Exploration of susceptible genes associated with Henoch–Schönlein purpura by whole exome sequencing

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Conflict of interest

None declared

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Abstract

Background. Henoch–Schönlein purpura (HSP) is a systemic small-vessel vasculitis caused by environmental and inherent factors. Although recent research has advanced our understanding of the role of genetic susceptibility in HSP, there are still significant gaps in our knowledge.

Objectives. In this study, we aimed to explore some susceptibility genes likely associated with HSP.

Material and methods. Three DNA samples from a family with HSP were used to perform whole exome sequencing with Illumina HiSeq 2500 high-throughput sequencing. The relevant single nucleotide variants (SNVs) were screened according to specific filter conditions and the screened SNVs were then verified with Sanger sequencing. The Sanger sequencing results were further screened according to available literature. Finally, candidate genes were validated in 92 samples from children with HSP, and also in 1 child with HSP from HSP family, using the polymerase chain reaction technique (PCR).

Results. Our analysis revealed that the *MIF* gene and the *MGAT5* gene related to immunity remained after screening. Among the 93 children with HSP, there were 3 patients with *MIF* mutations and 2 patients with *MGAT5* mutations.

Conclusions. Our findings are helpful for providing new methods and ideas for understanding the pathogenesis of HSP by detecting and analyzing gene mutations at the whole-exome level including multi-generation sequencing. *MIF* and *MGAT5* may be new susceptibility loci for HSP, but their roles in the pathogenesis of HSP are worthy of further study.

Key words: Henoch–Schönlein purpura, whole exome sequencing, *MIF*, *MGAT5*

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Introduction

Henoch–Schönlein purpura (HSP) is a small-vessel vasculitis that often occurs in children and can cause various clinical issues, such as skin purpura, arthritis, and/or arthralgia, abdominal pain, and renal involvement.¹ Some of the renal damage can progress to renal insufficiency, which determines the long-term prognosis of HSP.² Although the first HSP report was made nearly 200 years ago, the exact etiology and pathogenesis of the disease are still unclear.

Some studies have shown that the incidence of HSP has a tendency for familial aggregation.^{3–5} In other words, genetic factors may play an important role in the pathogenesis of HSP. The past few years have seen a tremendously large amount of research in quest for genetic variants which may influence HSP susceptibility. Candidate gene investigations have since revealed roles in HSP susceptibility of an increasing number of genes, including *TGF- β* , *MEFV* (E148Q), *RAS*, *P-selectin*, *C1GALT1*, *HSP70-2*, *TNF- α* , *IL-17* (rs2275913), *CTLA-4*, *PAF-AH*, *eNOS*, *iNOS*, *TLR-4*, *ACE*, etc., all of which have been validated in independent Chinese populations.^{6–17} The above studies, however, were basically designed to study the correlation between specific genes and HSP, but these selected genes are not necessarily related to the incidence of hereditary HSP.

Besides, the occurrence of HSP is related to immune disorders, such as a Th1/Th2 imbalance and overactive Th2 cells.¹⁸ Therefore, we set out to perform whole exome sequencing (WES) in a family with HSP to find genes related to immune abnormalities which are likely associated with HSP, so as to provide clues for researching the pathogenesis of HSP.

Material and methods

Patients

The study focused on a Chinese family, consisting of a boy with HSP, his father (who has had HSP since he was 10 years old) and the boy's mother, who does not suffer from HSP. Other 92 samples from unrelated patients with HSP were recruited from the Department of Rheumatism at Shanghai Children's Medical Center. Henoch–Schönlein purpura was diagnosed according to criteria established by the European League Against Rheumatism (EULAR) in 2010.¹⁹

The 12-year-old boy (sample No. WES-741) presented with HSP on both of his lower limbs 2 days prior; this was accompanied by joint pain, but no renal involvement. His father (sample No. WES-742) had suffered from HSP since the age of 10, accompanied by abdominal pain and gastrointestinal bleeding, though his mother (sample No. WES-743) had no history of HSP.

The study was approved by the Medical Ethics Committee of Shanghai Children's Medical Center, Shanghai Jiaotong University School of Medicine, China, and informed consent was obtained from the parents of all participants.

Whole exome sequencing

The genomic DNA of the 3 samples was isolated from 2 mL of peripheral blood samples collected from the veins using a QIAamp Blood DNA Mini Kit® (Qiagen GmbH, Hilden, Germany). A total of 3 μ g of DNA from the sample was processed through shearing using a Covaris® M220 Ultrasonicator system (Covaris Inc., Woburn, USA) resulting in an amplicon of 150–200 bp. The genomic DNA library was prepared with an Agilent SureSelect Target Enrichment System (Agilent Technologies, Inc., Santa Clara, USA) according to the manufacturer's instructions. Both coding exons and the flanking intronic regions were enriched with SureSelect XT Human All Exon V6 (Agilent Technologies). Clusters were then generated with isothermal bridge amplification using an Illumina cBot station, and sequencing was performed on an Illumina HiSeq 2500 System (Illumina Inc., San Diego, USA).

Base calling and sequence read quality assessment were performed using Illumina Sequence Control Software with Real Time Analysis software (Illumina). The sequence reads to a reference human genome (Human 37.3, SNP135) were aligned using NextGENe (SoftGenetics, State College, USA). All single-nucleotide variants (SNVs) and indels were saved in VCF format and uploaded to Ingenuity Variant Analysis (Ingenuity Systems, Redwood City, USA) for biological analysis and interpretation.

Single nucleotide variant screening

The screening parameters of the candidate variants used with the Ingenuity software consisted of 5 parts: 1) exclusion of low confidence variants; 2) allele frequency under 1.0% in the 1000 Genomes Project, 1.0% of the NHLBI ESP exomes, 1.0% of the Allele Frequency Community, or 1.0% of the ExAC database, with an area of analysis including each exon and about 20 bp of exon–intron boundaries; 3) exclusion of benign variants, including synonymous, harmless missense predicted by PolyPhen-2 and SIFT software, when those predicted had no impact on splicing using MaxEntScan software; 4) a filtering index of clinical symptoms of immunity for analysis of the resulting candidate variants; and 5) taking the intersecting set of variants in the cases of WES-741 and WES-742.

Sanger sequencing verification

The primers for the amplicon of the screened SNVs gained by University of California, Santa Cruz (UCSC Genome Browser Home) were designed using Primer3 online

software (<http://genome.ucsc.edu/cgi-bin/hgGateway>) and synthesized with Map Biotechnology (Shanghai, China). The amplicon length was 300–500 bp and the annealing temperature was set at 60°C.

The exons and the exon–intron boundaries were amplified using polymerase chain reaction (PCR). The PCR products (5 µL) were examined on a 1% agarose gel and purified using an ExoSAP-IT Kit (GE Healthcare, Cleveland, USA). Sequencing reactions were prepared with the BigDye® Direct Cycle Sequencing Kit (Thermo Fisher, Waltham, USA). The final products were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen) and the capillary electrophoresis sequencing was performed by using an ABI Prism 3730XL Genetic Analyzer (Thermo Fisher) with the forward and reverse primers. The sequence data were analyzed using Mutation Surveyor® software v. 4.0.4 (SoftGenetics).

PCR of *MIF* and *MGAT5* and analysis of data

All exons of *MIF* and *MGAT5* were amplified using PCR from the genomic DNA of 93 children with HSP. The primers for the amplicons of *MIF* (GenBank accession No. NM_002415) and *MGAT5* (GenBank accession No. NM_002410) were designed using UCSC ExonPrimer online software (<http://genome.ucsc.edu/index.html>) and were synthesized using Map Biotechnology (Shanghai, China). The primers designed for *MIF* and *MGAT5* are listed in Table 1 (A and B). The exons and the exon–intron boundaries were amplified using PCR. The reaction mixture for each amplicon of *MIF* contained 1 × Premix Taq (Ex Taq v. 2.0; cat. No. RR 003; Takara/Biotechnology, Co. Ltd., Kusatsu, Japan), 100 ng of genomic DNA, and 1 µL of forward and reverse primer in a final volume of 25 µL. The reaction was performed under the following PCR conditions: initial denaturation at 95°C for 5 min; then 19 cycles of 95°C for 30 s, 65°C for 30 s and 72°C for 45 s; 14 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 45 s; and a final elongation step at 72°C for 5 min using a C1000™ Thermal Cycler PCR instrument (Bio-Rad Laboratories Inc., Hercules, USA). The PCR products were examined on a 1% agarose gel (Sangon Biotechnology Co. Ltd., Shanghai, China) with electrophoresis and purified using a QIAquick Gel Extraction kit (Qiagen). The purified DNA was sequenced using an ABI3730XL sequencer (Thermo Fisher) with forward and reverse primers. The sequence data was analyzed using Mutation Surveyor DNA Variant Analysis v. 4.0.4 (SoftGenetics). The same reaction system and conditions were used for the amplification of *MGAT5*.

After PCR analysis, we used Bioedit (V.7.1.3) (Tom Hall, <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) to find pathogenic predictions of all variants of *MIF* and *MGAT5*

Table 1A. The PCR primer sequence for the *MIF* gene

Primer name	Primer sequence (5'-3')
MIF-E1F	CAGGCGGTGACTTCCCA
MIF-E1R	CCCCGAACGTCCACTTCG
MIF-E2-3F	AGGGGACAGGAAGAGGGG
MIF-E2-3R	GACCTCGTCCCCAATATCC

Table 1B. The PCR primer sequence for the *MGAT5* gene

Primer name	Primer sequence (5'-3')
MGAT5-E1F	AGTGAAAACATGGCTTCTGGT
MGAT5-E1R	GGCTCTCTGTGTGAATTCA
MGAT5-E2F	CGGCCCTTGTATCTTTTCAG
MGAT5-E2R	ACTTATCTTTACAATGGCCATGC
MGAT5-E3F	TATATTGGAGCTGGAGGGGC
MGAT5-E3R	CATAGCCACAGACAAAGCCG
MGAT5-E4F	TGCACACCTGGCTATCTTGA
MGAT5-E4R	GGGAGAGTAATGAAGGACGGA
MGAT5-E5F	TCATCTGAGAGGGCACTTGT
MGAT5-E5R	TCCACAGAGACGAATACAGAAGT
MGAT5-E6F	CAGTTTGCAATCCTGGGACC
MGAT5-E6R	AGACTGAGCAGATTCACCTT
MGAT5-E7F	CCAAGGGCTGCAGTGATTTT
MGAT5-E7R	ATGAAGCCTCTGGAGCCTTC
MGAT5-E8F	TTTGAAAGGGCCATGCTGTC
MGAT5-E8R	GCAACACTCCATACAGCTGA
MGAT5-E9F	TCTTGTCTGGTCTGCTTCT
MGAT5-E9R	ACAGCACACACTTCTACAGC
MGAT5-E10F	ACTGGGAATGTGTGGGAGAG
MGAT5-E10R	GCAGGTACATACAGCCTTGG
MGAT5-E11F	TCCACAAAGTCCAGCCAGAT
MGAT5-E11R	GCTGCCATTGGTCTAGCAAA
MGAT5-E12F	AGCCTTCCAAAGTGAGGTGT
MGAT5-E13R	AGCCTCTATACCTCACCTT
MGAT5-E13F	TCAAGGGGAAGGACACAGTG
MGAT5-E13R	CCAAATCCTGCAGCTTGTT
MGAT5-E14F	GCTTGAGATCACGACTTGCA
MGAT5-E14R	GGTGACCATGACAGTGCTAC
MGAT5-E15F	CTCCAACTCTTCCCGTC
MGAT5-E15R	CTTATCCCCACAGCCAGTA
MGAT5-E16F	GATTGCAGACGAAGAGGGTG
MGAT5-E16R	GCAATGCCAATTCAGAACCTTC

and analyzed their conservation; the properties of amino acids were found using the Hphob./Kyte & Doolittle scale (<http://web.expasy.org/protscale/>).

Results

DNA sequencing

The 3 samples from the HSP family were screened for selected variants using WES. The sequencing quality parameters are listed in Table 2. After being screened for selected variants, the filtered SNVs were verified using Sanger sequencing; the results are listed in Table 3. Functional prediction and biological relevance analysis led to 2 genes: *MIF* and *MGAT5*. One variant was in *MGAT5* (c.673C>G; p.L225V) and the other variant was in *MIF* (c.295T>C; p.Y99H). The 2 variants were both missense mutations which were heterozygous in both the patient and his father (Fig. 1). The pathogenicity analysis of all variants of *MGAT5* and *MIF* are listed in Table 4.

PCR analysis of the *MGAT5* gene in 93 children with HSP

The sequencing results of *MGAT5* showed that there were 3 synonymous variants (c.141C>T, c.267A>G, and c.348A>G) and 2 missense variants (c.673C>G and c.1117A>G). The 3 synonymous variants do not cause changes in the corresponding amino acid and every synonymous variant exists in only 1 HSP patient. The other 2 missense variants are both heterozygous mutations and are located in the encoding region of *MGAT5*. Base mutation can cause amino acid change (c.1117A>G, p.Met373Val; c.C673G, p.Leu225Val). Also, each missense variant exists in only 1 HSP patient.

Conservation analysis of *MGAT5* amino acid sequences in different species was performed with Bioedit software (Fig. 2). The results showed that the above amino acid sites

Fig. 1. Sanger sequencing of *MGAT5* and *MIF* genes. The *MGAT5* c. 673C>G; p.L225V and *MIF* c.295T>C; p.Y99H variants were detected in the patient and his father. The patient's mother had no variants. The corresponding variant bases are indicated by arrows

<i>H. sapiens</i>	LHSIGKIGGA	QNRSYSKLLC	GLLAERLRIS	PDRVYINYYD	MNAANVGWNN	STFA
<i>P. troglodytes</i>	LHSIGKIGGA	QNRPHSKLLC	GLLAERLRIS	PDRVYINYYD	MNAANVGWNN	STFA
<i>S. scrofa</i>	LHSIGKIGGA	QNRSYSKLLC	GLLAERLRIS	PDRIYINYYD	MNAANVGWNG	STFA
<i>B. taurus</i>	LHSIGKIGGA	QNRSYSKLLC	GLLTERLRIS	PDRIYINFC	MNAANVGWNG	STFA
<i>R. norvegicus</i>	LHSIGKIGGA	QNRNYSKLLC	GLLSDRHLIS	PDRVYINYYD	MNAANVGWNG	STFA
<i>M. musculus</i>	LHSIGKIGGA	QNRNYSKLLC	GLLSDRHLIS	PDRVYINYYD	MNAANVGWNG	STFA
<i>G. gallus</i>	LYSIGKIGGQ	QNKTYTKLLC	DMIAKHLHVS	ADRVYINFD	INAANVGWNG	STFA
<i>T. sirtalis</i>	LHSIGKISAP	QNKAYSAILS	ALLAKRLHVP	ADRVYINFD	MAPANVGWNG	STFA
<i>X. laevis</i>	LCSIGKIGGP	QNKSYTKLLC	DILTKQLNIP	ANRVYINYYD	LNAANVGWNG	STFA
<i>D. rerio</i>	LTSIGKISGA	QNKQYSKLLM	GLLNKHLGVS	ADRIYINFD	MDPANVAWNN	STFG
<i>P. falciparum</i>	ITSIGGINRS	NNSALADQIT	KLLVSNLNVK	SRRIYVEPRD	CSAQNFASF	STFG

Fig. 2. Conservation analysis of mutations in *MIF*. One variant (c.295T>C, p.Y99H; in yellow) is well-conserved in vertebrates except *B. taurus* and *D. rerio*. Another variant (c.338C>T, p.T113I; in green) is well-conserved across species

Table 2. The sequencing quality parameters of the 3 cases included in this study

Sample	Total reads	Average read depth (x)	Percent of ROI with 1x [%]	Percent of ROI with 20x [%]	Unaligned reads	Off-target percent of reads [%]
Patient (WES-741)	59938725	101.95	96.897	99.873	2625970	28.88
Father (WES-742)	69050718	115.8	97.658	99.884	3382470	29.353
Mother (WES-743)	57841691	101.44	97.044	99.776	2191901	27.118

NA – not available; ROI – region of interest.

Table 3. The screened SNVs verified using Sanger sequencing

Gene	NM_ID	Transcript variant	Protein variant	PolyPhen-2 function prediction	Associated disease (OMIM)	Function
<i>KIF1B</i>	NM_015074.3	c.1717C>T	p.R573C	probably damaging	Charcot-Marie-Tooth disease type 2A1; neuroblastoma; pheochromocytoma	transport of mitochondria
<i>ABCA4</i>	NM_000350.2	c.3265A>T	p.T1089S	probably damaging	Stargardt disease; retinitis pigmentosa age-related macular dystrophy; fundus flavimaculatus	transport of an essential molecule (or ion) either into or out of photoreceptor cells
<i>MGAT5</i>	NM_002410.4	c.673C>G	p.L225V	possibly damaging	NA	catalyzes the addition of β -1,6-N-acetylglucosamine to the α -linked mannose of biantennary N-linked oligosaccharides
<i>MUC4</i>	NM_018406.6	c.11079G>T	p.Q3693H	NA	NA	protection of the epithelial cells and epithelial renewal and differentiation
<i>NMUR2</i>	NM_020167.4	c.451C>T	p.P151S	probably damaging	NA	regulation of food intake and body weight
<i>HIST1H2AE/HIST1H2BG</i>	NM_021052.2; NM_003518.3	c.-554C>A; c.223G>T	p.A75S	NA	NA	basic nuclear proteins that involved in the nucleosome structure of the chromosomal fiber in eukaryotes
<i>ITPR3</i>	NM_002224.3	c.6262A>T	p.M2088L	benign	NA	mediates the release of intracellular calcium
<i>SMARCA2</i>	NM_003070.4;	c.705_707 dupGCA	p.Q238dup	NA	Nicolaides-Baraitser syndrome	has helicase and ATPase activities and regulate transcription of certain genes
<i>PKP2</i>	NM_004572.3;	c.473G>A	p.R158K	benign	arrhythmogenic right ventricular cardiomyopathy	may regulate the signaling activity of beta-catenin
<i>MUC19</i>	NM_173600.2	c.4910-3C>A	NA	NA	NA	may be involved in disruption of the ocular surface in Sjögren syndrome
<i>F10</i>	NM_000504.3	c.706G>T	p.V236L	probably damaging	factor X deficiency	encodes the vitamin K-dependent coagulation factor X
<i>STRC</i>	NM_153700.2	c.5243G>A	p.R1748Q	probably damaging	autosomal recessive deafness 16	involved with mechanoreception of sound waves
<i>ZNF276</i>	NM_001113525.1	c.493C>T; c.268C>T	p.R165W; p.R90W	possibly damaging	NA	zinc finger protein 276
<i>FAM129C</i>	NM_173544.4	c.2013G>C	p.Q671H	possibly damaging	NA	unknown
<i>LIPE</i>	NM_005357.3	c.2200G>A	p.V734M	possibly damaging	familial partial lipodystrophy-6	converts cholesteryl esters to free cholesterol in steroidogenic tissues or hydrolyzes stored triglycerides to free fatty acids in adipose tissues
<i>BP1FA1</i>	NM_130852.2;	c.726T>G	p.I242M	possibly damaging	NA	has antibacterial activity against Gram-negative bacteria
<i>KRTAP19-3</i>	NM_181609.3	c.70_75dupGGCTAT	p.G24_Y25dup	NA	NA	unknown
<i>PI4KA</i>	NM_058004.3	c.2158A>G	p.I720V	NA	perisylvian polymicrogyria; cerebellar hypoplasia; arthrogryposis	catalyzes the first committed step in the biosynthesis of phosphatidylinositol 4,5-bisphosphate
<i>MIF</i>	NM_002415.1	c.295T>C	p.Y99H	possibly damaging	systemic juvenile rheumatoid arthritis	regulation of macrophage function in host defense through the suppression of anti-inflammatory effects of glucocorticoids

OMIM – Online Mendelian Inheritance in Man; NA – not available.

Table 4. Pathogenic predictions of all mutations of the *MGAT5* and *MIF* genes

Gene	Transcript ID	DNA change	AA change	Translation Impact	Mutation Taster	PolyPhen2	SIFT	PROVEN	HSF	MAF in various databases		
										ESP	gnomAD	1000genomes
<i>MGAT5</i>	NM_002410.4	c.673C>G	p.Leu225Val	missense	disease-causing	probably-damaging	deleterious	neutral	NA	NA	5.54E-05	NA
		c.1117A>G	p.Met373Val	missense	disease-causing	benign	tolerated	neutral	NA	NA	0.0003724	NA
		c.141C>T	p.Arg47Arg	synonymous	disease-causing	NA	NA	neutral	potential alteration of splicing	NA	2.86E-05	NA
		c.267A>G	p.Leu89Leu	synonymous	disease-causing	NA	NA	neutral	potential alteration of splicing	NA	NA	NA
		c.348A>G	p.Ser116Ser	synonymous	disease-causing	NA	NA	neutral	potential alteration of splicing	NA	4.47E-05	NA
		c.295T>C	p.Tyr99His	missense	disease-causing	probably-damaging	deleterious	deleterious	NA	NA	NA	NA
<i>MIF</i>	NM_002415.1	c.282-6C>G		Frameshift	NA	NA	NA	NA	potential alteration of splicing	0.1385	0.1924	0.174
		c.338C>T	p.Thr113Ile	missense	disease-causing	probably-damaging	deleterious	deleterious	NA	NA	0.0001558	0

were highly conserved among different species. This suggests that the mutation of the 2 conserved sites may be important factors affecting the functioning of *MGAT5*, so the mutations are not caused by natural selection in evolution.

According to the Hphob./Kyte & Doolittle scale, the individual values for methionine, valine and leucine are 1.9, 4.2 and 3.8, respectively. These amino acids are hydrophobic and their properties do not change significantly. Because of the 2 highly conserved sites, the 2 gene mutations may still cause structural and functional changes of the *MGAT5* protein.

PCR analysis of the *MIF* gene in 93 children with HSP

The sequencing results of *MIF* showed that the 3 variants, c.282-6C>G, c.295T>C and c.338C>T, were all heterozygous mutations. The c.282-6C>G variant (rs2070766) is located in the splicing region of *MIF*. Among the 93 children, 35 patients (37.6%) carried this mutation. These data are a close match to those of the European population (32.2%) and the East Asian population (28.6%) according to the ExAC database, and the allele frequency is greater than 3%, so these sites are the single nucleotide polymorphism (SNP) site.

The c.295T>C and c.338C>T variants are both located in the encoding region of *MIF*. Base mutation can result in a change of the corresponding amino acid (c.338C>T; p.Thr113Ile and c.295T>C; p.Tyr99His). Among the 93 patients with HSP, there were 2 (2.15%) c.338C>T variants and 1 (1.08%) c.295T>C variant.

Conservation analysis of *MIF* amino acid sequences in different species were also performed with Bioedit software (Fig. 3). The results showed that the 113th amino acid was highly conserved among different species. This suggests that the variant may be one of the factors that affect the functioning of *MIF*, rather than natural selection in evolution. And the 99th amino acid is conserved in other vertebrates except cattle and zebrafish, in which it is semi-conserved.

Using the Hphob./Kyte & Doolittle scale, the individual values for tyrosine, histidine, threonine, and leucine are -1.3, -3.2, -0.7, and 4.5, respectively. The p.Tyr99His variant leads to a significant change in the structure of the amino acid side chain, while p.Thr113Ile can cause its hydrophobicity to change greatly. The amino acid side chain structure changes as well. Therefore, the 2 amino acids change more significantly.

Discussion

Henoch-Schönlein purpura is one of the most common types of vasculitis in children, but its cause is still unknown and, so far, no specific genetic abnormality has been described in patients with HSP.²⁰⁻²² In this study, a WES analysis was performed in a family with HSP. Considering

<i>H. sapiens</i>	IRTD F NI L YS MMKKHEEFRW	<i>H. sapiens</i>	QFKKTLGPSW VHYQ C MLRVL DSFGTEPEFN
<i>P. troglodytes</i>	IRTD F NI L YS MMKKHEEFRW	<i>P. troglodytes</i>	QFKKTLGPSW VHYQ C MLRVL DSFGTEPEFN
<i>C. familiaris</i>	IRTD F NI L YS MMKKHEEFRW	<i>C. familiaris</i>	QFKKTLGPSW VHYQ C MLRVL DSFGTEPEFN
<i>B. taurus</i>	IRTDVNI L YS MMKKHEEFRW	<i>B. taurus</i>	QFKKTLGPSW VHYQ C MLRIL DSFGTEPEFN
<i>R. norvegicus</i>	IRTD F NI L YG MMKKHEEFRW	<i>R. norvegicus</i>	QFKKTLGPSW VHYQ C MLRVL DSFGTEPEFN
<i>M. musculus</i>	IRTD F NI L YG MMKKHEEFRW	<i>M. musculus</i>	QFKKTLGPSW VHYQ C MLRVL DSFGTEPEFN
<i>G. gallus</i>	IRINFDPLYK MMSRHEEFRW	<i>G. gallus</i>	QFKKTLGPSW VHYQ C MLRVL DSFGTEPEFN
<i>T. sirtalis</i>	IRTNFDLLYK MMSRHEEFRW	<i>T. sirtalis</i>	QFKKTLGPSW VHYQ C MLRVL DSFGTEPEFN
<i>X. tropicalis</i>	IRTD F DDLYK VMAKHEEFRW	<i>X. tropicalis</i>	QFKKTLGPSW VHYQ C MLRVL DSFGTEPEFN
<i>D. rerio</i>	VRTSFDELYR VMSRREEFRW	<i>D. rerio</i>	QFKKTLGPSW VHYQ C MLRVL DSFGTEPEFN

Fig. 3. Conserved analysis of mutations in *MGAT5*. The 2 variants (c.673C>G, p.L225V; left, in yellow, and c.1117A>G, p.M373V; right, in green) are both highly conserved across species

that HSP is associated with immune imbalance, we gained a total of 20 SNVs in the 20 different genes screened. Among the screened markers for immunity in our whole exome association study, *MGAT5* and *MIF* were of particular interest by functional prediction and biological relevance analysis.

The *MGAT5* gene, also known as *GNT-V*, encodes beta-1,6 N-acetyl-glucosaminyltransferase, an enzyme which catalyzes the addition of beta-1,6 GlcNAc- to N-glycans on glycoproteins. An *MGAT5* deficiency increases the number of T-cell receptors recruited to the antigen-presenting surface, thereby reducing the need for CD28 co-receptor engagement. *MGAT5* functions as a negative regulator of T-cell activation thresholds and susceptibility to immune diseases, such as multiple sclerosis or rheumatoid arthritis.^{23–25} The expression of beta-1,6 GlcNAc-branched N-linked glycans selectively inhibits Th1 cell differentiation and enhances the polarization of Th2 cells.²⁶ In our study, PCR analysis of *MGAT5* in the 93 HSP patients showed that there was 1 patient (1.08%) with the c.1117A>G variant and another 1 child (1.08%) with the c.673C>G variant. Our result for the c.673C>G variant (1.08%) is much higher than that of the East Asian population (0.83%) and the European population (0%). The frequency of the c.1117A>G variant in HSP patients (1.08%) is also far higher than that of the East Asian population (0.43%) and the European population (0%). According to the analysis of *MGAT5* gene sequence conservation and amino acid properties, these 2 amino acids did not change significantly, but because the 2 sites are highly conserved their mutation may still lead to structural and functional changes of the *MGAT5* protein. In addition, the HSP patient with the c.673C>G variant, whose father also had HSP in childhood, had joint pain at his initial diagnosis. Once he had received enough prednisone therapy, his rash was under control and his joint pain was relieved. The patient with c.1117A>G had a rash which lasted 2 months, with a longer course and kidney involvement, and he recovered after 1 year of prednisone treatment. We speculate that the mutation of the *MGAT5* gene may be related to the occurrence

of HSP, severity of HSP and the duration of glucocorticoid treatment through some unknown mechanism. Of course, more research is needed on the reliability of these findings and on the intrinsic mechanism of the disease.

Another interesting marker in our screening is macrophage migration inhibitory factor (*MIF*). It is a pro-inflammatory cytokine that is produced by immune T cells, macrophages and monocytes, and plays pivotal roles in innate and acquired immunity.²⁷ It has been reported to inhibit random migration and concentration of macrophages at sites of inflammation and to induce pro-inflammatory cytokine secretion, leading to an excessive inflammatory response.^{28,29} The functional effects of *MIF* gene polymorphisms have been identified and associated with susceptibility or severity of several acute, chronic and autoimmune inflammatory conditions and vasculitides.³⁰ The promoter region of the *MIF* -173G/C polymorphism has been identified as a functionally relevant variant of this gene.³¹ Our previous study and other studies have found increased levels of *MIF* in the urine and renal tissues of children with HSP.^{32,33} However, Nalbantoglu et al. and Amoli et al. showed that the *MIF* gene -173G/C polymorphism was not associated with HSP.^{34,35} The above results indicate that the -173G/C polymorphism in the *MIF* gene promoter does not appear to be a genetic risk factor for HSP, and that HSP may be related to abnormal regulation of inflammation by the *MIF* protein, abnormal levels of which are caused by other mutations in *MIF*. Our study demonstrated 2 new variants in the *MIF* gene. One is the c.295T>C variant; it was found in 1 patient (1.08%) with HSP and the site had never been reported before. Therefore, the gene frequency cannot be compared with the normal population of the ExAC database. The other variant is the c.338C>T variant. There were 2 children (2.15%) carrying this variant, and its frequency is much higher than that of the populations of East Asia (0.28%) and Europe (0%). According to the analysis of *MIF* sequence conservation and amino acid properties, the 99th amino acid is semi-conserved, the 113rd is highly conserved and the properties of the 2 amino acids change significantly. Therefore, the 2 variants

are likely to lead to changes in the structure and function of MIF, which is related with HSP.

Additionally, glucocorticoid (GC), which has a broad spectrum of anti-inflammatory and immune-regulatory effects on host immune responses, can inhibit the activation of immune cells and reduce the production of pro-inflammatory mediators, including cytokines, prostaglandins and reactive oxygen species.³⁶ Because of these properties, GC is effective in the treatment of some inflammatory diseases.³⁷ As a unique trans-regulator of GC, MIF can antagonize endogenous and exogenous glucocorticoid activity, so as to maintain a balance in immune regulation.³⁸ However, the research on the systemic lupus erythematosus (SLE) showed that *MIF* was associated not only with disease severity,³⁹ but also with steroid resistance.⁴⁰ In our study, there were 2 children with HSP and the c.338C>T variant. One was diagnosed with HSP nephritis, which was refractory and lasted for 2 years. The proteinuria was relieved after prednisone treatment combined with immunosuppressant. The other was a girl with severe rash (both lower limbs and both upper limbs) at her initial diagnosis who was treated with prednisone. The patient with the c.295T>C variant had joint pain at his initial diagnosis, and his rash was controlled after sufficient prednisone treatment. Thus, we speculate that *MIF* may be related to the severity of HSP. The specific mechanism between *MIF* and the occurrence of HSP, the relationship between *MIF* and the severity of the disease, and the drug resistance of the hormone still need to be studied further.

Because the sample size in this study is small, it needs to be verified in larger samples. At the same time, it also needs to be further studied in gene function and animal models. So far, few studies have reported a relationship between *MGAT5* or *MIF* and the pathogenesis of HSP, but our study provides a new direction for future research.

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