Heat shock protein 70-2 and tumor necrosis factor-α gene polymorphisms in Chinese children with Henoch-Schönlein purpura

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Background: **Henoch-Schönlein purpura (HSP) or IgA-associated vasculitis is related to immune disturbances. Polymorphisms of the heat shock protein 70-2 gene (*HSP70-2*) and the tumor necrosis factor-α gene (*TNF-α*) are known to be associated with immune diseases. The purpose of this study was to investigate the likely association of *HSP70-2* (+1267A/G) and *TNF-α* (+308A/G) gene polymorphisms with HSP in children.**

Methods: **The polymerase chain reaction restriction fragment length polymorphism method was used to detect the *HSP70-2* and *TNF-α* polymorphisms in 205 cases of children with HSP and 53 controls; and the association of these polymorphisms with HSP and HSP nephritis (HSPN) was analyzed.**

Results: **The G/G genotypic frequencies at the +1267A/G position of *HSP70-2* in the HSP group (22.9%) were significantly higher than those in the healthy control group (9.4%) (χ^2=4.764, *P*<0.05). The frequencies of the A/A, A/G and G/G genotypes of *HSP70-2* in patients in the nephritis-free group and the HSPN group showed no statistically significant difference. The A/A genotype frequency at the +308G/A position of *TNF-α* in the HSP group was 8.3%, which was higher than that in the control group (χ^2=6.447, *P*<0.05). The A allele frequency of *TNF-α* in the HSP group was higher than that in the control group, with a statistically significant difference (χ^2=7.241, *P*<0.05).**

Conclusions: **The *HSP70-2* (+1267A/G) and *TNF-α* (+308G/A) gene polymorphisms were associated with HSP in children. The G/G homozygosity of *HSP70-2* and the A/A homozygosity of *TNF-α* may be genetic predisposing factors for HSP.**

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***Key words*: gene polymorphism; heat shock protein 70-2; Henoch-Schönlein purpura; Henoch-Schönlein purpura nephritis; tumor necrosis factor-α**

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Introduction

Henoch-Schönlein purpura (HSP), which is characterized clinically by purpura, joint pain, gastrointestinal symptoms, and renal disease, is the most common type of small-vessel vasculitis in children.[1,2] The incidences of HSP and HSP nephritis (HSPN) have increased in recent years, and the incidence rate of HSP varies from 10 cases to 30 cases per 100 000 children.[2-4] HSPN, which develops in 30%-50% of patients with HSP, is likely to be associated with the recurrence of purpura.[5-7] In certain patients, HSPN progresses to a long-term disease and develops into end-stage renal disease. The Renal Group of the Chinese Academy of Pediatrics has investigated the medical records of 105 hospitals nationwide and demonstrated that HSPN is the third most serious threat to children's renal health, which is secondary to acute nephritis and primary nephrotic syndrome.[8] HSP is a type of immune disorder that is frequently induced by infection and inflammation. The polymorphisms of cytokine genes, such as interleukin (*IL*)-*1*, *IL-8*, transforming growth factor (*TGF*)-*β*, and angiotensinogen, are associated with susceptibility, pathogenesis, progression, and prognosis of HSP or HSPN.[9-13]

Genes encoding human heat shock protein 70 (HSP70) and tumor necrosis factor-α (TNF-α) reside in

the human histocompatibility leukocyte antigen (HLA) class III region on chromosome band 6p21.3, where the gene *C4* is located. The frequencies of C4A*Q0 and C4B*Q0 genotype of *C4* gene are associated with HSP in children.[13] The 1267 position in the *HSP70-2* gene exhibits a G-A polymorphism[14] that has been associated with the occurrence and development of immune system diseases, such as ankylosing spondylitis,[15] systemic lupus erythematosus (SLE),[16] and diabetes mellitus.[17] Kroeger et al[18] demonstrated that *TNF-α* (+308A/G) polymorphism can affect the *TNF-α* gene transcriptional activity. To date, few studies have investigated the *TNF-α* (+308A/G) and *HSP70-2* (+1267A/G) polymorphisms in HSP patients. The present study was aimed to explore the correlation between *HSP70-2*, *TNF-α* polymorphisms and HSP in children, and to provide novel ideas and methods for the clinical diagnosis and treatment as well as the prevention of HSP in children.

Methods

Research subjects

Two hundreds and five children who were diagnosed with HSP between September 2008 and May 2009 at Nanjing Children's Hospital, were enrolled in the study, including 121 males and 84 females. All of the subjects in our study were ethnic Han Chinese from different families and had no blood relationship. The subjects ranged in age from 2 years 1 month to 14 years 7 months, with an average age of 8.2±2.7 (mean±standard deviation) years. The HSP diagnoses were performed according to the criteria for the classification of HSP by the American College of Rheumatology.[19] HSP patients with secondary renal involvement (hematuria, proteinuria and impaired renal function) were diagnosed as HSPN. The patients who developed renal impairments within 3 to 6 months from the onset of HSP were included in the HSPN subgroup. There were 121 patients with HSP who did not develop HSPN (the nephritis-free subgroup), including 67 males and 54 females. The HSPN subgroup comprised 84 patients (54 males and 30 females). The frequency matching method was applied to select outpatients or hospitalized patients as controls who had a history of trauma, who underwent debridement or general surgery, and who had no kidney diseases or rheumatic immune diseases. Additionally, the controls were selected to match the features of gender, age, and area of residence with the patients in the case group. There were a total of 53 unrelated control patients (33 males and 20 females) with an average age of 8.0±2.5 years. In these groups, there were no significant differences in gender or age (P>0.05). This study was performed according to the guidelines of Nanjing Medical University, which abides by the Helsinki Declaration on ethical principles for medical research involving human subjects.

DNA extraction

Five milliliters of venous blood was collected from each subject. The Promega reagent kit (Promega Corporation, Madison, USA) was used to extract DNA from whole blood samples according to the manufacturer's manual. The samples were stored at -20°C until subsequent usage.

Detection of the *HSP70-2* and *TNF-α* gene polymorphisms

Tris and ethylene diamine tetraacetic acid buffer was added to a 2 μL DNA solution to make an 80 μL solution. The purity of the extracted genomic DNA was determined by calculating the optic density (OD)260/OD280 ratio. According to the methods reported in the literature, a 50-500 ng DNA template was added to each 20 μL polymerase chain reaction (PCR) sample. The gene sequences obtained from GenBank and PubMed were used to design primers using the Primer 5.0 software (Premier, Canada). The upstream primer used to amplify the *HSP70-2* gene was 5'-AAGGTGCAGAAGCTGCTGCA-3', and the downstream primer was 5'-GGACTTGTCCCCCAT-3'. The upstream primer sequence for the amplification of the *TNF-α* gene was 5'-AGGCAA TAGGTTTTGAGGGCC AT-3', and the downstream primer sequence was 5'-TCCTCCCTGCTCCGATTCCG-3'. The volume of each PCR sample was 20 μL, including 0.25 μL of upstream primer, 0.25 μL of downstream primer, 10 μL of master mix, and 2 μL of DNA. The PCR protocol included an initialization step at 94°C for 5 min followed by 38 cycles of 94°C for 40 s, 58.5°C for 40 s, and 72°C for 40 s, followed by a final elongation step at 72°C for 10 min.

Enzymatic digestion reaction

According to the instructions regarding enzymatic digestion, each 20 μL digestion reaction contained 15 μL PCR product and 15 U PstI restriction endonuclease (TAKARA, Otsu Shiga, Japan). The digestion was conducted at 37°C for 16 hour. The products from the restriction digests were subjected to 2.5% agarose gel electrophoresis and ethidium bromide staining. The results were photographed using a gel documentation system. The length of the PCR fragment of the *HSP70-2* gene was 123 bp. After enzymatic digestion, the G/G genotype carriers had two fragments (103 bp and 20 bp), the G/A genotype carriers had three fragments (123 bp, 103 bp, and 20 bp), and the A/A genotype carriers had a single 123-bp fragment. The length of the PCR fragment of the *TNF-α* gene was 107 bp. After enzymatic digestion, the G/G genotype carriers had two

fragments (87 bp and 20 bp), the G/A genotype carriers had three fragments (107 bp, 87 bp and 20 bp), and the A/A genotype carriers had a single 107 bp fragment.

Data analysis

The SPSS13.0 software package (IBM SPSS, North Castle, USA) was used to perform the statistical analysis. The coincidence of the *HSP70-2* and *TNF-α* genotypes in the HSP group and the control group with Hardy-Weinberg equilibrium and the genotypic and allelic frequencies in patients were compared between groups using the χ^2 test. $P<0.05$ was considered to be statistically significant.

Results

Distribution and frequency of *HSP70-2* genotypes in the HSP and control groups

The A/A, A/G, and G/G genotypic frequencies at the +1267A/G position of the *HSP70-2* gene were 25.9%, 51.2%, and 22.9% in the HSP group and 30.2%, 60.4%, and 9.4% in the control group. The frequency of the G/G genotype of *HSP70-2* in the HSP group was significantly higher than that in the control group ($\chi^2=4.764$, $P<0.05$). The A and G allele frequencies of *HSP70-2* were 51.5% and 48.5% in the HSP group and 60.4% and 39.6% in the control group. The difference between the groups was not significant ($\chi^2=2.689$, $P>0.05$). The results are presented in Table 1.

Distribution and frequency of each *HSP70-2* genotype in the HSPN subgroup and the nephritis-free subgroup

The A/A, A/G, and G/G genotypic frequencies of *HSP70-2* were 24.8%, 52.9%, and 22.3% in the nephritis-free subgroup and 27.4%, 48.8%, and 23.8% in the HSPN subgroup. The differences between them were not statistically significant ($\chi^2=0.063$, $P>0.05$). The G and A allele frequencies of *HSP70-2* were 51.2% and 48.8% in the nephritis-free subgroup and 51.8% and 48.2% in the HSPN subgroup; there was no significant difference between the nephritis-free subgroup and the HSPN group ($\chi^2=0.012$, $P>0.05$). The results are presented in Table 2.

Distribution and frequency of *TNF-α* genotypes in the HSP and control groups

The G/G, G/A, and A/A genotype frequencies at the +308A/G position of *TNF-α* were 59.5%, 32.2%, and 8.3% in the HSP group and 77.4%, 20.8%, and 1.9% in the control group. The A/A genotype frequency in the HSP group was higher than that in the control group ($\chi^2=6.447$, $P=0.040$), with a significant difference. The G and A allele frequencies of *TNF-α* were 75.6% and 24.4% in the HSP group and 87.7% and 12.3% in the control group. The A allele frequency in the HSP group was higher than that in the control group with a significant difference ($\chi^2=7.241$, $P=0.007$). The results are presented in Table 3.

Distribution and frequency of each *TNF-α* genotype in the HSPN subgroup and the nephritis-free subgroup

The G/G, G/A, and A/A genotype frequencies of *TNF-α* were 64.5%, 26.4%, and 9.1% in the nephritis-free subgroup and 52.4%, 40.5%, and 7.1% in the HSPN subgroup. The differences between them were not significant ($\chi^2=4.474$, $P=0.107$). The G and A allele frequencies of *TNF-α* were 77.7% and 22.3% in the nephritis-free subgroup and 72.6% and 27.4% in the HSPN subgroup. There was no significant difference in either the A or G allele between the nephritis-free subgroup and the HSPN group. The results are presented in Table 4.

Table 1. Comparison of *HSP70-2* genotypic frequencies in the HSP group and the control group

Groups	*n*	Genotypic frequency			Allele	
		AA, *n* (%)	AG, *n* (%)	GG*, *n* (%)	A, *n* (%)	G†, *n* (%)
HSP group	205	53 (25.9)	105 (51.2)	47 (22.9)	211 (51.5)	199 (48.5)
Control	53	16 (30.2)	32 (60.4)	5 (9.4)	64 (60.4)	42 (39.6)

HSP: Henoch-Schönlein purpura. *: $\chi^2=4.764$, $P<0.05$; †: $\chi^2=2.689$, $P>0.05$.

Table 2. Comparison of *HSP70-2* genotypic frequencies in the nephritis-free group and the HSPN group

Groups	*n*	Genotypic frequency			Allele	
		AA, *n* (%)	AG, *n* (%)	GG*, *n* (%)	A, *n* (%)	G†, *n* (%)
Nephritis-free	121	30 (24.8)	64 (52.9)	27 (22.3)	124 (51.2)	118 (48.8)
HSPN	84	23 (27.4)	41 (48.8)	20 (23.8)	87 (51.8)	81 (48.2)

HSPN: Henoch-Schönlein purpura nephritis. *: $\chi^2=0.063$, $P>0.05$; †: $\chi^2=0.012$, $P>0.05$.

Table 3. Comparison of *TNF-α* genotypic frequencies in the HSP group and the control group

Groups	*n*	Genotypic frequency			Allele	
		GG*, *n* (%)	GA, *n* (%)	AA, *n* (%)	G, *n* (%)	A†, *n* (%)
HSP group	205	122 (59.5)	66 (32.2)	17 (8.3)	310 (75.6)	100 (24.4)
Control	53	41 (77.4)	11 (20.8)	1 (1.9)	93 (87.7)	13 (12.3)

HSP: Henoch-Schönlein purpura. *: $\chi^2=6.447$, $P<0.05$; †: $\chi^2=7.241$, $P>0.05$.

Table 4. Comparison of *TNF-α* genotypic frequencies in the nephritis-free group and the HSPN group

Groups	*n*	Genotypic frequency			Allele	
		GG, *n* (%)	GA, *n* (%)	AA*, *n* (%)	G, *n* (%)	A†, *n* (%)
Nephritis-free	121	78 (64.5)	32 (26.4)	11 (9.1)	188 (77.7)	54 (22.3)
HSPN	84	44 (52.4)	34 (40.5)	6 (7.1)	122 (72.6)	46 (27.4)

HSPN: Henoch-Schönlein purpura nephritis. *: $\chi^2=4.474$, $P>0.05$; †: $\chi^2=0.240$, $P>0.05$.

Discussion

In this study, we demonstrated that the *HSP70-2* gene polymorphism (+1267A/G) and *TNF-α* (+308G/A) gene polymorphism are associated with HSP in children. Both G/G in *HSP70-2* and A/A genotype of *TNF-α* may be genetic susceptibility factors of HSP pathogenesis. No significance was observed between the HSP nephritis subgroup and the HSP nephritis-free subgroup in both gene polymorphisms.

The HSP70 family is the largest and most highly conserved group of heat shock proteins. Additionally, the HSP70 family gets the most abundant family of chaperones in the majority of organisms. Synthesized under stress conditions, HSP70 is involved in renal cell survival and matrix remodeling of acute and chronic renal diseases.[20] The major HSP70 is encoded by three genes, *HSP70-1*, *HSP70-2* and *HSP70-Hom*.[21] Previous researches have shown that *HSP70-2* gene polymorphisms are closely associated with autoimmune diseases.[15-17] Additionally, *HSP70-2* polymorphisms have been reported to be associated with various kidney diseases, such as diabetic nephropathy,[22] kidney and urinary malformations,[23] acute renal failure in premature neonates,[24] and renal allograft rejection.[25] Nearly all of the polymorphisms mentioned above focus on the location of *HSP70-2* +1267 and G allele is associated with increased pathological risk. However, the data regarding the relationship between this allele polymorphism and HSP are still lacking.

Previous studies have shown that HSP70 plays a role in autoimmune diseases via two potential mechanisms.[26] First, the pathogen and the host could have the HSP70 family with the same antigenic epitope, so the former could effectively induce autoimmune diseases by the host immune tolerance mechanism. Second, the HSP70 of pathogens binds to pathogen antigens or connects with MHC class I or class II molecules to become immunodominant antigens, which are presented to the autoimmune reactive T-cells without any inhibition. Diversely, our study showed that the frequency of the G/G homozygous genotype in the HSP group was significantly higher than that observed in the control group (χ^2=4.764, P<0.05), suggesting that the G/G homozygous genotype, the low HSP-70 producer, may be a genetic susceptibility factor for HSP. Located in the coding region, the *HSP70-2* gene polymorphism (+1267 A/G) scarcely affects the amino acid sequence of the protein but regulates the mRNA expression of *HSP70*. Specifically, the expression of *HSP70* mRNA is lower in G/G homozygotes than in the A/A and A/G genotypes.[27] The decreases in the *HSP70* expression impair the cell response to stress, resulting in intracellular accumulation and transport defects of denaturalized protein.[22] Thus, our results introduced a new perspective concerning HSP pathogenesis, which must be confirmed in further research. No significant differences in genotypic frequencies of *HSP70-2* (+1267A/G) were observed between the nephritis-free subgroup and the HSPN subgroup, suggesting that the risk of nephritis does not increase with the genotype of *HSP70-2* (+1267A/G). Currently, no other studies have reported the association of *HSP70-2* with HSP and HSPN. Future studies are required to clarify whether the down-regulation of *HSP70-2* expression increases the susceptibility to HSPN and to explore whether other genetic or environmental factors might influence the renal outcomes in patients with immune diseases.

TNF-α plays a critical role in innate and adaptive immune responses.[28] The considerable production of TNF-α leads to leukocyte activation, inflammation and tissue injury.[29,30] Increased serum TNF-α level can lead to abnormal changes in glomerular cell morphology and abnormal accumulation of extracellular matrix in the mesangial area during the acute phase of HSPN.[31-34] Additionally, TNF-α can regulate the function of glomerular epithelial cells.[35] However, the low concentration of TNF-α impairs initial immune responses and, consequently, promotes a severe inflammatory reaction, which is observed in *TNF-α* gene knock-out mice with infection.[36] This phenomenon is highly suggestive of the yin and yang theory of *TNF-α* expression.

TNF-α gene polymorphism is associated with acute kidney injury,[37,38] allograft nephropathy,[39] contrast-induced nephropathy,[40] SLE nephritis,[41] renal cell carcinoma,[42] and so on. However, whether it may be an independent risk factor of IgA nephropathy,[43,44] which mimics the pathogenesis of HSP nephritis, remains controversial. Among the reported locus, the gene polymorphic variation 308 bp upstream of the *TNF-α* gene promoter is a hot spot; however, the risk allele varies depending on the specific diseases. To date, no studies on the correlation between the *TNF-α* gene polymorphism at the -308 loci and HSPN have been reported. The results revealed that the A/A genotype and the A allele frequencies in the HSP group were higher than those in the normal control group with significant differences (P<0.05), suggesting a correlation between the *TNF-α* gene polymorphism at the -308 locus and HSP susceptibility. However, Yang et al[45] conducted a comparative study of *TNF-α* gene polymorphisms at the -308 locus in 29 children with HSP and in 36 healthy children; they did not observe a correlation between the *TNF-α* gene polymorphism at the -308 locus and HSP. This conclusion was not consistent with our experimental results because their study likely had a small sample size and did not achieve statistical

significance. Furthermore, it might be attributed to the difference of clinical manifestations between the two studies, such as joint and gastrointestinal manifestations that were described in the study by Yang et al but were not provided in the current study, which needs to be further investigated. Our study also revealed that there were no significant differences in the genotypic and allelic frequencies between the nephritis-free subgroup and the HSPN subgroup, suggesting little correlation between the *TNF-α* gene polymorphism at the -308 locus and the development of kidney damage. The 308 A-allele carrier in the promoter is related to high promoter activity and enhances the *TNF-α* production both *in vivo* and *in vitro*.[18,46] HSP, a type of IgA-associated vasculitis,[1] can be triggered by infections and is secondary to an immune response to antigens. Therefore, it is likely that enhanced TNF-α production (-308 A-allele carrier) might have a stronger immune system activation and more serious tissue injury.

To further clarify our results in this study, several limitations are presented. Our study was limited by its small sample size and mono-centric data, which may have biased the results. Therefore, in the future, it is necessary to expand the sample size and perform a multi-center clinical trial to verify the results.

In conclusion, the *HSP70-2* (+1267A/G) and *TNF-α* (+308G/A) gene polymorphisms were associated with HSP in children. The G/G homozygosity of *HSP70-2* and the A/A homozygosity of *TNF-α* may be genetic predisposing factors for HSP.

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Competing interest: We declare no competing interests in this study.
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