loci ($P > 0.05$; Supplementary Table S4 online), suggesting that these SNPs act independently of *FLG*-null mutations towards the contribution of atopic disease. However, we note that our power to detect interaction may be limited by insufficient sample numbers (Purcell *et al.*, 2003).

In summary, our findings validate a strong association at chr10q21.2 and a weaker one at 20q13.33 with AD among Singaporean Chinese subjects, but suggests that genetic association at 5q22.1 might be influenced by the interaction with environmental factors. The identification of new validated AD loci is important to further dissect the genetic basis of this complex disease and account for the large number of AD cases that do not have *FLG*-null mutations. Clinically this is also vital as more genome-targeted therapies are developed.

URLs

Genetic Power Calculator: http://pngu.mgh.harvard.edu/~purcell/gpc/

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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Mutations in the SASPase Gene (*ASPRV1*) Are Not Associated with Atopic Eczema or Clinically Dry Skin

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TO THE EDITOR

A key event during epidermal differentiation is the proteolytic breakdown of profilaggrin into ''free'' filaggrin monomers. A recent study has shown that the skin-specific retroviral-like aspartic protease (SASPase) has a key role in profilaggrin–filaggrin processing (Matsui *et al.*, 2011). SASPase cleaves the linker peptide between the individual filaggrin monomers of profilaggrin, and on a *hairless* mouse background loss of SASPase leads to dry, scaly skin with reduced stratum corneum hydration accompanied by accumulations of profilaggrin–filaggrin intermediates but an absence of filaggrin monomers (Matsui

Abbreviations: ASPRV1, *SASPase gene; SASPase, skin-specific retroviral-like aspartic protease*

Table 1. Demographic and clinical data relating to eczema and dry skin cases and population controls

Demographic and clinical features	South African atopic eczema cases	Irish atopic eczema cases	Irish population controls	Scottish dry skin cases	Scottish population controls
Total number (% male)	102 (51.0)	442 (63.6)	460 (30.7)	178 (34.8)	100 (50.0)
Age in years, mean (SD) and range	8.9 (9.9)	3.3 (11.8)	35.4 (9.2)	45.5 (48.1)	⩾18
	0.7–50.9	0.25–17.0	19–69	18–86	
Eczema severity score, mean (SD) and range	10.4 (2.7)	11.3 (6.4)	0	NA	NA
	5–15	3–15			
Reported childhood eczema (%)	NA	NA	NA	16.3	NA

Abbreviations: NA, not applicable; SD, standard deviation.
All patient studies conformed to the Declaration of Helsinki Principles, and written informed consent was obtained. Irish population controls represent healthy adults from the population-based Trinity Biobank control samples; Scottish population controls are derived from adults attending hospital for hematological investigations; eczema severity is scored using the Nottingham Eczema Severity Score (Emerson *et al.*, 2000); dry skin was defined using a previously reported scoring system (Sergeant *et al.*, 2009) and corresponds to visible fine scale (noted by a trained observer) on one/more body sites, self-reported use of a moisturizer more than once weekly, or self-reported dry skin ''moderately'' to ''a lot''.

Table 2. dbSNP minor allele frequencies of *ASPRV1* polymorphisms identified in the discovery cohorts

ASPRV1 mutation	dbSNP minor allele	rs number	Irish atopic eczema cases (*n*=92)	South African atopic eczema cases (*n*=90)	Scottish dry skin cases (*n*=93)
c.145 A>G[1] p.T49A	A	rs3796097	*A*=0.418	*A*=0.083	*A*=0.435
c.155 G>A p.R52Q	NA	rs151323610	*A*=0.005	0	0
c.220 G>A p.V74I			*A*=0.005	0	0
c.259 G>A p.G87R	NA	rs148290351	0	0	*A*=0.005
c.618 C>T p.P206P	T	rs114182672	0	*T*=0.005	0
c.973 C>T p.L325 L	T	rs115036001	0	*T*=0.111	0
c.998 C>T p.S333F			0	0	*T*=0.005

Abbreviations: dbSNP, Single-Nucleotide Polymorphism Database; *n*, the number of fully sequenced samples, NA, minor allele not ascertained.
[1]G is the ancestral allele and A is the designated minor allele for this mutation.The *ASPRV1* gene was amplified for sequencing using forward primer 5′-ATGTGGTAGGAGCTCAGTACATGTAAAC-3′ and reverse primer 5′-AGAAGAGCAAGAGTTGATAAGCAGACTG-3′ to generate a 1,532-bp product. 50 ng of genomic DNA was amplified in a 25 μl reaction using 0.5 U AmpliTaq Gold polymerase (Applied Biosystems). For PCR amplification, an annealing temperature of 65°C and a 3 minute extension at 72°C was used (35 cycles). PCR products were purified and sequenced using overlapping primers in both directions: Forward 1 5′-TTCCTTCACTGGCTGATGAC-3′; Forward 2 5′-TTGCTGCTGAGGTTCCAGAG-3′; Forward 3 5′-TCACTGATGGCGATCTGGAC-3′ and Reverse 1 5′-AGAAGAGCAAGAGTTGATAAGC-3′; Reverse 2 5′-CCCAGGATCTTCATTTCAGC-3′ Reverse 3 5′-GATGACTTCAAAGCTGTGCAG-3′.

et al., 2011). In this same study, several missense mutations in the SASPase gene in atopic eczema patients and controls were identified, some of which were shown to have a detrimental effect on the ability of SASPase to cleave the profilaggrin linker peptide. Given the important role of filaggrin in skin barrier function and maintaining stratum corneum hydration (O'Regan and Irvine, 2010), these results prompted us to question whether aberrant profilaggrin–filaggrin processing due to altered SASPase activity could provide an alternative pathogenic mechanism for atopic eczema or clinically dry skin.

To answer this question, the entire coding region of the SASPase gene, *ASPRV1*, was amplified by PCR in a single fragment and fully sequenced. To maximize our chances of finding mutations that might be associated with atopic eczema or clinically dry skin, we sequenced *ASPRV1* from three discovery cohorts; 96 pediatric atopic eczema cases from Ireland, 96 atopic eczema cases from the Cape Town region of South Africa (Xhosa people), and 99 cases of clinically dry skin from patients referred to dermatology clinics in Glasgow, Scotland. Atopic eczema in the Irish pediatric cases was diagnosed using the UK Diagnostic criteria (Williams *et al.*, 1994); atopic eczema in the Xhosa people was diagnosed by experienced dermatologists. Clinically dry skin was defined using a previously published scoring system (Sergeant *et al.*, 2009). Demographic and clinical data relating to the discovery cohorts are shown in Table 1.

Sequencing of the *ASPRV1* gene in the discovery cohorts identified a total of five non-synonymous mutations and two synonymous mutations (Table 2). None of the *ASPRV1* mutations identified in a previous Japanese study (Matsui *et al.*, 2011) were detected in our discovery cohorts. We then investigated some of these mutations further by screening an additional 259 Irish atopic eczema cases and 167 Scottish dry skin cases (which included the original 93 cases from the discovery cohort) using custom-designed TaqMan allelic discrimination assays for the mutations V74I, G87R, and S333F

(Supplementary Table S1 online). The G87R mutation was identified only in a single case of atopic eczema, and the V74I and S333F mutations were not detected in any of these additional cases. In the 167 cases of dry skin, the G87R and S333F mutations were found only in single cases (i.e., in the original discovery cohort) and the V74I mutation was not found in any of the cases. We then carried out two independent case–control studies to investigate any association between the T49A mutation and atopic eczema and clinically dry skin (Supplementary Table S2 online). Overall, 442 Irish atopic eczema cases (which included the original 92 cases from the discovery cohort) and 458 Irish population controls were screened using a TaqMan allelic discrimination assay. There was no association between the T49A mutation and atopic eczema in the Irish study: χ^2-test $P=0.415$, odds ratio $=0.98$ (95% confidence interval 0.81–1.18). Similarly, screening of 167 clinically dry skin cases and 100 Scottish population controls failed to reveal any association between the T49A mutation and dry skin: $P=0.479$, odds ratio $=0.90$ (0.63–1.28). Power calculations showed that the eczema case–control study had $>80\%$ power to detect an odds ratio of 1.5 or above and the dry skin case–control study had $>70\%$ power to detect an odds ratio of 2.0 (Quanto 1.2.4, University of Southern California, http://hydra.usc.edu/gxe/). As *FLG*-null mutations are known to have such a strong effect on eczema risk, it is possible that the effect of *ASPRV1* mutations may only be apparent in *FLG* wild-type individuals. Therefore, the four most prevalent *FLG*-null mutations (R501X, 2282del4, R2447X, and S3247X) were screened in each of the cases and controls using methods described previously (Sandilands *et al.*, 2007; Kezic *et al.*, 2011). The statistical analyses for each study were repeated after excluding individuals carrying *FLG*-null mutations, but there was still no evidence of association between *ASPRV1* mutation T49A and eczema or clinically dry skin (Supplementary Table S3 online).

With the exception of T49A and to a lesser extent L325L, the remaining *ASPRV1* mutations that we identified were rare ($<1\%$) and therefore unlikely to be significant on a population level, although it is still possible that these rare mutations could contribute significantly to individual disease risk. Mutations P206P and L325L result in synonymous changes and are therefore unlikely to be pathogenic. All of the non-synonymous mutations we identified (Supplementary Figure S1 online) affect amino acid residues outside the active protease site of SASPase (Bernard *et al.*, 2005); however, the effect of these mutations on SASPase activity remains to be determined experimentally.

Finally, we used custom-designed TaqMan allelic discrimination assays (Supplementary Table S1 online) to screen for the V187I and V243A mutations, which reduce and abolish SASPase-mediated profilaggrin cleavage, respectively (Matsui *et al.*, 2011). However, we failed to detect these mutations in any of the discovery cohorts, nor in the Irish atopic eczema cohort, indicating that these mutations are likely to be specific to the Japanese population.

Although our results failed to find an association between *ASPRV1* gene mutations and atopic eczema or clinically dry skin in the European populations that we studied, they do not exclude the possibility that an association exists in other ethnicities. In the populations that we studied, other factors that modulate SASPase activity could contribute instead, such as the actions of protease inhibitors that provide a powerful counterbalance against excessive protease activities (Hewett *et al.*, 2005). Profilaggrin-filaggrin processing is a tightly regulated process involving not just SASPase but multiple proteases such as elastase 2 (Bonnart *et al.* 2010) and the serine proteases matriptase/MT-SP1 (List *et al.*, 2003) and prostasin (Leyvraz *et al.*, 2005). A greater understanding of the proteases and inhibitors involved in profilaggrin-filaggrin processing will be required to fully appreciate their contribution to skin barrier dysfunction.

CONFLICT OF INTEREST

WHIM and CSM have filed patents related to genetic testing and therapy development for the filaggrin gene. The other authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

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Lack of Evidence for Association of VEGF Polymorphisms in Swedish Patients with Psoriasis

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TO THE EDITOR

The vascular contribution to the pathogenesis of psoriasis has generally received limited attention, although abnormalities in cutaneous vessels in psoriatic lesions are a prominent feature. The dermal blood vessels in psoriatic lesions are found to be tortuous, enlarged, and hyperpermeable (Creamer *et al.*, 2002; Heidenreich *et al.*, 2009). Interestingly, these microvascular changes occur very early in the development of the psoriatic lesion, suggesting that angiogenesis is one of the key features of psoriasis pathogenesis (Creamer *et al.*, 2002). In 1994, vascular endothelial growth factor (VEGF) was identified as a major epidermis-derived growth factor that was strongly upregulated in skin lesions of psoriasis (Detmar *et al.*, 1994). Previous studies have demonstrated increased serum levels of VEGF in psoriasis patients that correlated to disease severity (Creamer *et al.*, 1996; Nielsen *et al.*, 2002; Heidenreich *et al.*, 2009). Recently, serum levels of VEGF and its soluble receptor sVEGFR1 were significantly increased in psoriasis, with the highest levels in patients with a psoriasis area and severity index score higher than 20 (Flisiak *et al.*, 2008). In contrast, we and others have demonstrated no significant increase in VEGF serum levels in patients with mild-to-moderate psoriasis (Barile *et al.*, 2006; Anderson *et al.*, 2010). Moreover, VEGF-overexpressing transgenic mice displayed an increased density of tortuous capillaries and were shown spontaneously to develop an inflammatory skin disorder resembling psoriasis (Xia *et al.*, 2003).

The increasing evidence for the role of VEGF in the pathogenesis of psoriasis has led to interest in VEGF as a modifier gene in psoriasis. Certain genetic variants of VEGF may represent an ''angiogenic constitution'' with an increased risk of developing psoriasis. In line with this hypothesis, recent studies have indicated that distinct single-nucleotide polymorphisms (SNPs) in the VEGF gene are linked with reduced or increased expression levels (Watson *et al.*, 2000; Stevens *et al.*, 2003).

In a large and well-defined family-based patient sample, we have aimed comprehensively to characterize variations throughout the VEGF gene. Haplotype-tagging SNPs were selected for genotyping from a 19.7 kb region using the HapMap release 27 data (www.hapmap.org) and the Tagger utility of Haploview (www.broad.mit.edu/mpg/tagger). The 19.7 kb region

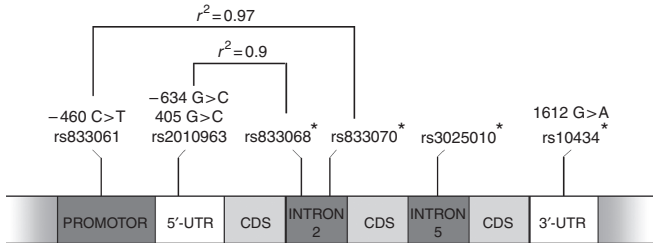

Figure 1. Structure of the *VEGFA* gene and the positions of the tSNPs analyzed in this study. tSNP, tag single-nucleotide polymorphism; UTR, untranslational region.

Abbreviations: SNP, single-nucleotide polymorphism; VEGF, vascular endothelial growth factor