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Enrichment of Rare Variants of Hemophagocytic Lymphohistiocytosis Genes in Systemic Juvenile Idiopathic Arthritis

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Abstract

Objective: To evaluate whether there is an enrichment of rare variants in familial hemophagocytic lymphohistiocytosis (HLH)-associated genes among patients with systemic juvenile idiopathic arthritis (sJIA) with or without macrophage activation syndrome (MAS).

Methods: Targeted sequencing of HLH genes (*LYST*, *PRF1*, *RAB27A*, *STX11*, *STXBP2*, *UNC13D*) was performed in sJIA subjects from an established cohort. Sequence data from control subjects were obtained *in silico* (dbGaP:phs000280.v8.p2). Rare variant association testing (RVT) was performed with sequence kernel association test (SKAT) package. Significance was defined as $p<0.05$ after 100,000 permutations.

Results: Sequencing data from 524 sJIA cases were jointly called and harmonized with exome-derived target data from 3000 controls. Quality control operations produced a set of 480 cases and 2924 ancestrally-matched control subjects. RVT of cases and controls revealed a significant association with rare protein-altering variants (minor allele frequency [MAF]<0.01) of *STXBP2* ($p=0.020$), and ultra-rare variants (MAF <0.001) of *STXBP2* ($p=0.006$) and *UNC13D* ($p=0.046$). A sub-analysis of 32 cases with known MAS and 90 without revealed a significant difference in the distribution of rare *UNC13D* variants ($p=0.0047$) between the groups. Additionally, sJIA patients more often carried ≥2 HLH variants than did controls ($p=0.007$), driven largely by digenic combinations involving *LYST*.

Conclusion: We identified an enrichment of rare HLH variants in sJIA patients compared with controls, driven by *STXBP2* and *UNC13D*. Biallelic variation in HLH genes was associated with sJIA, driven by *LYST*. Only *UNC13D* displayed enrichment in patients with MAS. This suggests that HLH variants may contribute to the pathophysiology of sJIA, even without MAS.

Introduction

Systemic juvenile idiopathic arthritis (sJIA) is a severe inflammatory condition with onset in childhood. It is characterized by recurrent episodes of high-spiking fevers, a classic salmon-colored rash, arthritis, generalized lymphadenopathy and serositis.[1] Up to a third of the cases are complicated by macrophage activation syndrome (MAS), a secondary form of hemophagocytic lymphohistiocytosis (HLH), with up to 40% of patients having subclinical forms of the disease.[2,3] Previous small studies have identified an enrichment of variants in genes associated with familial forms of HLH in patients with sJIA and a history of MAS. However, whether these variants associate with sJIA in general has never been evaluated Therefore, we performed a large, targeted sequencing study to investigate the role of HLH variation in sJIA.

Patients and Methods

Samples and Sequencing

Study populations: We studied sJIA patients from the International Childhood Arthritis Genetics (INCHARGE) Consortium (Supplementary Table 1). The patients were diagnosed with sJIA by pediatric rheumatologists from 9 countries according to International League of Associations for Rheumatology (ILAR) criteria,[1] as previously described.[4,5] In a subgroup of patients, referring rheumatologists confirmed whether patients had ever had a history of macrophage activation syndrome by Ravelli criteria.[2] Sequencing data from the Atherosclerosis Risk in Communities (ARIC) Cohort, obtained *in silico* from the database of Genotypes and Phenotypes (dbGaP; accession number phs000280.v8.p2), were utilized as population control data. The ARIC cohort was a large collection of individuals between 45 and 65 years of age assembled from United States to investigate cardiovascular risk factors within communities. The present study was conducted as non-human subjects research, as determined by the Institutional Review Board of the National Institutes of Health.

Sequencing: The sJIA patients underwent targeted sequencing of genes associated with familial forms of HLH (*LYST, PRF1, RAB27A, STX11, STXBP2, UNC13D*) using custom Illumina Nextera Capture Assays. Libraries were sequenced via Illumina HiSeq or MiSeq sequencers. Population controls had undergone whole exome sequencing at the Human Genome Sequencing Center at the Baylor College of Medicine using Illumina HiSeq 2000 platform.[6] Data from the target region were extracted from the whole exome sequencing (Supplementary Data 1).

Variant Calling: All cases and controls were jointly called using a variant discovery pipeline modelled after the Genome Analysis Toolkit (GATK 4.0.12.0) best practices (Broad Institute), and variants were filtered to only include high-quality variants that were well covered on both cases and controls. Details of variant-level quality control are described in the supplementary methods.

Ancestral matching and exclusion of related individuals: The INCHARGE sJIA cohort was predominantly individuals of European ancestry.[4,5] Therefore, the control cohort was composed of ARIC study participants of European ancestry. The INCHARGE and ARIC cohort datasets were integrated with data from the 1000 Genomes Project reference populations to facilitate ancestral matching. Principal components analysis (PCA) was performed with SNP & Variation Suite 8.3.0 (Golden Helix, Bozeman, MT) to identify and exclude all ancestrally dissimilar individuals, as previously described.[4,5] Case-control matching was evaluated by calculating the genomic control inflation factor (λ_{GC}). Cryptically related sample pairs ($Pi_{hat} \geq 0.125$) were identified with PLINK (v1.9) and KING (v2.2), and one member of each pair was excluded. See supplementary methods for full details.

Rare Variant Association Testing

Selection of qualifying variants: Minor allele frequency (MAF) and variant function filters were applied to include rare (MAF < 0.01), exonic, protein altering variants that

mapped to the canonical Ensembl GRCh37.p13 transcript (Supplementary Table 2). As a sensitivity analysis, low-frequency (MAF < 0.05) or ultra-rare (MAF < 0.001) variant sets were also examined.

Statistical tests: Rare variant association testing (RVT) was performed in R (version 4.3.2) using the Sequence kernel association test package (SKAT, version 2.2.5). The primary RVT was performed at the gene and group level using the unweighted SKAT with 100,000 permutations.[7] SKAT is a variance-component test, which enables comparison of variant distributions between 2 groups with the least assumptions. As a sensitivity analysis, we used the SKAT package to perform burden testing. Significance was evaluated at a threshold of $P_{permutation} \leq 0.05$. Frequency of individuals with 2 or more HLH variants was analyzed using two-tailed Fisher's exact test in R.

Results

Targeted sequencing was performed in 525 sJIA patients and sequencing data from the target region were extracted from 3000 population control subjects. Joint variant calling of the 3525 samples revealed data corruption in 1 sJIA case, which was excluded. Subjects with dissimilar genetic ancestry were excluded (39 cases, 22 controls), as were those with cryptic relatedness (1 case, 48 controls) or data missingness over 10% (6 controls). Additionally, 4 sJIA cases were excluded after being found to have monogenic autoinflammatory disease. This produced a final study population of 480 sJIA patients and 2924 control subjects (Supplementary Figure 1). After joint calling, the dataset included 622 variants in 6 HLH genes. Coverage harmonization revealed 115 variants had unbalanced coverage between cases and controls, which were excluded (Supplementary Data 2). Eight additional variants did not meet the hard variant quality control thresholds and were excluded. The final dataset included 499 variants (Supplementary Figure 2, Supplementary Data 3).

Among these 499 variants, 369 were observed in control subjects and 161 were observed in sJIA cases. The sJIA variants included 64 synonymous variants, 5 variants of the 5' or 3' untranslated region and 92 protein-altering variants (91 nonsynonymous variants and 1 non-frameshift deletion). None of the HLH variants observed in cases were classified as pathogenic by ClinVar, whereas 11 variants in the control population were pathogenic, 2 of which were excluded for unbalanced coverage (Supplementary Data 2 and 3). Three of the variants observed in sJIA cases were not present in the gnomAD database. The first (*UNC13D* E1066D) was observed in a patient with known MAS, while the other 2 (*LYST* Q2628R and *LYST* K1729M) were observed in patients with unknown MAS status. There were no rare variant homozygotes among the sJIA patients, however three sJIA patients did carry homozygous, low-frequency variants. These included *UNC13D* R928C, which is listed as benign/likely benign on ClinVar; *PRF1* A91V, which has been shown to cause partial cytolytic dysfunction but is also often observed in healthy individuals,[3] and *UNC13D* A59T, which has been observed in homozygosity in a healthy individual without HLH.[8] Finally, another sJIA case who was excluded for dissimilar ancestry was homozygous for *UNC13D* A59T, while also carrying two *LYST* variants (D2228H and N1228S).

Rare/ultra-rare coding variants of *STXBP2* and *UNC13D* are associated with sJIA

In the primary subgroup of variants (MAF < 0.01), 480 sJIA cases carried 110 alternate alleles of 71 unique HLH gene variants, and 2924 control subjects carried 517 alleles of 198 variants (Supplementary Table 3). Visual comparison of the aggregated alternate allele frequencies of HLH genes revealed a higher frequency of rare, protein-altering variants in sJIA cases than in controls in each comparison (Figure 1). To evaluate whether these differences reflected a significant difference in the distribution of variants, we performed RVT of rare variants of HLH genes by SKAT (Supplementary Table 4). This revealed a significant association between sJIA and *STXBP2* ($p = 0.020$). When SKAT was repeated on the set of ultra-rare variants (MAF < 0.001), the association of *STXBP2* was even more significant ($p = 0.006$), an association with *UNC13D* variation was also identified ($p = 0.046$), and the HLH genes as a panel became significant ($p = 0.011$). We observed no significant association of sJIA with low frequency (MAF < 0.05) variants of HLH genes.

Development of MAS in JIA is associated with rare, protein-altering variants of *UNC13D*

To evaluate the relationship between rare HLH gene variants and MAS, we examined 122 sJIA cases in our cohort who were known to have either had MAS (MAS+; n = 32) or to have never had MAS (MAS-; n = 90) by Ravelli criteria.[2] (Supplementary Tables 5, 6, 7 and 8). Association testing with SKAT identified associations between MAS+ and rare *UNC13D* variation in comparison with either MAS- or healthy controls ($p = 0.0047$ and $p = 0.038$, respectively).

Full sJIA cohort and MAS+ cases have enrichment of rare, protein-altering variants of HLH genes

In situations where rare variants are expected to have the same directional effect on disease risk, burden testing provides greater statistical power to detect rare variant associations than SKAT. Given that HLH-causing variation collectively produces HLH through a reduction of cytolytic capacity,[3] we employed burden testing as a sensitivity analysis (Supplementary Tables 4 and 6). This revealed that rare, protein-altering variants of HLH genes as a panel were significantly enriched in children with sJIA, relative to control subjects ($p = 0.014$), and despite the smaller sample size, the same association was seen in MAS+ compared to controls ($p = 0.030$), but not on MAS- ($p = 0.151$). Rare variants of *PRF1* were significantly enriched in MAS- relative to controls by the burden test ($p = 0.029$), but not in the primary SKAT analysis.

Additionally, sJIA cases more often carried 2 or more rare, protein-altering HLH variants (13/480, 2.7%) than did control subjects (31/2924, 1.1%; $p = 0.007$, OR 2.6 [1.2, 5.2]; Figure 2). Although the MAS+ and MAS- patients carried 2 or more HLH variants at rates higher than the sJIA or control cohorts (2/32, 6.3% and 3/90, 3.3%, respectively), the differences were not statistically significant.

Variation in HLH genes causes congenital HLH in a recessive fashion, but it has also been suggested variants in different HLH genes could synergize to cause pathology in a digenic or oligogenic fashion.[9] We examined the set of variants that were detected in these combinations (Table 1, Supplementary Data 4) and observed that most combinations

involved *LYST* in both cases and controls. However, combinations involving *LYST* occurred significantly more commonly in sJIA (11/480, 2.2%) than in controls (24/2924, 0.8%; p = 0.007; OR 2.8 [1.2,6.0]). While sJIA patients also more often carried biallelic *LYST* variation than did controls (0.8% vs. 0.2%), the difference was not statistically significant.

Discussion:

This study identified an association of rare/ultra-rare variants of *STXBP2* and *UNC13D* in sJIA patients compared to controls. The association with *UNC13D* seems to have been indeed driven by the patients with MAS in the cohort, since the association was apparent in the subset of patients with a known history of MAS, despite the smaller size of this cohort. This finding aligns with previous studies that had identified an increased burden of HLH variants as a whole and an enrichment of *UNC13D* variants in sJIA patients with MAS.[3] In contrast, the *STXBP2* association was only found in the full sJIA cohort, and it was not identified in the MAS positive patients. This could have been due to a lack of power, given that the cohort of MAS patients was much smaller than the full sJIA cohort. However, the smaller size did not limit the identification of the *UNC13D* association, so it is possible that the association of *STXBP2* is truly being driven by the patients without MAS in the cohort, and it is playing a role in the pathophysiology of sJIA itself. *STXBP2* variants have been previously described in sJIA patients with MAS,[3] but the smaller sample size of the study did not allow for evaluation of associations of individual genes.

This study identified that sJIA patients in general and those with MAS have an increased burden of rare HLH variants as a panel compared to controls, which was even more pronounced in the ultra-rare MAF cutoffs. Furthermore, sJIA patients more often carried 2 or more HLH variants than controls, which was driven by *LYST* variants in combination with variants in other genes (or in *LYST*). It is notable that although *LYST* was not associated with sJIA by itself, its presence in combination with another HLH variant was significantly associated. This is consistent with the hypothesis that variants in *LYST*, which is involved in granule biosynthesis and generally leads to milder cytolytic defects, may interact with heterozygous variants in terminal granule processing genes and produce pathology.[9]

Similar to a previous study of MAS in lupus[10] and unlike a study of secondary HLH in adults,[11] we did not identify significant associations of low-frequency HLH variants in sJIA or MAS. The association of rare variants as a panel was only identified by burden tests, which could be attributable to its higher power to identify associations when all variants have the same directionality of effect. In this way, it would also be more sensitive to identify synergistic effects of combined hypomorphic variants. We did not identify a significant association of HLH variants in the MAS- group compared to controls in the primary analysis. However, we did identify an enrichment of rare *PRF1* variants in this group by burden test. Therefore, it is possible that in larger studies of sJIA patients without MAS, an association with *PRF1* could rise to significance.

This study did not recapitulate previous findings of an association of *PRF1* variants in patients with MAS.[12] Specifically *PRF1* A91V, which had been previously associated

with MAS in sJIA,[12] and with severity of secondary HLH,[11] was more common in sJIA patients without MAS than in patients with MAS in our cohort and had similar incidence in sJIA patients and controls. Functional studies of this variant have shown it to cause a partial loss of protein function and stability, however, it has been vastly reported in unaffected individuals as well.[3] We did identify one patient of unknown MAS status who was homozygous for *PRF1* A91V, which has been shown to cause transient cytolytic defects in the setting of HLH.[13] Our study helps to increase the understanding that even though *PRF1* A91V variants might predispose to HLH in the context of infections[13,14] or immune reconstitution syndrome,[15] this variant does not seem to confer an increased risk of development of MAS in the inflammatory background of sJIA. This could suggest that the increased susceptibility to HLH in the context of infection could be related to a component of immunodeficiency.

A major strength of this study is its power. As the largest examination of HLH gene variation in sJIA to date, it provides the best estimate of the burden of HLH variation among patients with sJIA and the first population-level assessment of these genes on an individual basis. Another advantage of this study design was its use of a large population control dataset. This enabled the study to move beyond investigations of sJIA/MAS and discover new relationships between HLH gene variants and sJIA, itself. Furthermore, the analysis of individual sequencing data allowed us to compare the frequency of digenic HLH variants in cases and controls, as well as the combination of variants.

This study also had some limitations. First, variation identified in the study was not validated by Sanger sequencing. Instead, strict quality cutoffs were systematically applied for variant calling to minimize the inclusion of false positive variants. Second, detailed clinical data that may have enabled informative sub-analyses were not available. For example, the INCHARGE sJIA cohort lacked granular clinical data that may have allowed examination of the relationship between HLH variation and disease severity. Similarly, the ARIC cohort lacked clinical data that may have allowed variant-phenotype analyses of variants classified as “pathogenic” or “likely pathogenic.” Third, the INCHARGE cohort was predominantly composed of individuals of European ancestry and this study examined an ancestrally matched European case-control collection. Because the distribution of rare variation differs between ancestries, it is necessary to independently evaluate the relationship between rare HLH gene variation and sJIA in non-European populations. Future studies of large cohorts of sJIA patients from diverse backgrounds will allow for the replication of these findings in patients with different ancestries. Furthermore, the INCHARGE cohort did not include parental sampling, therefore it was not possible to determine whether multiple variants were inherited in *cis* or *trans*. Ongoing efforts to assemble large cohorts with detailed clinical datasets that can be linked to sequencing data and biospecimens will help clarify further the associations of HLH variants in sJIA patients without MAS. Finally, we did not perform functional studies in our cohort, and with most variants being of unknown significance, it was not possible determine their pathogenicity. All the pathogenic variants in this cohort were identified among the controls, which might reflect a sampling bias in the relatively smaller sJIA cohort. It is also possible that the class of variants that are enriched in sJIA patients might cause disease through a different mechanism than what has been studied for HLH. This has been previously noted in the context of very early onset inflammatory bowel

disease, in which variants of *STXBP2* have been found to cause intestinal symptoms through other impacts in cell trafficking beyond cytolytic activity.[16] Future studies are needed to verify the functional effect of the individual variants identified in the sJIA cases, as well as the digenic combinations found in this group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data sharing statement:

"All data used in this research are already included in the article or the supplementary material."

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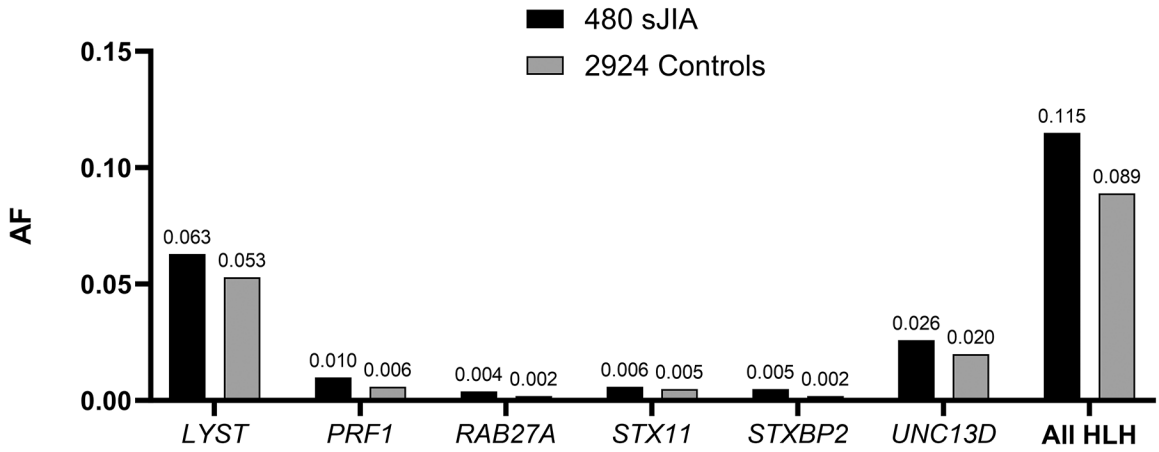

Figure 1. Aggregated allele frequencies from crude counts of rare qualifying variants in the sJIA cohort compared to controls.

Aggregated alternate allele frequencies were calculated as the total count of alternate alleles in all the variants in the gene, divided by the mean number of alleles identified in all the variants in the gene (to account for missing genotypes). Rare qualifying variants defined as minor allele frequency <0.01, exonic, non-synonymous, passing quality filters, present in the canonical transcript. Burden test showed significant difference in all HLH genes as a panel ($p = 0.014$) and SKAT showed significant association of *STXBP2* ($p= 0.020$).

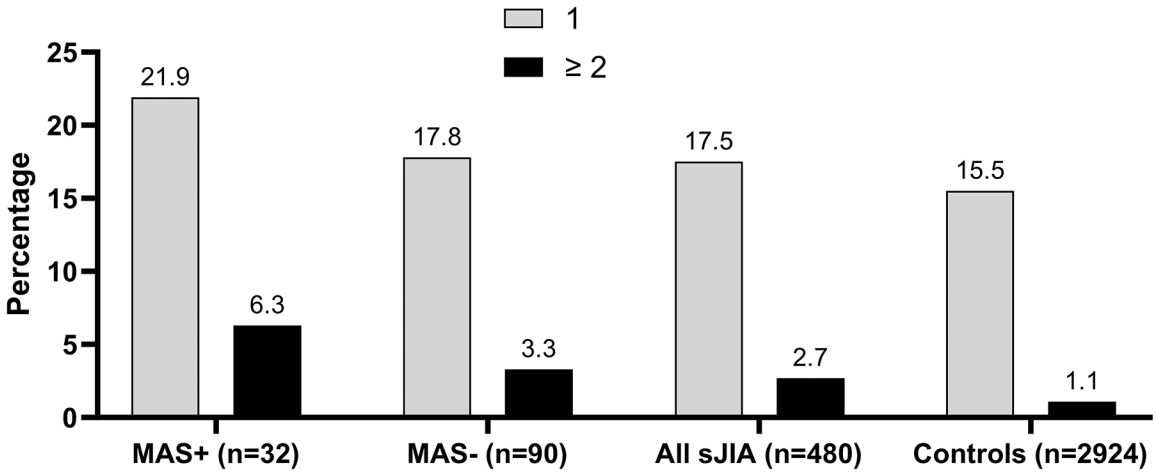

Figure 2. Distribution of individuals with one or more rare, protein-altering HLH variants.

A bar plot displays the proportion of patients with either 1 or ≥2 rare (MAF < 0.01), protein-altering variants on canonical HLH gene transcripts among sJIA patients with a history of MAS (MAS+), sJIA patients with no history of MAS (MAS-), the full sJIA cohort and the control collection. MAS, macrophage activation syndrome. MAF, minor allele frequency. HLH, hemophagocytic lymphohistiocytosis.

Table 1.

Combination of variants on sJIA patients with more than one rare qualifying HLH variant

MAS		*LYST*	*STX11*	*PRF1*	*RAB27A*	*UNC13D*	*STXBP2*
Positive	P1	Q562H				V779M	
Positive	P2	N2971K			R184Q		
Negative	P3	Y875C	L9R				
Negative	P4	N2971K, Q562H					
Negative	P5	V3775M, Q562H					
Unknown	P6	Q562H				R1074W	
Unknown	P7			T496I			A211T
Unknown	P8	R3509Q, N2971K					
Unknown	P9	N3158S				H224Y	
Unknown	P10	R1718Q					P573L
Unknown	P11		V197M	P501S			
Unknown	P12	R2624W, K1899N					
Unknown	P13	N2971K				R1074W	

Rare qualifying variants defined as minor allele frequency < 0.01, exonic, non-synonymous, passing quality filters, present in the canonical transcript.