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Macrophage Activation Syndrome in Systemic Juvenile Idiopathic Arthritis is Associated With MUNC13-4 Gene Polymorphisms

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Abstract

Objective—Systemic Juvenile Idiopathic Arthritis (SJIA) is associated with macrophage activation syndrome (MAS). MAS bears close resemblance to familial hemophagocytic lymphohistiocytosis (FHLH). The development of FHLH has been recently associated with mutations in MUNC13-4 gene. The purpose of this study was to assess for possible sequence alterations in MUNC13-4 gene in SJIA/MAS.

Methods—MUNC13-4 sequence was analyzed in 18 unrelated patients with SJIA/MAS using 32 primer pair sets designed to amplify the 32 exons and at least 100 base pairs of the adjacent intronic regions. DNA samples from unrelated 73 SJIA patients without MAS history and 229 healthy unrelated individuals were used as controls.

Results—Bi-allelic sequence variants in MUNC13-4 gene reported in FHLH were present in two of 18 patients. Further analysis of the MUNC13-4 sequences revealed an identical combination of

12 single nucleotide polymorphisms (SNP) in 9 of remaining 16 SJIA/MAS patients (57%). Additional analysis suggested that these 12 SNPs [154(-19)g>a, 261(+26)c>g, 388(+81)g>a, 388(+122)c>t, 570(-60)t>g, 888G>C, 1389(+36)g>a, 1992(+5)g>a, 2447(+144)c>t, 2599A>G, 2830(+37)c>g, 3198A>G] were inherited as an extended haplotype. In several patients, in addition to the described haplotype, there were other SNPs in the second allele of the MUNC13-4 gene. Moreover, one patient had a complex mutation with two changes, 2542A>C and 2943G>C in a cis configuration. The haplotype was present only in 27 of 229 (12%) healthy controls (Chi Square =23.5) and in 6 of 73 (8.2 %) SJIA patients without MAS history.

Conclusions—The data suggest an association between MUNC13-4 gene polymorphisms and MAS in SJIA.

Keywords

juvenile idiopathic arthritis; Still's disease; macrophage activation syndrome; hemophagocytic lymphohistiocytosis; MUNC13-4 gene; SNP polymorphism

Macrophage Activation Syndrome (MAS) is a severe, potentially fatal condition associated with excessive activation of macrophages and T cells leading to an overwhelming inflammatory reaction. The main manifestations of MAS include fever, hepatosplenomegaly, lymphadenopathy, severe cytopenias, serious liver disease, and disseminated intravascular coagulation [1,2]. The pathognomonic feature of MAS is often found in bone marrow: numerous, well-differentiated macrophages phagocytosing hematopoietic elements. Although MAS has been reported in patients with different rheumatic diseases, it is most strongly associated with Systemic Juvenile Idiopathic Arthritis (SJIA). In fact, it accounts for much of the morbidity and mortality seen in this form of JIA. At least 10% of the patients with SJIA develop MAS [3]. The true incidence of MAS might be much higher since there are no validated diagnostic criteria and mild instances of MAS are not always recognized [4,5].

It is now recognized that MAS bears close resemblance to a group of histiocytic disorders collectively known as hemophagocytic lymphohistiocytosis (HLH) [2,6]. HLH is a term that describes a spectrum of disease processes characterized by accumulations of well-differentiated mononuclear cells with a macrophage phenotype [7]. In the contemporary classification of histiocytic disorders, HLH is further subdivided into primary, or familial HLH, and secondary, or reactive HLH (ReHLH) [7]. Clinically, however, they may be difficult to distinguish from each other [9]. Familial hemophagocytic lymphohistiocytosis (FHLH) is a constellation of rare autosomal recessive immune disorders. The clinical symptoms of FHLH usually become evident within the first 2 months of life although initial presentation as late as 22 years of age has been reported [8]. Secondary HLH tends to occur in older children and more often is associated with an identifiable infectious episode, most notably Epstein-Barr virus (EBV) or cytomegalovirus (CMV) infection. The exact pathophysiological relationship between MAS and HLH is not understood. Some pediatric rheumatologists view MAS as ReHLH occurring in a setting of a rheumatologic disease [6].

The pathological mechanisms of HLH/MAS are not fully understood. In HLH, there is uncontrolled proliferation of T cells and macrophages that has been linked to decreased NK-cell and cytotoxic T-cell function [10] often due to mutations in the gene encoding perforin [11]. Perforin is a protein which cytolytic cells utilize to induce apoptosis of target cells such as tumor cells or cells infected by viruses. It has been hypothesized by some authors that abnormal cytotoxic cells may fail to provide appropriate apoptotic signals for removal of activated macrophages and T cells during the contraction stage of certain immune responses [12]. Our recent observations suggest that as in HLH, MAS patients have profoundly depressed NK-cell function, often associated with abnormal perforin expression [13].

More recently, mutations in another gene, MUNC13-4, have been implicated in the development of hemophagocytic lymphohistiocytosis in about 10-30% of patients with inherited HLH [14]. The protein encoded by the MUNC13-4 gene is an essential effector of the cytolytic secretory pathway. MUNC13-4 protein is involved in vesicle priming function which follows granule docking and precedes plasma granule membrane fusion [14]. Therefore, it is an important player in the intracellular transport of perforin. Although the cytolytic cells of the patients with FHLH caused by MUNC13-4 mutations produce sufficient amounts of perforin, the poor ability to deliver perforin to the surface of the cells leads to profoundly decreased cytolytic activity against target cells. Here we present new data that suggests an association between MAS in SJIA and specific mutations and/or haplotypes in MUNC13-4 gene.

MATERIALS AND METHODS

Patients

Ninety one unrelated patients included in the study met the ELAR diagnostic criteria for Systemic Juvenile Idiopathic Arthritis [15]. 18 of 91 SJIA patients had developed MAS at some point during the course of the disease. The retrospective chart review of the remaining 73 patients did not reveal findings suggestive of MAS. The diagnosis of MAS in 17 patients was established by managing clinicians based on the combination of cytopenias, coagulopathy and liver dysfunction. One patient was included in this group based mainly on the presence of hemophagocytic macrophages in the bone marrow aspirate. Overall, diagnostic bone marrow aspiration was performed in 10 of 18 patients, and hemophagocytosis was demonstrated in all 10 samples. Ten of the 18 patients were seen in the Cincinnati Children's Hospital Medical Center (CCHMC) Rheumatology clinic. The remaining eight patients were from other pediatric institutions; their DNA samples were submitted to the laboratory of Human Genetics at CCHMC to rule out FHLH. All 18 patients have been analyzed for PRF1 gene mutations in Exons 2 and 3 (coding regions) as previously described [13], and no mutations were identified. The patients were of diverse ethnic origins, including 14 Caucasians, 2 African Americans, 2 Latin American, one Native American, and one Asian American individuals from different regions of the United States and Canada. The subjects enrolled in this study at CCHMC provided written informed consent approved by the Institutional Review Board. A total of 229 healthy individuals from Southern Ohio population were included in the study as controls. The control group included 34 individuals of the African American descent.

Mutational Analysis by PCR and direct sequencing

Genomic DNA was isolated from peripheral blood or buccal swabs using standard techniques. To assess for possible sequence alterations in the MUNC13-4 gene, 32 primer pair sets were designed based on the genomic sequence of the MUNC13-4 gene to amplify the 32 exons (Figure 1) and included at least 100 base pairs of the adjacent intronic regions [14]. Amplified exons from patients and control subjects were purified by the EXOSapit (USB, Cleveland, Ohio) and sequenced by cycle sequencing using BigDye Terminator Sequencing Kit. Labeled products were separated by ABI 3730 automated DNA sequencer (Applied Biosystem, Forester city, California). Raw sequence data were then analyzed by Sequencher® 4.7 according to the consensus sequence retrieved from NCBI.

RESULTS

DNA samples from eighteen patients with SJIA and a history of MAS were sequenced as described in Methods. Bi-allelic sequence variants in MUNC13-4 gene reported in FHLH were present in two of these 18 patients. Both patients were of African American descent and shared one allele with the mutation that affects +3 position at a splicing donor site in intron 9 of the

MUNC13-4 gene. Mutations at this position could result in abnormal alternative splicing of exon 9 during mRNA processing. Exon 9 is located in the C2A domain, which contain a conserved Ca^{2+} binding motif. Mutations in C2A domain modulate the binding affinity to Ca^{2+} and/or protein co-factors interacting with that region. Both of these patients also carried a second missense mutation (3145C>G and 1579 C>T, respectively). Interestingly, the latter patient had hemophagocytosis in the bone marrow, but did not meet the clinical criteria for the diagnosis of FHLH. This patient had been described in detail elsewhere as a case report (Hazen et al, A&R, in press). None of the MUNC13-4 mutations were found in normal controls.

Sequence information for the 16 remaining patients is summarized in Table 1 and reveals a common pattern of sequence variant for 9 of these 16 patients. This common pattern is highlighted in yellow in Table 1 and includes an identical combination of 12 single nucleotide polymorphisms (SNP) in the MUNC13-4 gene. The specific pattern of interest is defined as 154 (-19) g>a, 261 (+26) c>g, 388 (+81) g>a, 388 (+122) c>t, 570 (-60) t>g, 888 G>C, 1389 (+36) g>a, 1992 (+5) g>a, 2447 (+144) c>t, 2599 A>G, 2830 (+37) c>g, 3198 A>G (See Figure 1).

One of the SJIA/MAS patients (#10) was homozygous for all 12 SNPs suggesting that these SNPs might be inherited as an extended haplotype (two alleles highlighted by yellow shading in Table 1). To confirm this, the genomic sequence of the MUNC13-4 gene was then analyzed in the parents of one of the SJIA/MAS patients who was heterozygous for the 12 SNPs. This additional analysis revealed the presence of the same series of SNPs in one of the parents, suggesting that these SNPs are indeed likely to be inherited as an extended haplotype (designated “*2” in Table 1 with “*1” being the major allele). In several patients, in addition to the presence of the described haplotype, there were other SNPs in the second allele of the MUNC13-4 gene. Moreover, Patient #4 (Table 1) had a complex mutation with two changes, 2542 A>C (I848L) and 2943 G>C (A995P) in a cis configuration. This complex mutation has been seen in patients with FHLH [Zhang et al, unpublished observations].

Next, the minor allele frequency for the 12 SNPs was determined in a set of 229 local control DNAs. Haplotype determination in these samples was performed using direct sequencing. The minor allele frequencies and the total alleles assayed are shown in the last row of Table 1. Overall, the described haplotype (designated “*2” in Table 1) was present in 9 of the 16 MAS patients (57%) and only in 27 of 229 (12%) controls (Chi Square =23.5). In the control group, the proportion of Caucasian individuals carrying the haplotype (23 /195 or 12%) was similar to that in African American controls (4/34 or 12%).

To assess whether the described haplotype was associated with not only MAS, but also with SJIA in general, the study was expanded to include patients with SJIA without MAS history. The frequency of the haplotype in this group of patients was 6/73 (8.2%) compared to 27/229 (12%) in healthy controls (Chi Square=1.4), suggesting the association between the presence of the haplotype and MAS, but not SJIA in general.

DISCUSSION

The examination of the sequence of the MUNC13-4 gene in patients with Systemic Juvenile Idiopathic Arthritis revealed bi-allelic mutations previously reported in FHLH in two of 18 patients with MAS presenting as a complication of SJIA. The presence of bi-allelic mutations in the MUNC13-4 gene in these two patients is sufficient to establish the definite diagnosis of Familial HLH [9]. In addition these two patients also satisfied the criteria for systemic JIA [15] suggesting that there may be an overlap between the two diseases.

The analysis of the sequence of the MUNC13-4 gene in the remaining 16 MAS patients revealed a common pattern of sequence variants for 9 of these 16 patients. Increased frequency

of the SNPs within the MUNC13-4 gene in MAS patients inherited as a unique haplotype is highly intriguing. It provides further support to the concept that there are pathophysiologic pathways common to both Familial Hemophagocytic Lymphohistiocytosis and SJIA. Given striking similarities in the clinical presentation of FHLH and MAS, the described MUNC13-4 gene polymorphisms may be relevant to the development of the predisposition to MAS in systemic JIA. Since the frequency of the described haplotype in SJIA patients without MAS history is similar to controls, its presence may help identify SJIA patients at long term risk for MAS.

Another important question is whether the observed polymorphism in the MUNC 13-4 is associated with abnormal function of the MUNC13-4 protein and, thus, directly contributes to the development of MAS. Another possibility is that the described haplotype may extend either upstream or downstream of the MUNC13-4 gene and involve additional polymorphisms in other immunologically relevant genes. Therefore, further studies are necessary to establish the extent of the MAS-associated haplotype. Another important question to address in the future studies is whether the frequency of the described haplotype is also increased in Reactive HLH.

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References

1. Hadchouel M, Prieur AM, Griscelli C. Acute hemorrhagic, hepatic, and neurologic manifestations in juvenile rheumatoid arthritis: possible relationship to drugs or infection. *J Pediatr* 1985;106:561–6. [PubMed: 3981309]
2. Grom AA. NK dysfunction: a common pathway in systemic onset juvenile rheumatoid arthritis, macrophage activation syndrome, and hemophagocytic lymphohistiocytosis. *Arthritis Rheum* 2004;50:689–698. [PubMed: 15022306]
3. Sawhney S, Woo P, Murray KJ. Macrophage activation syndrome: A potentially fatal complication of rheumatic disorders. *Arch Dis Child* 2001;85:421–6. [PubMed: 11668110]
4. Bleesing J, Prada A, Villanueva J, Siegel DM, Olson J, Ilowite N, Brunner HI, Griffin T, Graham TB, Sherry D, Passo MH, Ramanan AV, Filipovich A, Grom AA. The diagnostic significance of soluble CD163 and soluble IL2R α chains in macrophage activation syndrome and untreated new onset systemic juvenile idiopathic arthritis. *Arthritis Rheum* 2007;56:965–71. [PubMed: 17328073]
5. Behrens EM, Beukelman T, Paessler M, Cron RQ. Occult macrophage activation syndrome in patients with systemic juvenile idiopathic arthritis. *J Rheumatol* 2007;34:1133–8. [PubMed: 17343315]
6. Athreya BH. Is macrophage activation syndrome is a new entity? *Clin Exp Rheumatol* 2002;20:121–23. [PubMed: 12051388]
7. Favara BE, Feller AC, Pauli M, Jaffe ES, Weiss LM, Arico M, Bucskey P, Egeler RM, Elinder G, Gardner H, Gresik M, Henter JI, Imashuku S, Janka-Schaub G, Jaffe R, Ladisch S, Nezelof C, Pritchard J. Contemporary classification of histiocytic disorders. The WHO Committee On Histiocytic/Reticulum Cell Proliferations. Reclassification Working Group of the Histiocyte Society. *Med Pediatr Oncol* 1997;29:157–66. [PubMed: 9212839]
8. Clementi R, Emi L, Maccario R, Liotta F, Moretta L, Danesino C, Arico M. Adult onset and atypical presentation of hemophagocytic lymphohistiocytosis in siblings carrying PRF1 mutations. *Blood* 2002;100:2266–7. [PubMed: 12229880]
9. Henter JI, Horne A, Arico M, Egeler RM, Filipovich AH, Imashuku S, Ladisch S, McClain K, Webb D, Winiarski J, Janka G. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer* 2007;48:124–31. [PubMed: 16937360]
10. Sullivan KE, Delaat CA, Douglas SD, Filipovich AH. Defective natural killer cell function in patients with hemophagocytic lymphohistiocytosis and in first degree relatives. *Pediatr Res* 1998;44:465–8. [PubMed: 9773832]

11. Stepp SE, Dufourcq-Lagelouse R, Le Deist F, Bhawan S, Certain S, Mathew PA, Henter JI, Bennett M, Fischer A, de Saint Basile G, Kumar V. Perforin gene defects in familial hemophagocytic lymphohistiocytosis. *Science* 1999;286:1957–9. [PubMed: 10583959]
12. Menasche G, Feldmann J, Fischer A, de Saint Basile G. Primary hemophagocytic syndromes point to a direct link between lymphocyte cytotoxicity and homeostasis. *Immunol Rev* 2005;203:165–79. [PubMed: 15661029]
13. Grom AA, Villanueva J, Lee S, Goldmuntz EA, Passo MH, Filipovich A. Natural Killer cell dysfunction in patients with systemic-onset juvenile rheumatoid arthritis and macrophage activation syndrome. *J Pediatr* 2003;142:292–6. [PubMed: 12640378]
14. Feldmann J, Callebaut I, Raposo G, Certain S, Bacq D, Dumont C, Lambert N, Ouachee-Chardin M, Chedeville G, Tamary H, Minard-Colin V, Vilmer E, Blanche S, Le Deist F, Fischer A, de Saint Basile G. MUNC13-4 is essential for cytolytic granules fusion and is mutated in a form of familial hemophagocytic lymphohistiocytosis (FHL3). *Cell* 2003;115:461–73. [PubMed: 14622600]
15. Petty RE, Southwood TR, Baum J, Bhattay E, Glass DN, Manners P, et al. Revision of the proposed classification criteria for juvenile idiopathic arthritis: Durban, 1997. *J Rheumatol* 1998;25:1991–5. [PubMed: 9779856]

Figure 1. Schematic representation of the MAS-associated haplotype in MUNC13-4 gene

The upper panel represents the structure of the MUNC13-4 protein (adapted from [14]). The middle panel shows the corresponding genomic organization (the shaded areas correspond to the 32 exons). The lower panel shows the sites of the 12 single nucleotide polymorphisms (SNP) present in the MAS-associated haplotype described in the text. Note that only one of the 12 SNPs (i.e. 2595A>G) is non synonymous.

Table 1

MUNC13-4 SNPs observed in SJIA patients with MAS.

Patient ID	Genotype	117(+30)g>a	154(-19)g>a	261(+26)c>g	388(+81) g>a	388(+122) c>t	570(-60)t>g	754(-31)c>t	888 G>C	1055(+47)c>t	1389(+36)g>a	1596(+36)a>g	1728(-48)t>c	1992(+5)g>a	2299(-46)c>t	2447(+144)c>t	2448(-42)g>a	2448(-8_-9)insc	2542A>C	2599A>G	2709(+48)c>t	2782C>T	2830(+37) c>g	2943G>A	3151(+82)g>a	3198 A>G	Haplotype designation
1	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
	Allele 2	g	g	c	g	c	t	c	G	t	g	a	t	a	c	t	g	-	A	G	c	C	g	G	g	G	*35
2	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
	Allele 2	a	g	c	g	t	g	t	C	c	g	g	c	g	t	c	g	c	A	G	t	C	c	A	g	G	*7
3	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	A	g	A	*1
	Allele 2	g	a	g	a	t	g	c	C	c	a	a	t	a	c	t	g	-	A	G	c	C	g	G	g	G	*2
4	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	C	A	c	C	c	C	g	A	1848L+A995P
	Allele 2	g	a	g	a	t	g	c	C	c	a	a	t	a	c	t	g	-	A	G	c	C	g	G	g	G	*2
5	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
	Allele 2	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
6	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
	Allele 2	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	T	c	G	g	A	*4
7	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	a	-	A	A	c	C	c	G	g	A	2448(-42)g>a
	Allele 2	g	a	g	a	t	g	c	C	c	a	a	t	a	c	t	g	-	A	G	c	C	g	G	g	G	*2
8	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	t	C	c	G	g	A	2709(+48)c>t
	Allele 2	g	a	g	a	t	g	c	C	c	a	a	t	a	c	t	g	-	A	G	c	C	g	G	g	G	*2
9	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	a	A	3151(+82)g>a
	Allele 2	g	a	g	a	t	g	c	C	c	a	a	t	a	c	t	g	-	A	G	c	C	g	G	g	G	*2
10	Allele 1	g	a	g	a	t	g	c	C	c	a	a	t	a	c	t	g	-	A	G	c	C	g	G	g	G	*2
	Allele 2	g	a	g	a	t	g	c	C	c	a	a	t	a	c	t	g	-	A	G	c	C	g	G	g	G	*2
11	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
	Allele 2	g	a	g	a	t	g	c	C	c	a	a	t	a	c	t	g	-	A	G	c	C	g	G	g	G	*2
12	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
	Allele 2	g	a	g	a	t	g	c	C	c	a	a	t	a	c	t	g	-	A	G	c	C	g	G	g	G	*2
13	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
	Allele 2	g	a	g	a	t	g	c	C	c	a	a	t	a	c	t	g	-	A	G	c	C	g	G	g	G	*2
14	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
	Allele 2	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
15	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
	Allele 2	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
16	Allele 1	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1
	Allele 2	g	g	c	g	c	t	c	G	c	g	a	t	g	c	c	g	-	A	A	c	C	c	G	g	A	*1

Capital letters indicate nucleotide substitutions in the coding regions, small case letters indicate changes in the introns or non-coding regions

The common pattern (highlighted in yellow) includes an identical combination of 12 SNPs in the MUNC13-4 gene inherited as an extended haplotype (designated “*2” with “*1” being the major allele). Note that patient #10 is homozygous for all 12 SNPs. In several patients, in addition to the presence of the described haplotype, there are other SNPs in the second allele of the MUNC13-4 gene (highlighted in grey). Patient #4 has a complex mutation with two changes, 2542 A>C (I848L) and 2943 G>C (A995P) in a cis configuration.