ISSN: 2476 - 2415

Research Article

International Journal of Clinical & Experimental Dermatology

Il-18 Overproduction Associated with NLRP1 Single Nucleotide Polymorphisms Linked to Risk for Vitiligo

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Submitted: 11 Sep 2021; **Accepted:** 20 Sep 2021; **Published:** 24 Sep 2021

Citation: *Laura J. Westhoff, Savannah J. Hughes, Erin Gill, Trent Walker, Brian D. Poole, et al. (2021) Il-18 Overproduction Associated with NLRP1 Single Nucleotide Polymorphisms Linked to Risk for Vitiligo. International Journal of Clinical & Experimental Dermatology 6(2), 1-4.*

Abstract

Background

The NLRP1gene is central to the NLR inflammasome. Variants to the NLRP1 gene are associated with vitiligo and other autoimmune diseases. We examined the effects of two single nucleotide polymorphisms (SNP) son cytokine levels and NLRP1 gene expression in 50 human volunteers.

Methods

NLRP1 was genotyped at SNPs rs2670660 and rs12150220, and participants who were homozygous at one or more SNP were analyzed. Plasma IL-18 and IL-1β levels were quantified using ELISA. NLRP1 gene expression was measured using real-time PCR.

Results

Participants with the risk genotype had significantly higher levels of plasma IL-18 than participants with protective genotype (0.439 ng/μL compared to 0.152 ng/μL, p = 0.024). Genotypes rs2670660 and rs12150220 were strongly linked in this population (p = 2.33 x 10-13).

Conclusions

Increased production of IL-18, suggests that at least one of the AA variants of rs2670660 or rs12150220 increases NLRP1 activity. rs2670660 and rs12150220 are strongly linked.

Key Words: Vitiligo, *NLRP1*, Il-18, Genetic Risk

Abbreviations used

NLRP1: Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing
NLR: nucleotide-binding domain leucine-rich repeat containing
IL-1β: Interleukin-1 Beta
IL-18: Interleukin 18
PBMC: Peripheral blood mononuclear cells
SNP: Single nucleotide polymorphism

Introduction

Variants in the Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing (*NLRP1*) gene are associated with increased risk for autoimmune disease, especially vitiligo [1]. This correlation is even stronger when vitiligo is present in concert with other autoimmune diseases such as autoimmune thyroid disease, latent autoimmune diabetes in adults, rheumatoid arthritis, psoriasis, pernicious anemia, systemic lupus erythematosus, or Addison's disease [1-3]. *NLRP1* is the sensor protein in the nucleotide-binding domain leucine-rich repeat containing (NLR) inflammasome. Inflammasomes are multi protein oligomers responsible for inflammatory responses that are essential to the innate immune system [4]. Components and homologues of the NLR inflammasome have been implicated in many auto inflammatory diseases, examples of which include inflammatory bowel disease, Blau syndrome, Muckle-Wells syndrome, and neonatal onset multisystem inflammatory disease [5-8].

Cytokine dysregulation is a likely mechanism for the influence of *NLRP1* on autoimmune and auto inflammatory disease. *NLRP1* regulates the activation of interleukin 1 beta (IL-1β) and interleukin 18 (IL-18) [9-12]. The release of these interleukins by the inflammasome has been linked to the development of vitiligo [13, 14], as well as other autoimmune diseases. For example, IL-18 is likely to play a role in Hashimoto's thyroiditis, and is found in higher than average levels in patients with rheumatoid arthritis and systemic lupus erythematosus [15-17]. Several *NLRP1* variants have been associated with autoimmune diseases [1, 4, 10]. In their foundational 2007 study on *NLRP1* and vitiligo-associated autoimmune disease, Jin et al. identified 19 SNPs that were associated with autoimmune and auto inflammatory diseases, including rs12150200 and rs2670660, the SNPs investigated in this study. rs12150220 is an L115H substitution located in the protein-coding region of *NLRP1*, while rs2670660 is located in the promoter region [1]. Of these, rs12150220 has been studied most extensively for its effects on autoimmune disease [9, 18, 19]. However, the current literature lacks consensus on the effects of rs2670660 on mRNA expression, cytokine expression, or protein function of *NLRP1*, and neither SNP has been explicitly tested for effects on IL-18 expression [10]. The purpose of this study was to characterize the effects of two specific *NLRP1* gene variants, at SNPs rs2670660 and rs12150220, on *NLRP1* IL-1β and IL-18 secretion.

Materials and Methods

Participants

Volunteers without autoimmune diseases were selected for this study. Demographics of the participants are listed (TABLE 1). After informed consent, blood was collected from each participant via venipuncture into tubes with sodium citrate anticoagulant. The study was approved by the Brigham Young University Institutional review board, protocol # X19041.

Sample collection

Plasma was stored at -80° C for cytokine analysis. DNA was isolated from whole blood using Qiagen DNeasy Blood & Tissue Kit, following the manufacturer's protocol. Peripheral blood mononuclear cells (PBMCs) were separated from red blood cells using Corning Lymphocyte Separation Medium according to the manufacturer's directions. RNA was isolated using the RNAqueous system (Ambion) according to the manufacturer's directions.

Genotyping

The *NLRP1* SNP rs2670660 has A or G variants, and SNP rs12150220 has A or T variants. These were genotyped for each volunteer using Applied Biosystem TaqMan SNP Genotyping Assays for rs2670660 and rs12150220 following the manufacturer's protocol. TaqMan qPCR was performed using the Applied Bio systems Step OnePlus Real-Time PCR System and Step One Software v2.3.

Cytokine analysis

Plasma was used for IL-1β and IL-18 ELISA analysis. IL-18 analysis was performed using eBio science Human IL-18 Platinum ELISA Kit, following the manufacturer's protocol. IL-1β analysis was performed using Invitrogen Human IL-1beta Uncoated ELISA Kit, following the manufacturer's protocol.

Gene expression analysis

cDNA was synthesized from total RNA using Invitrogen Super Script IV First- Strand Synthesis System following the manufacturer's protocols, using oligo-DT primers. Real-time PCR primers for *NLRP1* spanned the exon-exon junction created by the splicing of the first intron of the *NLRP1* gene to select for the amplification of mature mRNA and exclude DNA. Forward primer sequence: CTACGTTGGCCACTTGGGAT. Reverse primer sequence: AGGTGAAGGTACGGCTATGC. SYBR-green real-time PCR was performed on cDNA with GAPDH endogenous controls using Applied Bio systems Power SYBR Green PCR Master Mix following the manufacturer's protocol.

Statistical analysis

Demographic data was collected at the time of blood collection. Significance of the linkage of AA/AA and GG/TT genotypes was evaluated by calculating expected genotype outcomes and then using a chi-squared test. Significance in cytokine results was analyzed using Student's t-test. Student's t-test was also used to evaluate *NLRP1* expression.

Results

Demographics and Genotypes

Sample demographics are shown in (Table 1). TaqMan genotyping determined that at SNP rs2670660, 11 participants were homozygous AA, 27 participants were heterozygous AG, and 12 participants were homozygous GG, comprising 22%, 54%, and 24% of the sample, respectively. This resulted in allele frequencies at this SNP of 0.49 for A and 0.51 for G. Furthermore, 11 participants were homozygous AA, 28 participants were heterozygous AT, and 11 participants were homozygous TT at SNP rs12150220, comprising 22%, 56%, and 22% of the sample, respectively. This resulted in allele frequencies at this SNP of 0.50 for A and 0.50 for G (Table 2).

Table 1: Sample demographics. Participants were largely white, female, and between 18-29 years of age.

Demographics		N = 50	(%)
Sex	Female	35	70%
	Male	15	30%
Race	White	45	90%
	Hispanic	2	4%
	Asian	3	6%
Age	18-29	44	88%
	30-39	4	8%
	50-59	2	4%

Table 2: Genotypes and allele frequencies for rs2670660 and rs12150220

	Genotypes, n (%)			Alleles, frequency	
rs2670660	AA	AG	GG	A	G
	11 (22)	27 (54)	12 (24)	0.49	0.51
rs12150220	AA	AT	TT	A	T
	11 (22)	28 (56)	11 (22)	0.50	0.50

The genotyping at rs2670660 and rs12150220 showed very strong indications of linkage (Table 3). Genotypes will be written as rs2670660/rs12150220. Homozygous genotypes AA/AA and GG/TT were much more common than homozygous genotypes AA/TT or GGAA, with 10 AA/AA and 10 GG/TT participants and zero AA/TT or GG/AA participants. Mixed homozygous/heterozygous genotypes were also rare, with 1 each of AA/AT, AG/AA, and AG/TT genotypes, and 2 of GG/AT. There were 25 participants who were heterozygous at both rs2670660 and rs12150220. If rs2670660 and rs12150220 assorted independently, the expected distribution of genotypes would be as follows for 50 samples: 3.001 AA/AA, 6.248 AG/AA, 3.251 GG/AA, 6.003 AA/AT, 12.495 AG/AT, 6.503 GG/AT, 3.001 AA/TT, 6.248 AG/TT, and 3.251 GG/TT. Comparing these expected values with the observed genotypes via chi-squared test showed substantial linkage ($p=2.33 \times 10^{-13}$) (Table 3).

Table 3: Genotypes of rs2670660/rs12150220 show strong linkage. AA/AA and GG/TT are the strongest homozygous haplotypes, with no AA/TT or GG/AA homozygous haplotypes present. There are also more heterozygotes than would be expected in independent assortment based on the observed allele frequencies $= 2.33 \times 10^{-13}$.

		rs2670660, n (%)		
		AA	AG	GG
rs12150220, n (%)	AA	10 (20%)	1 (2%)	0 (0%)
	AT	1 (2%)	25 (50%)	2 (4%)
	TT	0 (0%)	1 (2%)	10 (20%)

Cytokine analysis

Plasma IL-18 was significantly increased in samples from the AA/AA genotype compared to the GG/TT ($p=0.024$). The AA/AA genotype had a mean of at least 2.88-fold higher levels of IL-18 than any of the other genotypes (Figure 1). Plasma IL-18 concentration was also compared independently for each SNP. The AA rs2670660 genotype had a mean IL-18 concentration of 0.41 ng/μL, significantly higher than the GG genotype at 0.145 ng/μL ($p=0.022$). The AA genotype of 12150220, showed a mean IL-18 concentration of 0.408 ng/μL, while the TT genotype was 0.157 ng/μL ($p=0.030$) (Figure 1). Although the standards and controls performed as expected, no plasma IL-1β was detected for any sample.

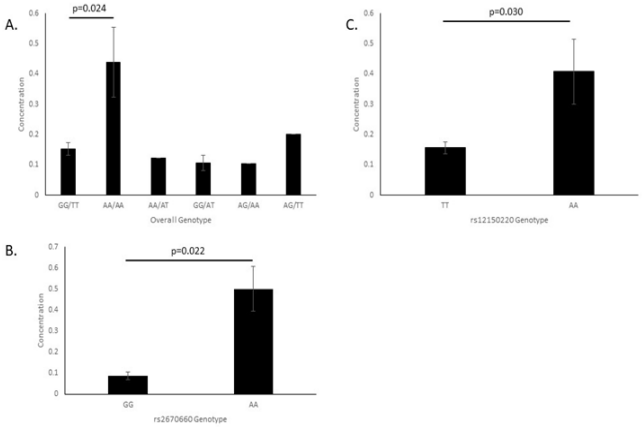

Figure 1: Significantly higher concentrations of IL-18 displayed in AA/AA genotype and individual AA genotypes. a) Comparison of IL-18 concentrations across all present haplotypes. $p = 0.024$ for AA/AA vs. GG/TT. Statistical significance was not calculated for other haplotypes due to small sample sizes. b) Comparison of IL-18 concentrations for GG to AA variants of rs2670660. $p = 0.022$. c) Comparison of IL-18 concentrations for homozygous allele variants of rs12150220. $p = 0.030$.

NLRP1 gene expression

Quantitative PCR was used to determine differences in *NLRP1* gene expression for each genotype. No significant difference was observed in *NLRP1* expression between any genotype.

Discussion

SNPs in the *NLRP1* gene are associated with increased risk for autoimmune disease [1]. Here we find that the genotype AA/AA of SNPs rs2670660 and rs12150220 is strongly associated with an increased concentration of plasma IL-18 in otherwise healthy individuals. There is consensus that the risk variant for autoimmune disease for rs12150220 is A, however the literature is less clear concerning the risk variant for rs2670660 [2, 18, 20]. In a European population, Magitta et al. found variant A at rs2670660 to have a trend towards association with Addison's disease and type 1 diabetes compared to healthy controls [18]. Therefore, it is possible that our AA/AA genotype represents two homozygous risk variants, with the GG/TT genotype representing two homozygous protective variants.

The NLRP1 protein recruits caspase-1, which activates IL-18 by cleaving the IL-18 precursor [4, 21]. In this context, the significant increase in IL-18 concentration found in the AA/AA likely represents increased processing of IL-18. This could come about either by increased protein activity or higher gene expression. rs12150220 is an L115H substitution located in the protein-coding region of *NLRP1*, while rs2670660 is located in the promoter region [10]. As such, we would expect rs2670660 to have more of an influence on levels of gene expression than rs12150220, while rs12150220 would be expected to alter the activity of the protein itself. Our results support this hypothesis, since there was no significant difference in expression levels of *NLRP1* but there was still a significant increase in plasma IL-18 in the risk genotype.

A pivotal study of *NLRP1* genotypes [9] showed that the L115H amino acid substitution at rs12150220 led to an increase in plasma IL-1β despite no increase in gene expression. Our current findings are in agreement, although we did not find IL-1β to be increased in the plasma. The absence of detectable plasma IL-1β is not surprising for a population without autoimmune diseases, as elevated levels of IL-1β related to *NLRP1* has only been observed in autoimmune populations [9]. The chief limitation to the study is also one of the findings, that the two polymorphisms are tightly linked in this population. Therefore, we were unable to definitively determine which polymorphism was responsible for the higher IL-18 phenotype. The two polymorphisms, which are found in different haplotype groups [9], being so strongly linked argues for caution in interpreting results from any study of individual haplotypes or single nucleotide polymorphisms in the *NLRP1* gene.

Acknowledgments: This work was funded by an internal research grant from Brigham Young University for Westhoff, L.J.

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