Targeted Deep Sequencing Identifies Rare 'loss-of-function' Variants in *IFNGR1* for Risk of Atopic Dermatitis Complicated by Eczema Herpeticum

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Abstract

Background—A subset of atopic dermatitis (AD) is associated with increased susceptibility to eczema herpeticum (ADEH+). We previously reported that common single nucleotide polymorphisms (SNPs) in interferon-gamma (*IFNG*) and receptor 1 (*IFNGR1*) were associated with ADEH+ phenotype.

Objective—To interrogate the role of rare variants in IFN-pathway genes for risk of ADEH+.

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Methods—We performed targeted sequencing of interferon-pathway genes (*IFNG*, *IFNGR1*, *IFNAR1* and *IL12RB1*) in 228 European American (EA) AD patients selected according to their EH status and severity measured by Eczema Area and Severity Index (EASI). Replication genotyping was performed in independent samples of 219 EA and 333 African Americans (AA). Functional investigation of 'loss-of-function' variants was conducted using site-directed mutagenesis.

Results—We identified 494 single nucleotide variants (SNVs) encompassing 105kb of sequence, including 145 common, 349 (70.6%) rare (minor allele frequency (MAF) <5%) and 86 (17.4%) novel variants, of which 2.8% were coding-synonymous, 93.3% were non-coding (64.6% intronic), and 3.8% were missense. We identified six rare *IFNGR1* missense including three damaging variants (Val14Met (V14M), Val61Ile and Tyr397Cys (Y397C)) conferring a higher risk for ADEH+ (*P*=0.031). Variants V14M and Y397C were confirmed to be deleterious leading to partial *IFNGR1* deficiency. Seven common *IFNGR1* SNPs, along with common protective haplotypes (2 to 7-SNPs) conferred a reduced risk of ADEH+ (*P*=0.015-0.002, *P*=0.0015-0.0004, respectively), and both SNP and haplotype associations were replicated in an independent AA sample (*P*=0.004-0.0001 and *P*=0.001-0.0001, respectively).

Conclusion—Our results provide evidence that both genetic variants in the gene encoding *IFNGR1* are implicated in susceptibility to the ADEH+ phenotype.

CAPSULE SUMMARY—We provided the first evidence that rare functional *IFNGR1* mutations contribute to a defective systemic IFN-γ immune response that accounts for the propensity of AD patients to disseminated viral skin infections.

Keywords

IFNGR1; genetic variants; atopic dermatitis; eczema herpeticum

INTRODUCTION

Atopic dermatitis (AD) is the most common chronic inflammatory skin disease affecting up to 17% of children in industrialized countries[1-6]. Eczema herpeticum (EH), a rare but serious complication of AD, occurs in less than 3% of AD patients[7]. Although it is well known that the primary predisposing factor for a history of EH (ADEH+) is herpes simplex virus-1 (HSV-1) exposure[8], previous studies have demonstrated that genetic susceptibility (*e.g.*, null mutation R501X in the *FLG* gene[9, 10]) contributes to risk.

Interferons (IFNs) are involved in many host defense functions including antiviral and antimicrobial response, apoptosis, cell cycle control and mediating the action of other cytokines[11]. There are three classes of IFNs, type I, II and III. IFN gamma (IFN-γ), the only Type-II interferon, is a proinflammatory cytokine that modulates many immune-related genes[12, 13]. Furthermore, IFN-γ is a T helper type 1 (Th1) cytokine that plays a major role in the host innate and adaptive immune responses by activating macrophages, enhancing natural killer cell activation, promoting T-cell differentiation, as well as regulating B-cell isotype switching, and is implicated in the pathogenesis of allergic diseases[14-16]. IFN receptors are required for IFNs to exert their biological activity and therefore play a critical role in IFN signaling[17, 18].

Previous studies in the NIH/NIAID supported Atopic Dermatitis Research Network (ADRN) found that patients with ADEH+ have markedly reduced levels of IFN-γ compared with AD patients without a history of EH (ADEH−). We also observed that reduced IFN-γ production was associated with *IFNG* (gene encodes IFN-gamma) and interferon gamma receptor 1 (*IFNGR1*) SNPs[6]. Moreover, global gene expression analysis showed that both *IFNGR1* and interferon alpha receptor 1 (*IFNAR1*) were downregulated in vaccinia virus-challenged ADEH+ PBMCs[6]. Separately, we have observed that PBMCs from patients with ADEH+ respond poorly to interferon alpha and gamma, and some are poor responders to IL-12 stimulation[19].

To further interrogate the role of genetic variation in IFN-pathway genes and risk of ADEH +, we performed targeted deep sequencing in a sample of AD patients with (ADEH+) and without EH (ADEH−) to identify common and rare variants associated with ADEH+ susceptibility. Included in the sequencing was IFN-γ (*IFNG*), interferon receptors (Type-I IFN receptor *IFNAR1* and Type-II IFN receptor *IFNGR1)* and *IL12RB1*, the gene encoding the b1 subunit of the IL-12 receptor expressed on NK and T cells[20]. Validation of variants significantly associated with ADEH+ was performed by either genotyping or Sanger sequencing, and exploration of the functional relevance of damaging variants was interrogated by *in vitro* studies including gamma-IFN activation sequence (GAS) reporter gene assays, testing of IFN-γ responsiveness in IFNgR1 (−/−) cells reconstituted with mutant constructs, and STAT1 phosphorylation upon IFN-γ stimulation.

METHODS

Study subjects and phenotypes

Study subjects for discovery sequencing were recruited through the NIH/NIAID-supported ADRN and included 228 unrelated European American AD patients selected according to their EH status (clinical definition stated in reference 7) and severity measured by Eczema Area and Severity Index (EASI) scores: ADEH+ subjects with the highest Eczema Area and EASI scores (n=121) and AD without a history of EH (ADEH−) subjects with the lowest EASI (n=107, Table 1 and Supplementary Figure 1 in the Online Repository). The total EASI score is calculated by combining information on intensity (redness, thickness, scratching and lichenification) and percentage area affected by eczema within each of the 4 body regions (head and neck, upper limbs, trunk, and lower limbs). To determine whether significantly associated SNVs were specific to risk of ADEH+, we genotyped or sequenced an additional 60 European American ADEH− patients, 159 non-atopic controls, and an independent sample of 169 African American ADEH− patients and 164 non-atopic, healthy controls also participating in ADRN, and for whom DNA samples were available.

AD was diagnosed using the US consensus conference criteria[21]. ADEH+ subjects were defined as subjects with AD who had a history of at least 1 EH episode. ADEH− subjects were defined as subjects with AD with a negative history of EH. Healthy, non-atopic controls were defined as having no personal history of chronic disease including atopy. All study participants were further evaluated by a detailed history and physical examination as well as a questionnaire to assess history of cutaneous viral infections and concomitant medication use.

The study was approved by the Institutional Review Boards at the National Jewish Health, Johns Hopkins University School of Medicine, Oregon Health and Science University, University of California, San Diego, Children's Hospital of Boston, Ann & Robert H. Lurie Children's Hospital of Chicago, and University of Rochester. All subjects gave written informed consent before participation.

DNA sequencing

DNA was extracted using standard protocols. Targeted deep sequencing (>40x) of 4 prioritized genes in the interferon pathway was performed on an Illumina HiSeq2000 at the Center for Inherited Disease Research (CIDR) at Johns Hopkins University (*see* Supplementary methods). Standard Agilent protocols were used to design the target capture for a total of ~105 kb of sequence representing the four interferon pathway genes (the gene itself and 2 kb both up and down-stream), excluding repeat elements. DNA fragmentation was performed on 100ng-1ug of genomic DNA using a Covaris E210 system and 'libraries' were prepared; the Agilent Bioanalyzer (HiSensitivity) was used for quality control (fragment size and DNA quality). The high quality next-generation sequencing (NGS) data were processed (*e.g.*, quality control (QC) and variant calling) via an automated pipeline CIDRSeqSuite 2.0, a set of software tools designed to perform secondary and initial tertiary analysis on NGS data from the Illumina HiSeq instrument (*see* Supplementary methods). The GVS annotation server[22], maintained at the Seattle SNPs at the University of Washington, was used for variant annotation. Variants were annotated for 1) position (based on their hg19 position); 2) functional prediction (intronic, coding synonymous, missense, splice site, UTR); 3) novelty (previously reported or not, compared to the HapMap[23] (phase 2, release 24) CEU (Utah residents with ancestry from northern and western Europe from the CEPH collection) and YRI (Yoruba in Ibadan, Nigeria) samples and to pilot data from the 1,000 Genomes Project[24]); and 4) rarity (variants with a minor allele frequency (MAF) <5% were considered as rare). Results were cataloged based on annotation at a gene-based level.

Genotyping

Validation genotyping was performed on both the common and rare variants identified by targeted sequencing in the same discovery cohort (n=228). Replication genotyping was performed to test for association with risk of AD in two independent samples of AD patients (167 European American and 169 African American) and healthy controls (159 European American and 164 African American) who participated in the ADRN. Three out of seven common *IFNGR1* variants (rs11914 (coding-synonymous), rs10457655 (intronic) and rs28515059 (C-1179T)), which had not been previously reported for association with ADEH +, were selected for validation (Table 3). Two variants (rs11914 and rs10457655), along with the three rare missense variants (Val14Met, Val61Ile and Tyr397Cys) were validated using TaqMan Allelic Discrimination Assays on the 7900HT Sequence Detection System (Applied Biosystems), as previously described[6]. Promoter variant rs28515059 (C-1179T) was not compatible with the TaqMan assay and was detected by Sanger sequencing. Briefly, amplicon was designed using Primer3 (http://frodo.wi.mit.edu/primer3/) with default parameters (forward primer sequence: 5′-ACCGTTTAGGCGTCTATGGAG-3′, reverse primer sequence: 5′-CCCATTGCTCTCCTTGGGAAT-3′). PCR amplification was carried

out in 25-µL reactions with 1X Taq Gold Buffer, 0.6 µM dNTPs, 0.125 µM forward and reverse primers, 40 ng of DNA, and 1.25 UTaq Gold polymerase (Life Technologies). The reaction was then cycled with the following conditions: initial denaturation at 94°C for 12 min; 40 cycles at 94°C for 20 sec, 54°C for 20 sec, and 72°C for 20 sec; final extension at 72°C for 10 min. PCR products were purified using Exonuclease III and Shrimp Alkaline Phosphatase (Promega, Madison, WI). Automated sequencing was performed by capillary electrophoresis on an ABI3700 (Applied Biosystems) at the Johns Hopkins Genetic Resources Core Facility and all variants were manually called by visual inspection of the electropherogram, using Sequencher software (Gene Codes Corp., Inc.).

Functional testing of *IFNGR1* 'loss-of-function' variants

Site-directed mutagenesis—Mammalian expression construct of wild type IFNgR1 (IFNgR1WT) in pCMV6-entry vector (RC202761) was purchased from Origene Technologies, Inc. (Rockville, MD). We then used restriction endonuclease Sgl I and Mlu I cut IFNgR1WT cDNA fragment and sub-cloned into pLenti-C-Myc-DDK vector (Origene Technologies, Inc.). We used pLenti-C-Myc-DDK-IFNgR1WT as backbones and QuickChangeII XL Site-directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) to generate constructs expressing IFNgR1 gene variants. The primers for making IFNgR1Y397C were: forward, 5′-cgctttaaactcgT**G**Tcactccagaaattg-3′; reverse, 5′-caatttctggagtgacacgagtttaaagcg-3′. The primers for making IFNgR1V14M were: forward, 5′-gtcatgcagggt**A**TGagcagggctg-3′; reverse, 5′-cagccctgctcataccctgcatgac-3′. IFNGR1V14MY397C was made by the two sets of primers. All constructs were sequence confirmed and plasmids were purified by Endofree Plasmid Maxi Kit (Qiagen).

GAS reporter gene assay—293 FT cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5g/L glucose, L-glutamine, sodium pyruvate, 10% of fetal bovine serum and penicillin/streptomycin (50 units/ml/50ug/ml) (Life Technologies). The cells were seeded in 24 well dishes the day before transfection. 1 µg plasmids of WT, IFNgR1Y397C, IFNgR1V14M, and IFNGR1V14MY397C with 0.1 µg GAS reporter plasmid and 0.04 ug RSV-Renilla luciferase control vector were co-transfected into each well of 293FT cells using Lipofectamine® 2000 reagent (Life Technologies) as the manufacturer instructed. Luciferase assays were carried out using the Dual-Glo® Luciferase Assay System (Promega) following the manufacture's guideline. The luciferase intensity was read by a GloMax-Multi+Microplate Multi-mode Reader (Promega).

STAT1 phosphorylation upon IFN-γ stimulation—50 µg plasmids of IFNgWT, IFNgR1Y397C, IFNgR1V14M, and IFNGR1V14MY397C were transfected into 4×10^7 human IFNgR1 deficient EBV-line (a kind gift from Dr. Jean-Laurent Casanova, Rockfeller University)[25] using an electroporation approach. The cells were suspended in Hepes-buffered saline and transferred into gap 0.4cm cuvettes, then pulsed at voltage 0.300kV and capacitor 960µF using a Gene Pulser Xcell Electroporation Systems (Bio-RAD). After the pulse, cells were recovered in RPMI 1640 with 20% fetal bovine serum and penicillin/streptomycin (50 units/ml/50ug/ml) (Life technologies) for two days. The cells were then stimulated with serial dilution of recombinant human IFN-γ (R&D systems) for 30 minutes at 37°C. After stimulation, the cells were lysed in RIPA buffer supplemented with protease

inhibitor and phosphatase inhibitor (Sigma-Aldrich). 100 µg protein from each sample was used for western-blot to detect phosphorylation of STAT1 and wild-type and mutated receptors. Antibodies recognizing phospho-STAT1 and total STAT1 were purchased from Cell Signaling Technology, IFNgR1 antibody (90 Kd) was purchased from Santa Cruz Biotechnology, Inc. (catalog number SC-700). The densitometry analyses of phospho-STAT1 and STAT1 western blots were measured by ImageJ program provided by National Institute of Health (NIH) website.

Statistical analysis

Clinical characteristic summary statistics and comparisons between ADEH+ and ADEH− groups (chi-square test for gender, t-test for age, $\log_{10}$ (total IgE), and $\log_{10}$ (EASI+1)) were calculated using STATA 11.0 (StataCorp. StataCorp LP. College Station, TX).

All variants and subjects passing QC were included in the sequencing data analysis. Additional QC on each variant was performed with PLINK[26], including an assessment of deviation from the Hardy-Weinberg equilibrium (HWE). Genetic association analysis for targeted sequencing data was conducted for all variants identified (both common and rare) and haplotype association tests were carried out for the seven common SNPs associated with ADEH+ phenotype. The single-variant tests for *IFNG*, *IFNGR1*, *IFNAR1* and *IL12RB1* and risk of ADEH+ was performed among 121 ADEH+ subjects and 107 ADEH− subjects using logistic regression (for the common variants (MAF 5%)) and rare variants with MAF<5% were tested under the Fishers exact test framework. Burden tests were also performed collapsing on the rare missense variants in each gene for the combined effects of rare variants. Pairwise linkage disequilibrium (LD) based on the D' statistic was measured using HaploView (http://www-genome.wi.mit.edu/personal/jcbarret/haplo). LD blocks were defined using the default algorithm which applies the definition of Gabriel *et al*[27].

For additional genotyping in the African American cohort, the Cochran–Armitage trend test was used to test for association between each individual SNP (under an additive model) and risk of ADEH− using PLINK. Tests for association with a P-value of <0.05 were further adjusted by the PLINK permutation test (10,000 permutations), which provided a framework for correction for multiple testing.

RESULTS

Patient characteristics

Targeted sequencing included a total of 121 ADEH[+] (case) and 107 ADEH[−] (control) European American subjects for analysis. Baseline characteristics are presented in Table 1. The ADEH+ group consisted of more male subjects (55.4% vs 23.4%) who were younger (22.0 vs 38.9 years) compared to the ADEH− group. As observed elsewhere[7], total serum IgE levels and mean AD severity EASI scores were significantly higher among subjects in the ADEH+ group (1836 vs 81.4 kIU/L and 10.4 vs 1.4, respectively). Detailed information on participants in the ADVN/ADRN has been previously described.[6]

Sequencing discoveries

As shown in Table 2, a total of 105kb targeting the four genes and 2 kb both up- and downstream were sequenced; 86,166 base pairs (bp) were successfully returned at a minimum of 40x coverage, after applying the quality control filtering procedure and variant calling. A total of 494 variants were identified across all four genes, including 145 common and 349 (70.6%) rare (minor allele frequency (MAF) <0.05) variants. Of these variants, 124 (25.1%) were private for ADEH+, 129 (26.1%) were private for ADEH−, and 86 (17.4%) were absent in dbSNP Build 141 (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi?view+summary=view+summary&build_id=141). The combined site-frequency spectrum of cases and controls is summarized in Figure 1A, indicating the majority of variants were rare in both the cases and controls (70.6%). Among all the variants detected (Figure 1B), 14 (2.8%) were coding-synonymous, 19 (3.8%) were missense, and 461 (93.3%) were non-coding (introns: 64.6%, 5′ UTR: 3.9%, 3′ UTR: 7.3%, 5′ upstream regions: 7.9%, and 3′ downstream regions: 9.7%). Proportions of variants identified in ADEH+ and ADEH− patients, are summarized according to the major functional categories in Figures 1C and 1D, respectively. All novel rare variants identified by targeted sequencing in 121 ADEH+ (n=37) or 107 ADEH− (n=40) patients are presented in Table E1 (A and B). In addition, six rare missense variants were identified in *IFNGR1* (Val14Met, Val61Ile, Val264Ile, His335Pro, Tyr397Cys and Leu467Pro; Figure 2), with 3 (Val14Met, Val61Ile and Tyr397Cys) as possibly damaging variants (with a score of 0.85 or higher on a scale of 0 to 1 (0 is benign)) according to Polymorphism Phenotyping v2 (PolyPhen-2; Table E2)[28]. Of note, Val14Met, Val61Ile and His335Pro are known *IFNGR1* variations (Figure 2) that have been associated with partial or complete *IFNGR1* deficiency[6, 29]. Only one ADEH+ carrier was identified for each of the two variants, Val14Met and Val61Ile, both carriers were positive for HSV-1. In contrast, variant His335Pro was seen in ADEH− patient (Table E2). Two additional rare missense variants Val264Ile and Tyr397Cys were also identified in ADEH+ patients only, but their potential functional role on *IFNGR1* deficiency remains unknown. The only carrier identified for the potentially damaging Tyr397Cys variant was positive to HSV-2 and had an EASI score of 40.6. In contrast, the one ADEH+ patient carrying the Val264Ile variant was negative for HSV-1 and HSV-2, and had an EASI score of 6. Thus, we targeted the Tyr397Cys variant in the downstream functional validation. However, no subject was found in our cohort carried a compound heterozygous mutation comprising the *IFNGR1* missense variants. As shown in Table 2, missense variants (both common and rare) were also identified in *IL12RB1* (n=9) and *IFNAR1* (n=3); two of the *IL12RB1* rare missense variants (Ser74Arg and Ser320Pro) were possibly damaging and one was novel (Thr517Met) (Table E2). However, no further evidence for association was observed between risk of ADEH+ and missense variants identified in either *IL12RB1* or *IFNAR1* genes. In contrast, no missense variant was discovered in the *IFNG* gene (Table 2), for which a region of 8.97kb was sequenced compared to a region of 25.95kb for the *IFNGR1* gene.

Common *IFNGR1* variants and haplotypes associated with protection for ADEH+

Single marker analyses—Association tests were performed between variants identified by targeted sequencing and EH status. Seven common variants in *IFNGR1* were

significantly associated with protection against ADEH+ (*P* = 0.015-0.002; Table 3 and Figure 3, *top* panel), four of which were reported previously (rs2234711, rs7749390, rs10457655 and rs1327475)[6] and no further validation was attempted. Of the three additional variants identified, 1 was coding-synonymous (rs11914), 1 was intronic (rs17175127) and 1 was in the *IFNGR1* promoter (rs28515059, C-1179T). Validation genotyping using TaqMan Assays and Sanger sequencing for these 3 variants described above has confirmed these findings (data not shown).

Haplotype analyses—In the sequencing discovery cohort, common protective haplotypes were identified from 2 to 7-SNP windows with an average prevalence of 12% in patients with ADEH+ compared with 23.7% in patients with ADEH− (Table 4 and Figure 3, *top*). The strongest association was for a common (25% in ADEH+ vs 12% in ADEH−) 2-SNP (rs10457655 and rs7749390) haplotype (AG) that conferred a reduced risk of ADEH+ (*P* = 0.0004) by over half (odds ratio, 0.41). These two variants are potentially functional: the GG genotype of rs7749390, located in the exon/intron splicing site of *IFNGR1*[30], has been associated with increased levels of IFN-γ production and reduced risk of ADEH+[6]; variant rs10457655 belongs to Category 2b (out of a category system of 1 to 6: 1=likely affect binding and linked to expression of a gene target; 2(a-c)=likely affect binding, subcategories demonstrate direct evidence of binding through ChIP-seq and DNase data, with additional annotations from the most to the least confident; 3=Less likely affect binding; 4-6=Minimal binding evidence) and is likely to directly affect binding of transcription factors according to RegulomeDB[31], a database that annotates variants with known and predicted regulatory elements (*e.g.*, DNAase hypersensitivity, binding sites of transcription factors, and promoter regions that have been biochemically characterized to regulate transcription) in non-coding regions[32]. A 3-SNP haplotype (AGG) also provided strong evidence for association (*P* = 0.0006) with one additional variant rs2234711 (A-56G or T-56C), which is a functional variant in the promoter associated with several infectious diseases[33, 34]. It is increasingly clear that a small percentage (7%) of disease-associated SNPs are located in protein-coding regions, and the majority (93%) are located in gene regulatory regions or in intergenic regions[35]. Because mutations in non-coding portions of a gene can affect how that gene is regulated, we further evaluated regulatory regions for *IFNGR1* using the Encyclopedia of DNA Elements (ENCODE) regulatory tracks[36]. Interestingly, the *IFNGR1* haplotype locus (centered on the 3-SNP haplotype) flanks the first exon and overlaps with many regulatory features including DNAaseI hypersensitivity clusters, transcription factors binding sites and enhancer histone H3K27Ac marks (which are often found near active regulatory elements) on 3 out of 7 cell lines: K562 (an immortalized cell line produced from a female patient with chronic myelogenous leukemia), NHEK (normal human epidermal keratinocyte) and NHLF (normal human lung fibroblasts) (Figure 3, *bottom*).

Tests for association with susceptibility of AD

To confirm that *IFNGR1* variants were specifically associated with risk of ADEH+ and not generic to risk of AD, tests for association between *IFNGR1* common and rare variants were performed in two independent samples of AD without EH. Four of the seven *IFNGR1* variants were significantly associated with protection against risk of ADEH− (*i.e.*, ADEH− vs non-atopic controls) among the African Americans (*P* = 0.004-0.0001; Table E3 in the

Online Repository). In contrast, no association was found for risk of ADEH− among the European Americans.

Similarly in the African American samples, we observed associations for protection against ADEH− and *IFNGR1* haplotypes; a 3-SNP haplotype CAA encompassing the entire *IFNGR1* gene provided the strongest evidence (rs11914, rs17175127 and rs28515059; $P = 5.9 \times 10^{-4}$). This strong protective haplotype for AD in the African American sample has not been previously observed[6].

Interestingly, a major linkage disequilibrium (LD) block was observed within *IFNGR1* for all 7 pairings of contiguous variants spanning 22kb among the African American non-atopic controls using the criteria of Gabriel *et al*[27] (see Supplementary Figure E2 in the Online Repository). No difference was observed for the LD structure among the European American non-atopic controls (Figure E2).

Rare damaging variants associated with risk of ADEH⁺ and functional validation

We observed the combined evidence of association with rare missense variants in *IFNGR1* ($P = 0.038$, n=6 (Table E2)) when we applied the burden test collapsing on these 6 rare variants, suggesting that rare variants contribute to ADEH+ susceptibility.

Upon binding to IFN-γ, IFNgR1 and IFNgR2 form as dimers and trigger the activation of JAK1/2. Activated JAK1/2 induces phosphorylation of STAT1, phosphorylated STAT1 then translocates from the cytoplasm into the nucleus to bind to gamma-IFN activation sequence (GAS) in the promoters of IFN-γ targeted genes (Figure 2)[37]. To confirm whether the two SNVs Val14Met and Tyr397Cys of *IFNGR1* alter the receptors' function, we subcloned these two putative 'loss-of-function' variants into mammalian expression plasmids using site-directed mutagenesis methods. We then used two different assays to evaluate the function of the two gene variants compared to the wild-type receptor: GAS report gene assay and western-blot to detect STAT1 phosphorylation upon IFN-γ stimulation. As shown by Figure 4, both V14M and Y397C gene variants had reduced GAS activation as compared to the wild-type receptor. IFNgR1 deficient EBV cells were re-introduced with wildtype IFNgR1, single mutation receptor V14M and Y397C, double mutation of V14M and Y397C. Protein lysates were made from these cells in the presence and absence of IFN-gamma stimulation, the protein expression levels of pSTAT1 and total STAT1 were detected by western blot method. pSTAT1/STAT1 ratio was determined by densitometry measurement. As shown in Figure 5, cells reintroduced with IFNgR1V14M and IFNgR1Y397C have reduced pSTAT1/STAT1 ratio as compared to wildtype receptors; As compared to the single mutated receptors, cells reintroduced with receptors of double mutations of V14M and Y397C have greater reduction of pSTAT1/STAT1 ratio, suggesting that double mutations confer increased damage to the function of the receptor as compared to a single mutation.

DISCUSSION

Our previous studies have demonstrated that genetic susceptibility plays a pivotal role in the development of ADEH+. A relatively uncommon null mutation in filaggrin (R501X), a

major skin barrier protein, was three times more prevalent in patients with ADEH+ than those ADEH– patients[9]. In addition, we have also previously implicated thymic stromal lymphopoietin (*TSLP*), an interleukin (IL)-7-like cytokine involved in the pathogenesis of allergic diseases, as a potential causal gene in ADEH+[38]. Using several different approaches (immunologic, genomic and genetic), we also demonstrated low *IFNG, IFNAR1* and *IFNGR1* expression in patients with ADEH+ as well as association between *IFNG* and *IFNGR1* tagging SNPs and the ADEH+ phenotype and levels of IFN-γ production[6]. Subsequently, we reported genetic variants in interferon regulatory factor 2 (*IRF2*) gene might contribute to risk of ADEH+ and the abnormal immune response to HSV[10]. These findings suggest genetic variations in the interferon signaling pathway regulate the level of interferon receptor gene expression, and therefore plays a critical role in IFN-γ responsiveness in the development of ADEH+.

It is recognized that a considerable proportion of heritable disease risk in complex traits is in fact associated with rare variants[39][40]. In this multicenter, case-control study, we conducted targeted deep sequencing with sufficient coverage (including regulatory regions) to discover both the common and rare variants, for four interferon signaling pathway genes with prior evidence for involvement in the immunopathogenesis and genetic risk of ADEH+. It is well known that genetic heterogeneity may limit the identification of risk loci in complex disorders. Therefore, genetic studies of clinically more homogeneous subforms of the disease may strengthen the possibility of identification of genetic susceptibility factors with a special interest for extreme phenotypes. Indeed, data have suggested that the most productive approach for identifying missing heritability in complex traits might be to combine the 'power of the extreme' in small, well-phenotyped cohorts, with targeted follow-up in case-control and population cohorts[41]. We selected 121 ADEH+ patients with the highest EASI score and 107 ADEH– patients with the lowest EASI in the discovery cohort for targeted deep sequencing. This approach represents one of the major strengths of our study, which successfully led us to the discovery of *IFNGR1* variants as the most prevalent genetic factors for ADEH+, including those novel deleterious variants previously we were unable to identify.

There are prior reports of the association between *IFNGR1* polymorphisms and susceptibility to infectious disease. *IFNGR1* promoter polymorphism rs2234711 (A-56G or T-56C) was associated with protection from development of or death from cerebral malaria in the Mandika, the major Gambian ethnic group[33]. Thye *et al*.[34] reported that allele T-56, along with two other *IFNGR1* coding sequence polymorphisms His335Pro and Leu467Pro, was associated with a higher rate of anti-Helicobacter pylori IgG antibodies, susceptibility to allergic disease and the production of high IgE titers[42]. Findings regarding pulmonary tuberculosis susceptibility and *IFNGR1* polymorphisms have also been described[43, 44] including recent results on an intronic SNP rs1327474 in African Americans[45] and rs7749390, located on the exon/intron splice site, in Han Chinese[30]. Defects in *IFNGR1* are a cause of mendelian susceptibility to mycobacterial disease, also known as familial disseminated atypical mycobacterial infection[46]. Jouanguy *et al*. reported a distinct group of exonic and intronic mutations in *IFNGR1* including ILE87THR (rs104893973)[46] and CYS77TYR (rs104893974)[47], along with *IL12RB1* deficiency. However, none of them

exists in AD patients we sequenced in the current study. Instead, we discovered 6 rare missense variants in *IFNGR1* including 3 known functional variants and 1 predicted damaging variant Y397C (Table E2). Moreover, we applied additional molecular biology approaches and confirmed that both V14M (a known functional variant) and Y397C (a predicted functional variant) of *IFNGR1* have impaired function in triggering IFN-γ signaling transduction as compared to the wild-type receptor. Thus, both common and rare *IFNGR1* variants contribute to susceptibility to ADEH+.

Significant genetic associations were observed in the European American patients by both single marker and haplotype analyses. We have identified common 2 and 3-SNP haplotypes that were protective against ADEH+. These haplotypes are comprised of potentially functional variants. The region harboring the 2 and 3-SNP haplotypes is also highly polymorphic and enriched with histone modification H3K27ac marks (Figure 3). Histone modification is an epigenetic mechanism that influences gene regulation in eukaryotes. In particular, histone modification H3K27ac is known as a promoter mark associated with transcriptional activation (*e.g.*, identifies active enhancers)[48, 49]. Recent evidence suggests that CpG islands under selective pressure are enriched with H3K27ac marks[50]. It has long been recognized that innate immunity genes such as *IFNGR1* are highly polymorphic or plastic in their promoters, suggesting that these regions of the genome have been under intense evolutionary selective pressure from infectious microorganisms[51]. Our study revealed the strongest association between genetic variants within the *IFNGR1* promoter or surrounding the first exon, where H3K27ac marks are peaked, and the ADEH+ phenotype. These findings suggest *IFNGR1* variants may affect gene expression by alternating regulatory elements and cause interferon receptor deficiency and impaired interferon response, which play a key role in the pathogenesis of ADEH+. Furthermore, the difference in significant associations (and frequencies) of the *IFNGR1* variants and haplotypes between the two ethnic populations was not entirely unexpected given the difference of population history and natural selection (*i.e.*, exposure to infectious microorganisms) that may shape patterns of genetic variation in the *IFNGR1* gene. We recognize the limitation of study that we were not able to replicate our targeted sequencing findings in an independent and similarly powered or larger cohort of ADEH+ patients, in addition to solely validating the findings using alternative platforms for genotyping. ADEH+, however, is an extremely rare phenotype involving <3% of AD, and it is noteworthy that the current study involves the largest cohort of such patients. Given our current limitations of a relatively modestly powered discovery sample, we only discovered 'loss-of-function' variants in the *IFNGR1* gene. We acknowledge that other genes (*e.g.*, *IFNG* and *IFNAR1*) in the same (IFN) or different (*e.g.*, skin barrier) pathways may also harbor genetic defects that could lead to impaired antiviral response against viruses such as HSV and vaccinia and increase the risk of ADEH+, but will require larger samples for discovery.

In summary, large-scale analyses of *IFNGR1* variants in diverse populations are warranted to confirm and quantify the proportion of ADEH+ risk that may be attributed to these variants. Second, functional validation of these variants applying additional molecular genetics approach helped us further understand the molecular mechanisms involved in AD complicated with viral dissemination. A defective systemic IFN-γ immune response that

fails to control viral replication plays a key role in the pathogenesis of ADEH+. Finally, genetic variation in these interferon-pathway genes may have broader implications in determining susceptibility to a range of common, chronic human diseases, which have an inflammatory component. In conclusion, our study provides the first evidence that rare functional *IFNGR1* mutations contribute to risk of ADEH+. Future studies in larger and diverse populations are warranted to improve our ability to identify patients at greatest risk for ADEH+ and ultimately lead to early intervention to prevent this devastating complication of AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Abbreviations

IFNGR1	interferon gamma receptor 1
AD	Atopic dermatitis
EH	Eczema herpeticum
ADEH−	Atopic dermatitis without a history of eczema herpeticum
ADEH+	Atopic dermatitis with a history of eczema herpeticum
EASI	Eczema Area and Severity Index
SNP	Single nucleotide polymorphism
MAF	Minor allele frequency
LD	Linkage disequilibrium
GAS	gamma-IFN activation sequence

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KEY MESSAGES

1. Targeted sequencing identified both common and rare genetic variations in the gene encoding *IFNGR1* that are associated with ADEH+ phenotype.

2. Both Val14Met and Tyr397Cys are 'loss-of-function' rare missense variants leading to partial *IFNGR1* deficiency and a defective systemic IFN-γ immune response in ADEH+ patients.

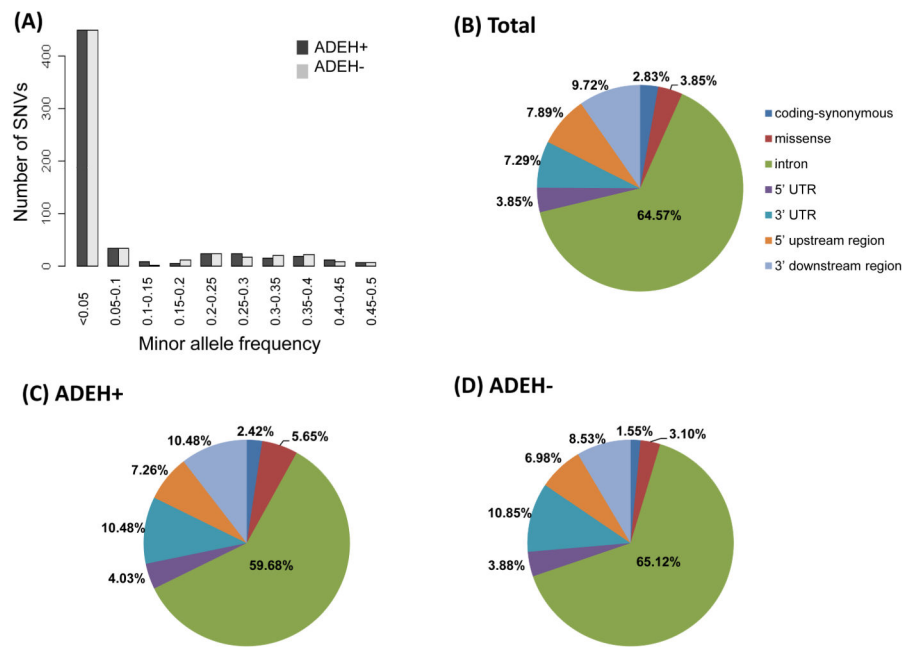3. Subjects with AD prone to disseminated viral skin infections have defects in their IFN responses.

Fig. 1.
(**A**) Combined site-frequency spectrum of ADEH+ and ADEH− patients for the 4 interferon-pathway genes. (**B**) Proportions of all variants identified in the 4 interferon pathway genes according to major functional categories. (**C**) Proportions of variants identified in ADEH+ and (**D**) ADEH− patients.
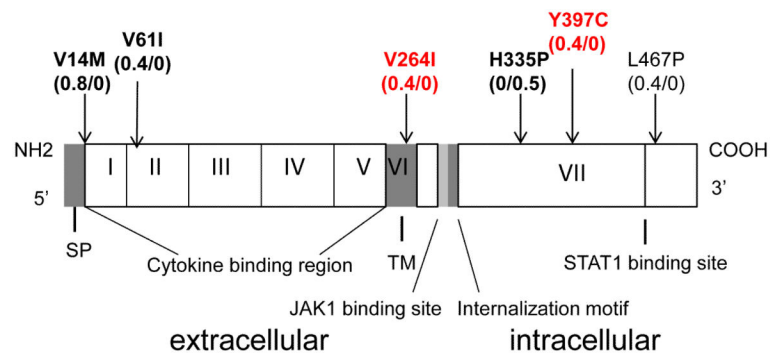
Fig. 2.
Schematic presentation of 6 rare missense variants in *IFNGR1*. On the top, the variations that identified by targeted sequencing are illustrated with allele frequencies (in ADEH+/ADEH− patients), 3 known functional variations are highlighted in bold and 2 novel variants are in red. On the bottom, the various domain and 7 exons in the 5′ to 3′ direction are indicated. SP=signal peptide, TM=transmembrane domain.
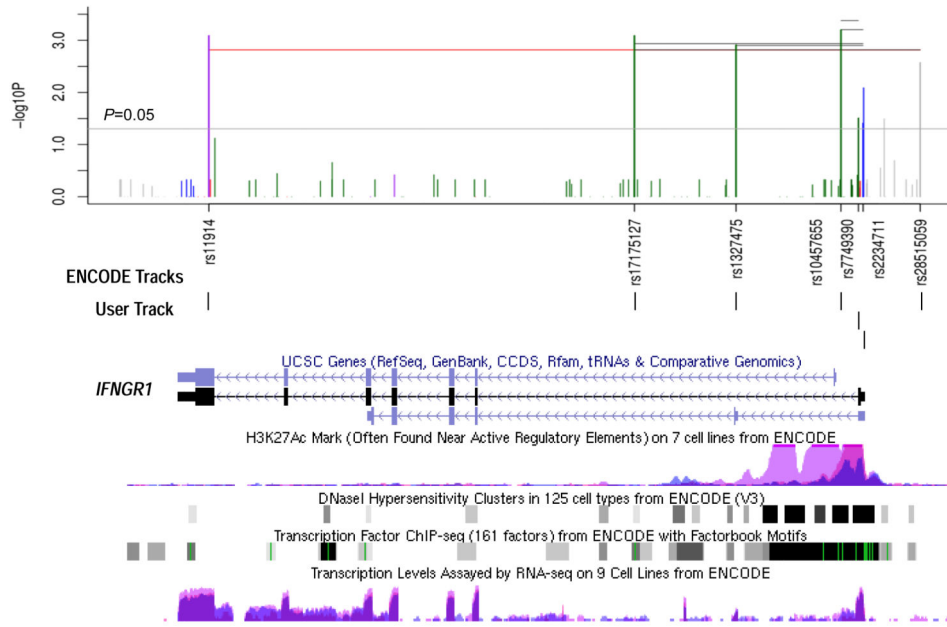
Fig. 3.

Single marker and haplotype association tests for European Americans and ENCODE regulation tracks on *IFNGR1* region (chr6: 137,516,621-137,542,567). SNVs identified by targeted sequencing significantly associated with ADEH+ phenotype (with *P* values great than 0.01) were illustrated (top panel). The *y* axis indicates the value of $-\log_{10}$ (*P*), and the *x* axis indicates the relative position for each SNV locus in *IFNGR1*, 3′ to 5′ direction. The vertical lines (green for intron, red for coding-nonsynonymous, purple for coding-synonymous, grey for near-gene-5 and blue for utr-5) represent the position of each SNV on the *x* axis, and height of the line on the *y* axis indicates the value of $-\log_{10}$ (P). Similarly, the most significant haplotype results from two to seven-marker windows across the *IFNGR1* gene were also illustrated by horizontal lines in black (except red color was used for the 7-SNP window) and a *P* value cutoff of 0.05 was depicted by the grey horizontal line. The bottom panel illustrates the *IFNGR1* structure, position of seven SNVs within *IFNGR1* gene region (User Track) and regulatory regions with ENCODE regulation tracks including UCSC Gene (IFNGR1), DNase Clusters, Transcription Factor ChIP-seq, Layered H3K27Ac and Transcription Levels on 3 cell lines (NHLF (in pink color), NHEK (in purple color), and K562 (in blue color).
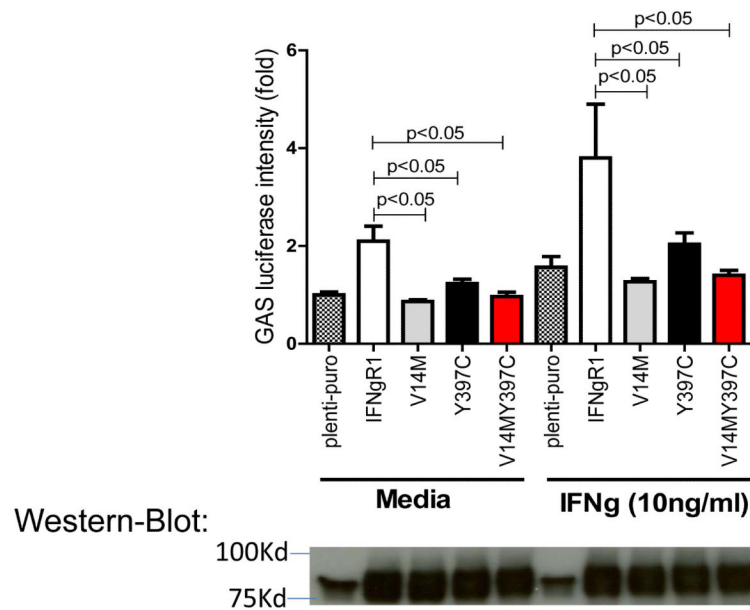
Fig. 4.
Reduced GAS activation by IFNgR1V14M and IFNgR1Y397C variants. Plasmids of empty-vector, IFNgR1WT, IFNgR1V14M, IFNgR1Y397C, and IFNgR1V14MY397C were cotransfected with GAS luciferase reporter plasmids into 293FT cells for overnight. A portion of cells was stimulated with IFN-γ (10ng/ml) for an additional 6 hours. The upper panel shows the luciferase intensity of GAS reporter; the lower panel shows the receptor levels corresponding to the samples shown in the upper panel. This is the representative result of three independent experiments.
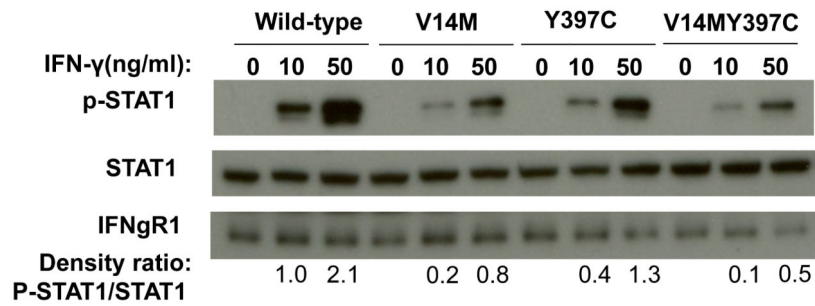
Fig. 5.

IFN-γ induced phosphorylation of STAT1 is reduced in IFNgR1(−/−). EBV cells reconstituted with IFNgR1V14M and IFNgR1Y397C variants. Plasmids of IFNgR1WT, IFNgR1V14M, IFNgR1Y397C, and IFNgR1V14MY397C were transfected into IFNgR1 deficient cells. The cells were then stimulated with recombinant human IFN-γ at concentration of 0, 10 ng/ml and 50ng/ml for 30 min. 100μg of protein per lane were loaded for the detection of pSTAT1, STAT1, IFNgR1 wildtype and mutants.

Table 1

Clinical characteristics of 228 European American subjects included in the targeted sequencing phase (discovery cohort).

Characteristic	Case (ADEH+)	Control (ADEH−)	P-value
N	121	107	
Males; N (%)	67 (55.4%)	25 (23.4%)	< 0.0001
Age (years); mean (range)	22.0 (1.3-80.7)	38.9 (18.7-73.6)	< 0.0001
IgE (kIU/L); Median (Q1, Q3)	1836 (300, 4987)	81.4 (25.2, 253)	< 0.0001
AD severity EASI score; Median (Q1, Q3)	10.4 (5.4, 16.2)	1.4 (0.6, 2.8)	< 0.0001

IgE calculated in kIU/L. The total EASI score is calculated by combining information on intensity (redness, thickness, scratching and lichenification) and percentage area affected by eczema within each of the 4 body regions (head and neck, upper limbs, trunk, and lower limbs).

Table 2

Summary of total number of variants identified by sequencing in four interferon-pathway genes, the number of private variants identified in ADEH+ or ADEH− patients and the break down according to rarity, novelty and major functional categories.

Gene	Target Region (bp)	Sequenced Base Pairs*	Total # of Variants	Common	Rare	Total # of Private Variants (ADEH+/ADEH−)	Novel dbSNP** (Total/ADEH+/ADEH−)	Coding synonymous (Total/ADEH+/ADEH−)	Missense (Total/ADEH+/ADEH−)	Non-Coding (Total/ADEH+/ADEH−)
IFNG	chr.12:68,546,538-68,555,255 (8,718)	2,508	41	12	29	7/13	8/3/5	0/0/0	0/0/0	41/7/13
IFNGR1	chr.6:137,516,664-137,542,633 (25,970)	18,821	120	18	102	47/31	23/9/11	5/2/0	7/5/1	108/40/30
IFNAR1	chr.21:34,695,765-34,734,206 (38,441)	36,448	199	59	140	41/58	35/15/15	1/0/0	3/1/1	195/40/57
IL12RB1	chr.19:18,168,352-18,199,532 (31,180)	28,389	134	56	78	29/27	20/11/9	8/1/2	9/1/2	117/27/23
All (%)	~105kb	86,166	494	145 (29.4%)	349 (70.6%)	124/129 (25.1%/26.1%)	86/38/40 (17.4%)	14/3/2 (2.8%/0.6%/0.4%)	19/7/4 (3.8%/1.4%/0.8%)	461/114/123 (93.3%/23.1%/24.9%)

*
The total number of sequenced base pairs that had passed the quality control filtering process.

**
Variants that were absent in the most recent dbSNP build 141 (the total number of novel variants including those seen in both ADEH+ and ADEH−; variants only seen in ADEH+; and variants only seen in ADEH−).

Table 3

Association between common variants in *IFNGR1* and protection against ADEH+ among European Americans (*P* 0.05)

Variants	Chr. 6 position (hg38)	Function	Major allele	Minor allele	MAF (ADEH+)	MAF (ADEH−)	P value
rs11914	137198451	coding-synonymous, +20,980	A	C	0.116	0.238	0.002
rs17175127	137212069	intron, +7362	G	A	0.116	0.238	0.002
rs1327475*	137215318	intron, +4113	G	A	0.116	0.234	0.002
rs10457655*	137218673	intron, +758	G	A	0.124	0.252	0.002
rs7749390*	137219233	splice site, +198	A	G	0.380	0.481	0.011
rs2234711*	137219383	promoter, −56	A	G	0.384	0.481	0.015
rs28515059	137221209	promoter, −1179	C	T	0.120	0.229	0.006

*
Denote markers previously reported.

Polymorphic positions are indicated with position 1 refers to the A of the start codon (ATG). Positive number suggests position after start codon, and negative number suggests position before start codon.

Table 4

Common 2 to 7-SNP haplotypes most significantly associated with protection against ADEH+ among EAs in the discovery cohort

Haplotype	Freq_ADEH+	Freq_ADEH−	CHISQ	OR	P	SNPs
AG	0.12	0.25	12.45	0.41	0.0004	rs10457655|rs7749390
AGG	0.12	0.25	11.70	0.43	0.0006	rs10457655|rs7749390|rs2234711
AAGG	0.12	0.23	10.41	0.44	0.0012	rs1327475|rs10457655|rs7749390|rs2234711
AAAGG	0.12	0.23	10.56	0.44	0.0011	rs17175127|rs1327475|rs10457655|rs7749390|rs2234711
AAAGGA	0.12	0.23	10.05	0.44	0.0015	rs17175127|rs1327475|rs10457655|rs7749390|rs2234711|rs28515059
CAAAGG						rs11914|rs17175127|rs1327475|rs10457655|rs7749390|rs2234711|rs2851505
A	0.12	0.23	10.05	0.44	0.0015	9