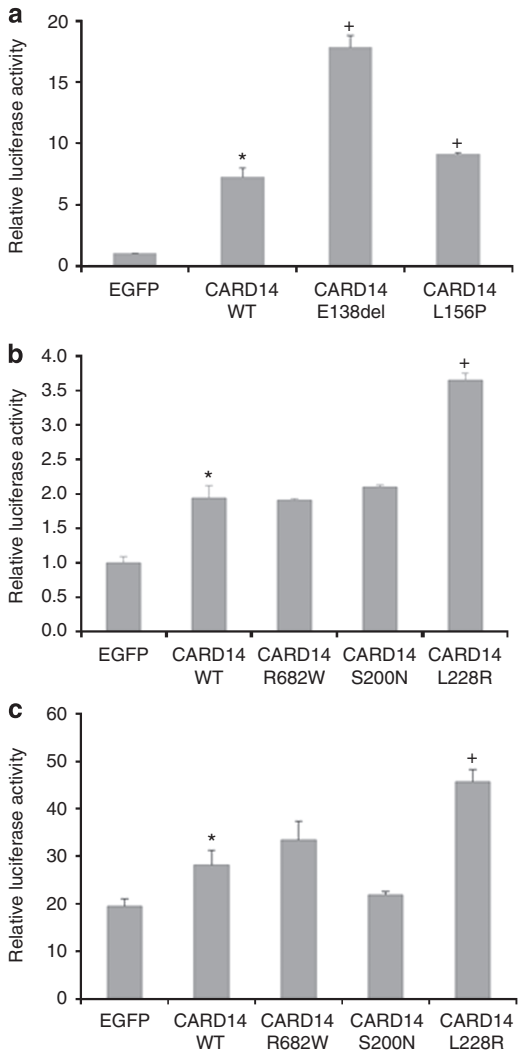

Figure 1. NF-κB activation by mutant CARD14 (caspase recruitment domain family, member 14) in cell culture systems *in vitro*. The cells were transfected with wild-type (WT) or mutant *CARD14* complementary DNA (cDNA) constructs using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA), followed by assay of luciferase activity after 24 hours of incubation. The *CARD14* cDNA (coding for 740 amino acids; GenBank BC018142) and EGFP cDNA as a control were cloned into pReceiver-M11 (Capital Biosciences, Rockville, MD) vector. (**a**) HEK293 cells were cotransfected with *CARD14* constructs together with κB-Luc plasmid (kindly provided by Professor Yinor Ben-Neriah, Hebrew University, Jerusalem, Israel) and *Renilla* luciferase plasmid expression vector. Luciferase activity was measured using Dual-Luciferase Reporter (DLT) Assay System (Promega, Mullion, WI). (**b**) HeLa cells stably expressing NF-κB luciferase reporter (Signosis) were cotransfected with the *CARD14* constructs together with pRSV-galactosidase expression plasmid as a control of transfection efficiency. (**c**) The cultures as in **b** were supplemented after 24 hours of incubation with 20 ng ml^{-1} of recombinant human tumor necrosis factor-α (TNF-α; PeproTech, Rocky Hill, NJ). After an additional 24 hours, luciferase activity was measured using Luciferase Assay System (Promega). The experiments were performed in triplicate cultures, and the values are expressed as mean ± SE. Statistical differences were evaluated by Student's two-tailed *t*-test: $^{*}P<0.05$ as compared with EGFP as a control construct; $^{+}P<0.05$ as compared with *CARD14* WT construct.

patient with definitive PRP, was capable of upregulation of the NF-κB-responsive element, as determined by the luciferase activity corrected for the transfection efficiency by β-galactosidase determination (Figure 1b). Mutations p.R682W and p.S200N were not capable of upregulation of NF-κB, consistent with previous observations (Jordan *et al.*, 2012a). This assay system was clearly functional and responsive to NF-κB activation, as incubation of the cells with recombinant human tumor necrosis factor-α (20 ng ml^{-1}), an activator of NF-κB, resulted in 10- to 20-fold upregulation of the NF-κB-responsive element (Figure 1c).

In conclusion, *CARD14* putative mutations were identified in a limited number (12.5%) of patients with sporadic PRP. This is consistent with a recent study wherein *CARD14* mutations were undetectable in eight cases of sporadic PRP (Hong *et al.*, 2014). However, recent studies on sporadic PRP, similar to sporadic psoriasis, have suggested that NF-κB signaling is activated in the epidermis of patients with PRP, even in the absence of pathogenic *CARD14* mutations (Eytan *et al.*, 2014a). There could be several explanations for the lack of identifiable mutations in the *CARD14* gene in most sporadic cases of PRP despite apparent activation of NF-κB. First, our mutation analysis is limited to exons and flanking intronic sequences, and does not detect possible mutations in the regulatory 5′-sequences or those embedded deeper in the introns. Second, it is possible that mutations in other components of the CARD14 signaling cascade, such as IKBKG/NEMO, can result in activation of NF-κB that is implicated in other genetic diseases (Conte *et al.*, 2014). Finally, NF-κB activation could occur in a CARD14-independent, noncanonical signaling pathway (Wullaert *et al.*, 2011). The importance of NF-κB signaling in the pathogenesis of PRP may have implications for development of specific therapies for the management of this therapeutically challenging disorder (Eytan *et al.*, 2014b). In summary, although NF-κB activation may be a common mechanism in inflammatory skin diseases, such as familial PRP, *CARD14* mutations may be rare in sporadic cases, and alternate mechanisms may be responsible for activation of the NF-κB signaling pathway.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by an NIH/NIAMS grant K01AR064766 and a Research Grant from Dermatology Foundation (to QL), by a grant from the Cove Charitable Trust of Boston (to JU), and donations of the Ram Family (to ES). We thank Carol Kelly for assistance with manuscript preparations, and Emad Alnemri, Richard Greene, Anthony Grieff, Ulrich Rodeck, and Joel Rosenbloom for helpful advice.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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IL-31 Induces Chemotaxis, Calcium Mobilization, Release of Reactive Oxygen Species, and CCL26 in Eosinophils, Which Are Capable to Release IL-31

Journal of Investigative Dermatology (2015) **135,** 1908–1911; doi:10.1038/jid.2015.106; published online 9 April 2015

TO THE EDITOR

Human eosinophils have an important role in the pathogenesis of allergic inflammatory diseases including atopic dermatitis (AD) and allergic diseases (Simon *et al.*, 2004; Rothenberg and Hogan, 2006; Raap and Wardlaw, 2008). Eosinophils secrete proinflammatory cytokines, chemokines, and proteins like eosinophil cationic protein (RNASE3), a protein known to correlate with disease severity in patients with AD (Kapp, 1993). Another cytokine correlating with disease severity in patients with AD is the pruritogenic IL-31 (Raap *et al.*, 2008; Raap *et al.*, 2012). IL-31 was shown to promote chronic dermatitis in mice through the induction of severe itch (Dillon *et al.*, 2004). Findings that a subpopulation of IL-31RA(+)/TRPV1(+)/TRAP1 (+) neurons mediates T-helper cell–dependent itch support the role of IL-31 in pruritus (Cevikbas *et al.*, 2014). In addition, skin IL-31 mRNA expression and IL-31 serum level correlate with Th2 cytokines including IL-4 and IL-13 in AD and acute allergic contact dermatitis (Neis *et al.*, 2006; Raap *et al.*, 2012).

Originally, IL-31 expression was shown in activated $CD4^+$ T-helper cells (Dillon *et al.*, 2004; Cornelissen *et al.*, 2012). We demonstrate the expression of IL-31 in human peripheral blood eosinophils (Figure 1a–h). Freshly isolated eosinophils from non-atopic patients, who gave written informed consent (approved by the ethics committee of the Hannover Medical School (MHH)), were cultivated with and without IL-3, and intracellular IL-31 was measured (Figure 1a, see Supplementary Material and Methods S1 online). Stimulation with IL-3, a cytokine that can enhance responses of eosinophils to various agonists (Simon *et al.*, 2004), increased the intracellular expression of IL-31 (Figure 1a). These results were confirmed with the western blot technique and the densitometric analysis of the western blot (Figure 1b and c). In addition, we determined IL-31 protein content in supernatants by ELISA (Figure 1d). The release of IL-31 increased during the time of incubation of eosinophils (Figure 1d). Similar to the densitometric analysis (Figure 1c) of the western blot, IL-3 significantly increased IL-31

Accepted article preview online 19 March 2015; published online 9 April 2015