Published in final edited form as:
Arthritis Rheumatol. 2014 December ; 66(12): 3486–3495. doi:10.1002/art.38793.

Whole Exome Sequencing Reveals Overlap Between Macrophage Activation Syndrome in Systemic Juvenile Idiopathic Arthritis and Familial Hemophagocytic Lymphohistiocytosis

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Abstract

Objective—Macrophage activation syndrome (MAS), a life-threatening complication of systemic Juvenile Idiopathic Arthritis (SJIA), resembles Familial Hemophagocytic Lymphohistiocytosis (FHLH), a constellation of autosomal recessive immune disorders resulting from deficiency in cytolytic pathway proteins. We hypothesized that MAS predisposition in SJIA could be attributed to rare gene sequence variants affecting the cytotolytic pathway.

Methods—Whole exome sequencing (WES) was used in 14 SJIA/MAS patients and their parents to identify protein altering SNPs/indels in the known HLH-associated genes. To discover new candidate genes, the entire WES data were filtered to identify protein altering, rare recessive homozygous, compound heterozygous, and *de novo* variants with the potential to affect the cytolytic pathway.

Results—Heterozygous protein-altering rare variants in the known genes (*LYST*, *MUNC13-4*, and *STXBP2*) were found in 5 of 14 SJIA/MAS patients (35.7%). This was in contrast to only 4 variants in 4 of 29 (13,7%) SJIA patients without MAS. Homozygosity and compound heterozygosity analysis applied to the entire WES data in SJIAMAS, revealed 3 recessive pairs in 3 genes, and 76 compound heterozygotes in 75 genes. We also identified 22 heterozygous rare protein altering variants that occurred in at least two patients. Many of the identified genes encode proteins with a role in actin and microtubule reorganization and vesicle-mediated transport. "Cellular assembly and organization" was the top cellular function category based on Ingenuity Pathways Analysis ($p<3.10E-05$).

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Conclusion—WES performed in SJIA/MAS patients identified rare protein altering variants in the known HLH associated genes as well as new candidate genes.

INTRODUCTION

Systemic juvenile idiopathic arthritis (SJIA) is a unique category of childhood chronic arthritis currently classified as a subtype of juvenile idiopathic arthritis [1,2]. It is characterized by arthritis, spiking fevers, characteristic rash, hepatosplenomegaly, lymphadenopathy and polyserositis. SJIA appears to be driven by continuous activation of innate immune pathways with dysregulated production of innate proinflammatory cytokines supporting the classification of SJIA as an autoinflammatory disorder [3]. A subset (~10–30%) of SJIA patients develop macrophage activation syndrome (MAS), a potentially life-threatening complication characterized by an overwhelming inflammatory reaction driven by excessive activation and expansion of T cells (mainly CD8+) and hemophagocytic macrophages [4,5]. Even with appropriate and timely treatment, MAS can be fatal. Reported motality rates reach 20–40% [6,7].

MAS bears striking clinical resemblance to a group of histiocytic disorders collectively known as hemophagocytic lymphohistiocytosis (HLH) [8,9]. HLH describes a spectrum of disease processes characterized by accumulations of well-differentiated mononuclear cells with a macrophage phenotype exhibiting hemophagocytic activity [9]. HLH is further subdivided into primary or familial (FHLH) and secondary or reactive (ReHLH) [10,11]. FHLH is a constellation of autosomal recessive immune disorders resulting from various genetic defects all leading to profoundly depressed cytolytic activity of natural killer (NK) cells and cytotoxic CD8+ lymphocytes (CTL). Its clinical symptoms usually become evident early in life. ReHLH tends to occur in older children and more often is associated with an identifiable infectious episode [8,9]. Based on clinical similarities between MAS and ReHLH, some rheumatologists view MAS as ReHLH that occurs in a setting of a rheumatic disease [8]. The distinctions between primary and secondary HLH, however, become increasingly blurred as new hypomorphic genetic variants are identified, some of which are associated with somewhat distinct clinical presentations occurring later in life [12].

Normally, NK cells and CTLs recognize and kill cells infected with viruses as well as tumorigenic cells. The killing is achieved through the regulated exocytosis of the cytotoxic effector molecules such as perforins and granzymes [13,14]. The lethal hit delivery to target cells involves the reorganization of the actin cytoskeleton, followed by the repositioning of the microtubule-organizing center (MTOC) and the centrosome beneath the immune synapse and by a microtubule cytoskeleton–directed movement of lytic granules toward the target cells.

In about 30% of FHLH patients the cytolytic dysfunction is due to mutations in the gene encoding perforin (*PRF1*) [15]. The proteins encoded by several other FHLH-associated genes (*MUNC13-4* [16], *Syntaxin 11 (STX11)* [17], and *syntaxin binding protein 2* (*STXBP2*, known as *MUNC18-2)* [18] are involved in the docking and fusion of the perforin-containing granules with the outer membrane. Defects in the exosome granule dependent cytotoxic functions of lymphocytes have also been implicated in two other genetic diseases

associated with the hemophagocytic syndrome. Thus, mutations in the gene encoding Rab27a, one of the MUNC13-4 effector molecules, have been linked to the development of Griscelli syndrome type 2 [19]. Mutations in the *LYST* gene have been identified as a cause of Chediak-Higashi syndrome [20]. HLH following exposure to EBV is the most frequent life-threatening complication of X-linked Lymphoproliferative Syndrome (XLP). XLP1 is caused by hemizygous mutations in the *SH2D1A* gene encoding SAP (SLAM-associated Protein), which leads to abnormal NK cell responses and invariant NKT cell deficiency [21]. XLP2 is caused by mutations in *BIRC4*, which encodes XIAP, and has been described as an X-linked form of familial HLH [22].

The exact pathway linking cytolytic abnormalities in MAS patients with excessive expansion of macrophages is not clear. One suggested mechanism is related to the fact that HLH/MAS patients appear to have a diminished ability to clear specific infections [23]. In this scenario, NK cells and cytolytic T cells fail to kill infected cells and, as a result, remove the source of antigenic stimulation. In turn, this leads to persistent antigen-driven activation and proliferation of T cells associated with production of cytokines that stimulate macrophages. Another explanation for macrophage expansion is based on the observations suggesting that in some circumstances cytotoxic cells may be directly involved in induction of apoptosis of activated macrophages and T cells. Failure to induce such apoptosis may delay the contraction stage of the immune response [24,25]. In both scenarios, persistent expansion of activated T cells and macrophages leads to escalating production of pro-inflammatory cytokines.

Although familial cases of MAS in SJIA have not been reported, as in FHLH, SJIA/MAS patients have functional defects in the granule dependent cytolytic pathway [26]. Furthermore, there have been several case reports describing MAS/SJIA patients who were heterozygous for rare variants in the FHLH-associated genes including *PRF1* [27], and *MUNC13-4* [28,29], suggesting that as in FHLH, genetic component may contribute to MAS predisposition in SJIA.

We hypothesized that predisposition to MAS in SJIA could be attributed to many individually rare variants affecting the granule dependent cytolytic pathway. Some of these variants may be *de novo*, while others are inherited, but cumulatively they account for most of the genetic component to MAS susceptibility. JIA is thought to represent a complex genetic trait [30]. Over the last several years, most focus has been placed on dissecting the genetic basis of complex diseases and traits such as JIA through genome wide association studies. In a recent genome wide association meta-analysis of 1,447,416 common SNPs in patients with SJIA, only a weak association was found with the 3 Mb interval that contains a range of genes involved in both innate and adaptive immunity including BTNL2 [31]. These observations prompted us to explore other potential sources of genetic susceptibility to MAS such as rare variants with larger effect sizes. Because of their low frequencies, these variants are very poorly assessed with available GWAS microarrays.

To address this, we took advantage of the *whole exome sequencing* methodologies that provide an unprecedented opportunity to detect rare variants both in the genes localized to a specific locus and in the genes from multiple loci involved in the same pathway [32–36].

First, we used this methodology to identify novel and previously reported rare protein altering SNPs/indels in the known HLH-associated genes. We then applied a family based *whole exome sequencing* approach to identify novel candidate genes. This was achieved through the identification of protein altering *de novo* variants as well as rare recessive homozygous and compound heterozygous variants. Particular attention was also given to candidate genes that had the potential to affect the cytolytic pathway.

MATERIALS AND METHODS

Patients

The study subjects were 14 SJIA/MAS patients who satisfied the ILAR criteria for SJIA [37], and had a positive history for MAS diagnosed using either Ravelli's SJIA-specific MAS criteria [38] or FHLH diagnostic criteria [11] (See Table 1). DNA samples from these patients as well as their parents were made available for the study through Cincinnati Pediatric Rheumatology Tissue Repository under approval of the Cincinnati Children's Hospital Medical Center (CCHMC) Internal review board. Twenty nine SJIA patients without MAS history were included as a comparison group.

NK-cell cytotoxicity

NK-cell cytotoxicity was analyzed as a part of the diagnostic evaluation at the time when MAS was suspected in the Diagnostic Immunology Laboratory at CCHMC. NK cytolytic activity was measured after co-incubation of PBMC with NK- sensitive K562 cell line as previously described [26]. Based on the normal range of NK cell cytolytic activity in pediatric controls established in the same laboratory, values below 2.6 LU are considered low.

Exome sequencing

Exon specific next generation sequencing was performed at the Novartis Institute for Biomedical Research. Briefly, DNA sequencing libraries were prepared using the NuGEN Ovation Ulralow DR Multiplex protocol. Capture of the 70Mb exome plus UTR sequences was performed using the Agilent SureSelectXT Target Enrichment System protocol (SureSelect Human All Exon V4+UTRs) protocol. Sequencing was performed on an Illumina HiSeq 2000 with a 2x 76bp read length. NGS reads were aligned to the human genome (HG19) using the Burrows-Wheeler Aligner (BWA).

Library Preparation and DNA Sequencing

100ng of dsDNA determined by Invitrogen Qubit high sensitivity spectrofluorometric measurement was sheared by sonication to an average size of 300bp on a Covaris E210 system. Library construction, amplification and size selection was performed as described in the NuGen Ovation Ultralow DR Multiplex protocol. Each library was uniquely indexed using the NuGen L2DR index series. Library capture was performed using the Agilent SureSelect XT V4+UTR capture kit with the addition of NuGen blockers and sequenced on an Illumina HiSeq2000 with a read length of 2x 76bp. Index demultiplexing was performed using the Illumina CASAVA suite and read QC was performed using the FASTQC package from the Babaram institute (Cambridge, UK).

Data Alignment and Analysis Methods

Alignment and variant calls were made using the Broad Institute's Genome Analysis Toolkit (GATK) following the pipeline described by De Pristo et al [32] and documented best practices. (http://www.broadinstitute.org/gsa/wiki/index.php/The_Genome_Analysis_Toolkit). Reads that passed Illumina's Chastity Filter were aligned with the Burrows Wheeler Aligner (BWA) [39] to the human genome reference HG19. The Picard MarkDuplicates utility was used to flag reads that appear to be artifacts of PCR bias. All reads that overlapped known or putative indels were re-aligned. Base quality scores were recalibrated to the empirical error rate derived from non-polymorphic sites. GATK's UnifiedGenotyper module was used to call variant sites (both single nucleotide and small indel) in all samples simultaneously. Finally, SNV calls were filtered using the variant quality score recalibration method described by DePristo et al. [32]. Indel calls were filtered with a set of hard filters.

Variant filtering and annotation

Variant calls were analyzed using Golden Helix SNP and Variation Suite ver 7.7.5 (http://www.goldenhelix.com). Variants were filtered based on individual sample and variant quality control measurements in the VCF files generated by GATK. Variants that had a read depth (DP) less than 15 and a genotype quality score (GQ) less than 20 were removed. For each genotype a ratio (alt-ratio) of the alternate allele read depth/(alternate allele read depth + reference allele read depth) was calculated. Homozygous reference genotypes with an alt-ratio greater than 0.15 and homozygous alternate alleles genotypes with an alt-ratio less than 0.85 were removed. Heterozygous genotypes with an alt-ratio less than 0.3 or greater than 0.7 were also removed. Variants passing these QC parameters were further filtered to coding exons with the ability to alter the amino acid sequence of a protein (synonymous SNPs were removed) using the UCSC genome browser build 37 human Reference Sequence Gene table.

Identification of candidate causative polymorphisms

Samples and sequence data that passed the above criteria were analyzed for polymorphisms in known FHLH genes. A trio (father, mother and proband) study design was used for all analyses. We defined *de novo* variations as polymorphisms in which both parents are homozygous for the reference allele and the proband contained a heterozygous genotype. For homozygous recessive variants we required both parents to be heterozygous for the variant and the proband to be homozygous for the rare allele. To further reduce the number of candidate variations we required that the minor allele frequency (MAF) in the general population (all races) was less than 1% or novel. This approach has been successfully used to identify candidate genes in other diseases [33,34]. The selection of the cut off value for MAF at less than 1% was based on the estimated prevalence of MAS. Since patients with SJIA account for approximately 10–15% of patients with JIA [1], and about 10–30% of SJIA develop MAS [40,41], the prevalence of MAS in the general population should be somewhere between 1:10,000 and 1:100,000. If MAF =1%, the probability of a recessive pair or a compound heterozygote is 1:10,000. Therefore, setting the cut of value for MAF at less than 1% should allow for capturing the relevant variants. Minor allele frequencies were based on the 1000 genomes project phase 1 v3 database and the NHLBI exome sequence

project ESP6500 variant frequency data. In addition, we generated and used an internal allele frequency table of 312 whole exomes analyzed at Cincinnati Children's Hospital Medical Center.

For compound heterozygous polymorphism we required the proband to contain at least two heterozygous polymorphisms in the same gene and neither parent could contain both polymorphisms. One variant could have a minor allele frequency in the general population up to 5% (based on the above data sources), however, the other polymorphism had to have an minor allele frequency below 1%. Functional predictions for variants identified in this study were generated using the dbNSFP functional predictions ver 2 table. All filtering and annotation was performed using the Golden Helix SVS software and data tables downloaded from the Golden Helix Website (with the exception of the CCHMC exome allele frequency table).

Validation of *de novo* variants by alternative methods of sequencing

Since current *whole exome sequencing* methodologies are associated with significant false positive rates, Sanger sequencing was used to confirm all de novo and some novel inherited variants. The sequencing was carried out with SNP flanking primers designed using BatchPrimer3 (primer sequence available upon request). The PCR reaction was performed in a 50ul reaction containing 5ul of AccuPrime *Pfx* Reaction master mix (Life Technologies, Grand Island, NY), 100ng of genomic DNA, and 0.5ul of each primer with a concentration of 200pm/ul. The resulting PCR products were cleaned up with Shrimp Alkaline Phosphatase (Sigma, St Louis, MO) (0.5U/ul) and ExoI Nuclease (New England BioLabs, Ipswich, MA) (2U/ul) prior to sequencing.

Pathway analysis

To identify prevailing biologic themes gene lists were analyzed using Ingenuity Pathways Analysis Software (Ingenuity Systems; Redwood City, CA).

RESULTS

Whole exome sequencing data was successfully generated and passed quality control for 14 trio sets of SJIA/MAS patients. The average read depth per sample ranged from 25.2X to 52.8X. Initially, 264,628 variants were found in the 14 SJIA/MAS families. Following QC filtering and annotation, 37,890 variants located in protein coding exons were identified.

Variants in the known genes

Initial efforts were focused on the identification of novel and previously reported rare protein altering SNPs/indels in the genes associated with primary hemophagocytic syndromes. Based on the described filtering criteria, rare protein-altering variants in FHLH associated genes were identified in 5 of 14 patients (Table 2). All these variants have been confirmed by Sanger sequencing. Two patients (PID 53 and 327) were heterozygous carries of the same rare variant in *STXBP2* (rs151257815/chr19/pos 7707311/795-4C>T, splice site, intronic). This variant has been previously reported in late onset FHLH [12]. One patient (PID 900) was a heterozygous carrier of a rare variant in *MUNC13-4* (rs35037984/

chr17/73826491/2782C>T, R928C). The functional significance of this variant is not known. Additionally, two patients (PID 155 and 7431) were heterozygous carries of rare variants in *LYST* (rs147794568/chr1/pos 235972178/T>G, Leu647Arg and rs115330112/chr1/pos 235938329/T>G, Ser1840Ala, respectively). Patient 155 also had a deletion in *STXBP2* at position 7705108 causing a frameshift in the coding region. The functional significance of these variants is not known.

To assess whether a similar degree of enrichment for rare variants in FHLH- associated genes is seen in SJIA in general, WES was performed in 29 SJIA patients without MAS history. Patients selected for this part of the study had been followed at CCHMC for at least 5 years and had never had clinical features suggestive of MAS. When the same filtering criteria were applied to this group of SJIA patients, only 4 variants were detected in 4 of 29 patients (13.7%). One patient (PID 70838) had protein-altering SNP in *PRF1*, and three patients (PIDs 2152, 2144, and 7464) had protein-altering SNPs in *LYST*. The observed variants are listed in Table 3. The functional significance of these variants is not known.

Recessive pairs

Clinically similar FHLH is a constellation of autosomal recessive disorders. Therefore, in a search for new candidate genes in MAS, using a trio approach in the SJIA group, the exome sequence data were filtered to identify inherited novel and previously reported homozygous, but rare (i.e. MAF<1%) protein altering SNPs/indels for which parents were heterozygous. Three variants met the filtering criteria. The product of the first gene *CCDC141* (coiled-coil domain containing 141 also known as *CAMDI*) has been shown to interact with MTOC associated proteins and cytoskeleton system [42]. This gene shows a ubiquitous pattern of expression. In contrast, the second gene, *FAM160A2*, is expressed mainly in hematopoietic cells with particularly high levels of expression in $CD8^+$ T cells and NK cells (www.biogps.org). A protein complex that includes FAM160A2 (also known as FTS and Hook-interacting protein) has been shown to promote vesicle trafficking and fusion [43]. The function of the third gene *LRGUK* (leucine-rich repeat and guanylate kinase domain-containing protein) is not known.

Compound heterozygotes

Since compound heterozygosity has been reported in FHLH [12], we attempted to identify rare protein altering variants for which the patient was a compound heterozygote while the parents contained at least one wild-type gene copy. In this analysis, the filtering criteria were relaxed: while the MAF for one allele remained at <1%, the MAF for the second allele was allowed to be at <5%. When these filtering criteria were applied, 76 compound heterozygotes were identified. The full lists of the genes and variants are shown in Supplemental Table 1. Of note, compound heterozygosity in *XIRP2* was observed in 2 patients. XIRP2 belongs to the Xin-repeat family of proteins. Xin-repeats in these proteins have been shown to bind to and stabilize the actin-based cytoskeleton [44]. *EXPH5* (also known as *SLAC2B*) was another interesting gene found as a compound heterozygote. The product of this gene has been shown to bind the GTP-bound form of Ras-related protein Rab-27A [45]. Loss of Rab27a impairs the docking of granules to the plasma membrane at the immune synapse leading to the development of Griscelli syndrome type 2 [19]. The

product of another compound heterozygote gene, *ARHGAP21*(Rho GTPase activating protein 21), is involved in regulation of CDC42 activity that affects both actin- and microtubule-dependent cellular processes [46].

FHLH patients who are heterozygous for the A91V perforin variation often have other heterozygous genetic defects [12]. This suggests the potential cumulative effect of heterozygous variants in multiple genes that affect the cytolytic function through a gene dosage effect. Accordingly, in the current study we identified genes with heterozygous rare protein altering variants that occurred in at least two patients. These genes are shown in Table 4. There are several interesting genes in this group. *MICAL2* encodes microtubule associated monooxygenase, calponin and LIM domain containing 2 protein. Recent discoveries of the ability of MICALs to bind and directly modify the actin cytoskeleton, implicate these proteins in the process of the docking and fusion of exocytotic vesicles [47]. MICALs also have anti-apoptotic properties. Two patients had the heterozygous variant in *XIRP2* (val762asp) that was also shared by the patients who were compound heterozygotes for variants in this gene. The MAF of this variant in the general population is 0.0005 according to the 1000 genomes project database. The *CADPS2* gene encodes a member of the calcium-dependent activator of secretion protein family, that regulate vesicle trafficking and exocytosis [48]. CADPS2 shows a ubiquitous pattern of expression. The protein encoded by the *FKBPL* gene has similarity to the immunophilin protein family, which play a role in immunoregulation and basic cellular processes involving protein folding and trafficking. Also it appears to have some involvement in the control of the cell cycle. The protein encoded by TNFRSF10B is a member of the TNF-receptor superfamily, and contains an intracellular death domain. This receptor can be activated by tumor necrosis factor-related apoptosis inducing ligand (TNFSF10/TRAIL/APO-2L) and transduces an apoptosis signal [49].

X-linked recessive variants in males

Patients with X-linked lymphoproliferative disease have been reported to have increased risk of HLH [22]. We attempted to identify the X-linked, rare and protein altering variants in male patients. These variants were required to satisfy the following criteria (1) located on X chromosome (2) MAF < 1% (3) predicted to alter the protein function. (4) heterozygous in the mother (5) hemizygous in the male proband. Filtering with the above criteria generated 13 variants in 13 genes across 6 of the 14 patients (see Supplemental Table 2). One interesting example is the *GDI1* gene. This gene encodes Rab GDP dissociation inhibitor alpha, a protein that belongs to the small GTP-binding proteins of the ras superfamily that are involved in vesicular trafficking of molecules between cellular organelles. Although it is expressed mainly in the neuronal tissue, and variants in this gene have been linked to non-specific mental retardation [50], *GDI1* expression has also been documented in CD8+ Tcells and NK cells (www.biogps.org).

De novo variants

This part of the analysis was based on the hypothesis that at least in some SJIA/MAS patients, MAS could be explained by *de novo* variants in the exome with the potential to alter the function of other proteins involved in the cytolytic pathway. A similar approach has

been successfully applied to identify causal variants in individuals with sporadic genetic diseases [35]. Full list of *de novo* variants validated by Sanger sequencing is shown in Table 5. Validation included sequencing both parents and the patient. The rate of de novo variants (0.57 per sample) is similar to rates observed in other exome projects for other phenotypes [36].

Biologic themes

When the Ingenuity Pathways Analysis was applied to the entire list of genes to discern biologic themes, the top cellular function categories included “Cellular Assembly and Organization” (Table 6).

DISCUSSION

Based on striking clinical resemblance between MAS in SJIA patients and autosomal recessive hemophagocytic syndromes caused by various genetic defects all leading to profoundly depressed CTL activity, the main purpose of the current study was to identify genetic variants with the potential to affect the granule dependent cytotolytic pathway in patients with MAS/SJIA. A large number of interesting rare variants with the potential to affect various parts of this pathway were identified. First, 5 of 14 patients (35.7%) had heterozygous protein altering variants in the known HLH associated genes. Two patients had the splice site variant in the *STXBP2* gene previously observed in adult onset FHLH [12], suggesting a role for this variant in the development of MAS. In addition, one patient had a protein altering variant in MUNC13-4 of unknown significance and 2 patients had variants in LYST. This was in contrast to only 4 variants of unknown significance in 4 of 29 (13,7%) SJIA patients without MAS history (p-value 0.098). The “enrichment” for heterozygous variants in the genes implicated in genetic hemophagocytic syndromes in our cohort of SJIA/MAS patients reinforces the concept that in addition to the striking clinical similarities, there is some pathophysiologic and genetic overlap between FHLH and MAS in SJIA.

The subsequent recessive homozygosity and compound heterozygosity analysis identified a large number of genes with several of them encoding proteins important for *intracellular vesicle transport.* In fact, when the Ingenuity Pathways Analysis was applied to the entire list of genes to the biologic themes, the top cellular function categories included “Cellular Assembly and Organization” and “Cellular Function and Maintenance”. One interesting candidate gene in this group is *SLAC2B*. SLAC2B binds Rab27a and does play a role in Rab27a dependent membrane trafficking of melanosomes [45]. It is expressed in lymphocytes but it is not clear whether it plays the same role in CTL degranulation. Another interesting gene in this group is *ARHGAP21*. Its product regulates vesicular traffic and intracellular organelle structure by recruiting coat proteins and modulating the structure of actin at membranes surfaces [46]. *MICAL2, XIRP2,* and *CADPS2* were among the genes with heterozygous rare protein altering variants that occurred in at least two patients. The products of these genes also have the ability to modify the actin cytoskeleton and influence the process of docking and fusion of exocytotic vesicles. The impact of the identified sequence variants on the cytolytic function and ultimately on the clinical phenotype now needs to be investigated in further mechanistic studies.

The general approach used in this study has a number of limitations. First, since only exons were sequenced, variants in the promoter and intronic regions that could affect the rate of transcription were not assessed. Due to the limitations of WES, large variants such as structure variants and copy number variations were not assessed either. Additional limitations were related to the somewhat artificially set filtering criteria. In this study, rare variants were defined as those with MAF <1% based on estimated prevalence of MAS in the general population in the range of 1:10,000 to 1:100,000. In clinically similar FHLH, the frequency of the relatively common *PRF1* A91V variant (MAF of about 3%) is increased among HLH patients suggesting a pathologic role. Similar variants could have been missed in our analysis. One also needs to consider the possibility of the potential cumulative effect of heterozygous variants in multiple genes that affect the cytolytic function through a gene dosage effect. Consistent with this idea, FHLH in patients who are heterozygous for the A91V perforin variation is often associated with other heterozygous genetic defects [12]. In the present study we identified only those heterozygous variants that occurred in at least two patients. Nevertheless, despite all these limitations WES performed in a small number of MAS patients identified rare protein altering variants in the known HLH associated genes as well as in new candidate genes reinforcing the role of the cytolytic pathway in MAS and providing additional support for the concept of genetic overlap between MAS in SJIA and FHLH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Substantial contributions to Study design and acquisition of data were provided by Edward J. Oakeley, Virginie Petitjean and Marc Altorfer, Novartis Institutes for Biomedical Research, Basel, Switzerland

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Table 1

Systemic JIA/MAS Patients

Family ID	Patient ID	Gender	Ethnicity	Age at SJIA onset	Number of joints involved	Number of MAS episodes	NK cell cytolytic activity* (LU)	HLH criteria met**	MAS criteria met***	HPS in bone marrow biopsy	Treatment	Outcome
21	53	F	Caucasian	2.4 y	18	>3	0.8	yes	yes	yes	CS, CyA	recovered
60	155	M	Caucasian	4.1 y	7	>3	0	yes	yes	yes	CS, CyA	recovered
88	241	M	Caucasian	2.8 y	2	>3	0.9	yes	yes	yes	CS, CyA	died****
119	327	M	Caucasian	2.8 y	16	1	0.8	no	yes	yes	CS, CyA	died
342	900	F	Caucasian	6.8 y	28	1	0	no	yes	N/D	CS,	recovered
3784	7431	F	Caucasian	5.5 y	13	1	6.6	no	yes	N/D	CS,	recovered
3790	7437	M	Caucasian	2.2 y	16	2	0.1	yes	yes	N/D	CS, CyA	recovered
3807	7472	M	Caucasian	15.7 y	28	1	0	yes	yes	yes	CS, IVIG, Rituximab	recovered
3932	7894	M	Hispanic	4.7 y	4	>3	N/D	yes	yes	yes	CS, CyA	recovered
4218	8463	F	Caucasian	13.7 y	16	1	2.4	no	yes	yes	CS, CyA	recovered
4272	8610	M	Caucasian	0.9 y	10	1	10.1	yes	yes	N/D	CS, CyA	Recovered
4388	8872	F	Caucasian	12 y	16	1	0.4	no	yes	yes	CS	Recovered
12778	70087	F	Caucasian	0.9 y	28	1	15.8	yes	yes	yes	CS, CyA	Recovered
12937	72575	F	Caucasian	16 y	2	1	N/D	yes	yes	yes	CS, CyA, ATG	Recovered

* NK cytolytic activity was measured after co-incubation of PBMC with NK- sensitive K562 cell line in the Diagnostic Immunology Laboratory at Cincinnati Children's Hospital as previously described [26]. Based on the normal range of NK cell cytolytic activity in pediatric controls established in the same laboratory, values below 2.6 LU are considered low.

** Based on the FHLH diagnostic guidelines [11]

*** Based on the preliminary MAS diagnostic criteria [38]

**** Patient PID241 recovered from several episodes of MAS, but eventually died from the later episode of MAS after the transfer to adult care

CS - corticosteroids; CyA – cyclosporine A; IVIG – intravenous immunoglobulin; ATG –anti-thymocyte globulin

HPS - hemophagocytosis

Table 2

Genes of Interest with Variations in SJIA/MAS Patients

Family/Patient ID	Known Genes Implicated in Clinically Similar Syndromes	New Genes		
	Heterozygous variants	Recessive Pairs		De Novo variants
		Homozygotes	Compound heterozygotes	
21/53	*STXBP2**		6 (XIRP2)	*C7orf70, ACIN1*
60/155	*LYST, STXBP2*	*CCDC141*	7	
88/241			3	
119/327	*STXBP2**		3	*NIPBL, ZIK1*
342/900	*MUNC13-4*	*FAM160A2*	4 (*ARHGAP21*)	
3784/7431	*LYST*		6	*WDR66*
3790/7437			7	*KIF24, GPR132*
3807/7472			4	
3932/7894			12 (XIRP2)	
4218/8463			3	
4272/8610			5	
4388/8872			10 (*SLAC2B)*	
12778/70087			3	
12937/72575		*LRGUK*	3	*CAMK1, OGG1*

Table 3

Variations in FHLH Associated Genes Assessed in 29 SJIA Patients Without MAS History.

PID	Chrom	Position	Gene	Polymorphism	DNA coding	Protein	Identifier
2152	1	2.36E+08	LYST	Nonsyn SNV	c.5186A>T	p.Lys1729Met	
2144	1	2.36E+08	LYST	Nonsyn SNV	c.1686G>C	p.Gln562His	rs77091385
7464	1	2.36E+08	LYST	Nonsyn SNV	c.1664A>C	p.His555Pro	
70838	10	72358591	PRF1	Nonsyn SNV	c.886T>C	p.Tyr296His	rs148237800

Table 4

Rare Variants Identified in Multiple Samples in SJIA/MAS Patients

Type	Chr	Position	Gene	Alternate	Class	Protein	Case Allele Count	1gk Freq	ESP Freq
Het	1	75684979	SLC44A5	T	Nonsyn	Gly410Glu	2	0.0005	0.000846
Het	2	97531651	SEMA4C	A	Nonsyn	Pro92Ser	2		0.001461
Het	2	168100187	XIRP2	A	Nonsyn	Val762Asp	2	0.0005	
Hmz/Het	2	179914649	CCDC141	A	Nonsyn	Pro7Leu	3	0.0037	
Het	2	219028997	CXCR1	T	Nonsyn	Arg313His	2		0.001
Het	4	5642249	EVC2	T	Nonsyn	Gly488Ser	2	0.0018	0.001615
Het	4	20715142	PACRGL	G	Nonsyn	Leu99Val	2	0.0009	0.002614
Het	6	30860313	DDR1	T	Nonsyn	Ile365Phe	2	0.0009	0.003998
Het	6	32021216	TNXB	T	Nonsyn	Gly2912Ser	2		0.000394
Het	6	32097318	FKBPL	AGACTTATGAGA	Ins	Ser80_His81ins SerHisLysSer	2	Novel	Novel
Het	6	70386413	LMBRD1	C	Nonsyn	Thr480Ala	2		7.70E-05
Het	6	80626456	ELOVL4	G	Nonsyn	Glu272Gln	2	0.0009	0.009534
Het	7	122303598	CADPS2	C	Nonsyn	Ala160Gly	2	Novel	Novel
Het	8	22880380	TNFRSF10B	A	Nonsyn	Ala376Val	2		0.000384
Het	11	3681478	ART1	C	Frameshift Ins	Phe246fs	2	Novel	Novel
Het	11	12270794	MICAL2	-	Splicing		2	Novel	Novel
Het	11	58034463	OR10W1	T	Nonsyn	Gly290Arg	2	0.0005	0.001539
Het	11	122968526	CLMP	T	Nonsyn	Asp55Asn	2	0.0005	0.002154
Het	19	41510282	CYP2B6	G	Nonsyn	Lys139Glu	2	0.0009	0.002691
Het	19	56320924	NLRP11	T	Nonsyn	Arg351Gln	2		0.001076

Table 5

Confirmed de novo variants in sJIA/MAS patients

ID	Gene	Chr/Position	Classification	HGVS coding	HGVS protein	Function	Expression pattern
53	C7orf70	7:6370406	Nonsyn SNV	c.380G>A	Arg127Gln		
53	ACIN1	14:23548787	Sub	c.1931delins GTTCACG	644delinsArg SerArg	Apoptotic chromatin condensation inducer	↑in CD8+ T cells and monomyelocyto id lineage
327	NIPBL	5:37006499	Del	c.3896_3898 delTTA	1299_1300de linsLeu	Contains HEAT repeats	↑in CD8+ T cells
327	ZIK1	19:58101966	Frameshift Del	c.787delG	263fs	Docking platform for protein interactions	
7431	WDR66	12:122359408	Nonsyn SNV	c.197G>A	Gly66Glu	WD repeat-containing family of proteins	
7437	KIF24	9:34256529	Nonsyn SNV	c.3076G>A	Gly1026Ser	Kinesin family, microtubule-dependent ATPases	Ubiquitous expression
7437	GPR132	14:105518013	Nonsyn SNV	c.461G>A	Arg154His	may react to LPS at sites of inflammation to limit the expansion of tissue-infiltrating cells	↑in CD8+ T cells and monomyelocyto id lineage
72575	CAMK1, OGG1	3:9807532	Nonsyn SNV	c.988C>T	Arg330Cys	DNA repair	Ubiquitous expression

Table 6

Ingenuity Top Bio Functions Analysis

Molecular and Cellular Functions	p-value	# of Genes
Cellular Assembly and Organization	3.10E-05 - 3.31E-02	16
Cellular Function and Maintenance	8.99E-06 - 3.31E-02	19
Cellular Compromise	7.37E-05 - 3.31E-02	9
Cell Morphology	1.26E-04 - 3.17E-02	20
Small Molecule Biochemistry	2.78E-04 - 3.31E-02	5