Targeted Resequencing and Functional Testing Identifies Low-Frequency Missense Variants in the Gene Encoding GARP as Significant Contributors to Atopic Dermatitis Risk

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Gene-mapping studies have consistently identified a susceptibility locus for atopic dermatitis and other inflammatory diseases on chromosome band 11q13.5, with the strongest association observed for a common variant located in an intergenic region between the two annotated genes *C11orf30* and *LRRC32*. Using a targeted resequencing approach we identified low-frequency and rare missense mutations within the *LRRC32* gene encoding the protein GARP, a receptor on activated regulatory T cells that binds latent transforming growth factor-β. Subsequent association testing in more than 2,000 atopic dermatitis patients and 2,000 control subjects showed a significant excess of these *LRRC32* variants in individuals with atopic dermatitis. Structural protein modeling and bioinformatic analysis predicted a disruption of protein transport upon these variants, and overexpression assays in $CD4^+CD25^-$ T cells showed a significant reduction in surface expression of the mutated protein. Consistently, flow cytometric (FACS) analyses of different T-cell subtypes obtained from atopic dermatitis patients showed a significantly reduced surface expression of GARP and a reduced conversion of $CD4^+CD25^-$ T cells into regulatory T cells, along with lower expression of latency-associated protein upon stimulation in carriers of the *LRRC32* A407T variant. These results link inherited disturbances of transforming growth factor-β signaling with atopic dermatitis risk.

Journal of Investigative Dermatology (2016) ■, ■–■; doi:10.1016/j.jid.2016.07.009

INTRODUCTION

Atopic dermatitis (AD) is the most common chronic inflammatory skin condition. Although the pathomechanisms of AD are not fully understood, it is widely agreed that both abnormalities of the epidermal barrier function and a dysregulation of T-cell immunity with an imbalance of effector and regulatory T cell (Treg) responses lead to chronic inflammation, which extends beyond the skin and contributes to an increased risk for inflammatory comorbidities such as asthma, inflammatory bowel disease, and arthritis (Weidinger and Novak, 2016).

The immune abnormalities observed in AD and its common comorbidities are in part genetically predetermined, as evidenced by shared susceptibility genes that regulate T-cell differentiation and effector function or encode components of the innate immune system. Among such shared susceptibility regions is the chromosome band 11q13.5 locus, where variants located between the two annotated genes, *C11orf30* (coding for the protein EMSY) and *LRRC32* (coding for the protein GARP), have been associated with AD, asthma, Crohn's disease, ulcerative colitis, allergic sensitization, allergic rhinitis, eosinophilic esophagitis, and alopecia areata (Anderson et al., 2011; Barrett et al., 2008; Betz et al., 2015; Bonnelykke et al., 2013; Ellinghaus et al., 2013; Esparza-Gordillo et al., 2009; Ferreira et al., 2011; Franke et al.,

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Abbreviations: AD, atopic dermatitis; C11orf30, chromosome 11 open reading frame 30; GARP, glycoprotein A repetitions predominant; LAP, latency associated protein; LRRC32, leucine rich repeat containing 32; PBS, phosphate-buffered saline; SNV, single nucleotide variant; TGF-β, transforming growth factor-beta; Treg, regulatory T cell; WT, wild type

Received 22 January 2016; revised 14 June 2016; accepted 5 July 2016; accepted manuscript published online 21 July 2016; corrected proof published online XXX

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2010; Kottyan et al., 2014; Paternoster et al., 2012; Ramasamy et al., 2011; Weidinger et al., 2013). The causal gene and variants underlying this association signal, however, are yet unknown. Both genes located next to the associated markers encode proteins that represent promising biological candidates. EMSY has been reported to regulate nuclear receptor-mediated transcription (Garapaty et al., 2009) and interferon-stimulated gene expression (Ezell et al., 2012), and GARP encodes a surface receptor on activated Tregs that binds latent TGF-β (transforming growth factor-β) and modulates peripheral tolerance and T effector cell function (Tran et al., 2009; Wang et al., 2012). We set out to refine the 11q13.5 disease association signals using a combination of sequencing and functional annotation.

RESULTS AND DISCUSSION

Targeted next-generation sequencing of 11q13.5 (chromosome 11:75,800,000−76,070,000; NCBI36/hg18) in 31 AD patients selected from the original genome-wide association study (Esparza-Gordillo et al., 2009) and enriched for low-frequency-risk haplotypes and subsequent validation by Sanger sequencing identified two missense variants (A407T/rs79525962, R518W/rs142940671) in *LRRC32* with a minor allele frequency less than 0.01 in the 1000 Genomes Project (The International Genome Sample Resource, 2016) and/or without annotation in dbSNP 132 (National Center for Biotechnology Information, 2016). An extended mutational screening of the coding regions of *LRRC32* in 100 independent AD patients identified four additional rare missense single nucleotide variants (SNVs): R312C/rs371900727, S411R/rs201431152, R414W (without annotation in dbSNP 132), and R652C/rs143082901. No rare missense variants fulfilling our filter criteria were identified in *C11orf30*. All six *LRRC32* variants were carried forward to frequency assessment and association testing in an independent set of 2,193 German AD patients and 2,197 control subjects from the German population-based PopGen cohort (Krawczak et al., 2006) (see Supplementary Table S1 online). Cumulative minor allele tests showed a significant excess of rare alleles in AD ($P = 0.002$, allele carriers AD = 6.1% vs. allele carriers control subjects = 4.2%), and a suggestive significant association for allergic sensitization as an intermediate trait ($P = 0.02$). The most frequent variant, A407T/rs79525962, showed an odds ratio of 1.46 (95% confidence interval = 1.11−1.92, $P = 0.007$). A407T/rs79525962 is in complete linkage disequilibrium ($D' = 1$) with previously reported genome-wide association study risk SNPs for different inflammatory traits within the 11q locus (see Supplementary Table S2 online).

To predict the functional consequences of the identified variants, we performed structural protein modeling based on the toll-like receptor regulator CD180. The 21 leucine-rich repeats of the GARP extracellular domain likely fold into an alpha/beta horseshoe shape, in which leucine-rich beta-strands form a characteristic concave inner surface (see Supplementary Figure S1 online). GARP disulfide links with latent TGF-β, but noncovalent bonds are also sufficient for protein-protein interaction (Wang et al., 2012), and several N-glycosylation sites have been predicted or verified for GARP (Chen et al., 2009).

Analysis of *LRRC32*/GARP mRNA expression levels in different tissues and cell types, including skin, fibroblasts, keratinocytes, and peripheral blood mononuclear cells showed an ubiquitous expression with, however, variable levels (see Supplementary Figure S2 online).

All six identified SNVs lead to changes in the physicochemical properties of the affected amino acids and may consequently interfere with proper protein folding and function. The loss of positive charge on the protein surface as a consequence of arginine mutations (R312C, R414W, and R518W) may disturb potential protein interactions. Further, missense variants may cause local structural rearrangements near N-glycosylation sites that may hamper posttranslational modifications. Therefore, the identified *LRRC32* missense variants are not assumed to directly interfere with TGF-β binding but rather obstruct proper protein folding or hamper posttranslational modifications necessary for protein transport.

The most frequent variant, A407T/rs79525962, was subjected to further functional studies. *LRRC32* mRNA expression did not differ significantly between mutant LRRC32 (A407T)− or wild-type (WT) LRRC32−transfected $CD4^+CD25^-$ T cells of four independent healthy donors (see Supplementary Figure S3 online). However, flow cytometric analyses (FACS) showed a significant lower expression on the cell surface (Bonferroni-Holm corrected P-value = 0.044) (Figure 1) and a significant higher intracellular amount of the protein (Bonferroni-Holm corrected P-value = 0.020) in A407T-LRRC32-transfected $CD4^+CD25^-$ T cells than in WT LRRC32-transfected T cells (see Supplementary Figure S4 online). Likewise, immunofluorescence microscopy analysis in Cos-7 cells indicated lower surface expression and intracellular retention of mutated GARP (Figure 2). These observations indicate that protein production per se is not affected by the mutation, but in line with bioinformatics predictions, the folding/stabilization or posttranslational modifications necessary for the transport of GARP to the cell surface are impaired. Simultaneous measurement of additional Treg markers showed a significantly lower expression of CTLA4 ($P = 0.034$) and a tendency toward reduced FOXP3 and neuropilin expression in A407T-LRRC32-transfected $CD4^+CD25^-$ T cells, indicating an overall dampening of the Treg gene signature (see Supplementary Figures S5 and S6 online).

FACS analyses of unstimulated T-cell subtypes obtained from AD patients (n = 13) showed a significantly lower extracellular GARP expression on $CD3^+$ ($P = 0.036$), $CD4^+CD25^-$ ($P = 0.028$), and $CD4^+CD25^+$ T cells ($P = 0.035$) for A407T mutation carriers (Figure 3). After treatment of $CD4^+CD25^-$ T cells obtained from AD patients carrying the A407T variant with TGF-β, $CD25^+FOXP3^+$ T cells showed a significantly lower surface expression of GARP ($P = 0.017$) than cells from WT carriers (see Supplementary Figure S7 online).

Further, we observed a lower conversion rate of $CD4^+CD25^-$ T cells into $GARP^+FOXP3^+$ Tregs ($P = 0.004$), and a lower expression of latency-associated protein (LAP) on $GARP^+FOXP3^+$ T cells ($P = 0.026$) in carriers of A407T after stimulation with soluble GARP (see Supplementary Figure S8 online).

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