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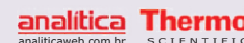
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Association of *MICA* gene polymorphisms with liver fibrosis in schistosomiasis patients in the Dongting Lake region

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Abstract

Major histocompatibility complex class I chain-related A (*MICA*) is a highly polymorphic gene located within the MHC class I region of the human genome. Expressed as a cell surface glycoprotein, *MICA* modulates immune surveillance by binding to its cognate receptor on natural killer cells, NKG2D, and its genetic polymorphisms have been recently associated with susceptibility to some infectious diseases. We determined whether *MICA* polymorphisms were associated with the high rate of *Schistosoma* parasitic worm infection or severity of disease outcome in the Dongting Lake region of Hunan Province, China. Polymerase chain reaction-sequence specific priming (PCR-SSP) and sequencing-based typing (SBT) were applied for high-resolution allele typing of schistosomiasis cases (N = 103, age range = 36.2-80.5 years, 64 males and 39 females) and healthy controls (N = 141, age range = 28.6-73.3 years, 73 males and 68 females). Fourteen *MICA* alleles and five short-tandem repeat (STR) alleles were identified among the two populations. Three (*MICA**012:01/02, *MICA**017 and *MICA**027) showed a higher frequency in healthy controls than in schistosomiasis patients, but the difference was not significantly correlated with susceptibility to *S. japonicum* infection ($P > 0.05$). In contrast, higher *MICA**A5 allele frequency was significantly correlated with advanced liver fibrosis ($P < 0.05$). Furthermore, the distribution profile of *MICA* alleles in this Hunan Han population was significantly different from those published for Korean, Thai, American-Caucasian, and Afro-American populations ($P < 0.01$), but similar to other Han populations within China ($P > 0.05$). This study provides the initial evidence that *MICA* genetic polymorphisms may underlie the severity of liver fibrosis occurring in schistosomiasis patients from the Dongting Lake region.

Key words: *Schistosoma japonicum*; *MICA*; NKG2D; Gene polymorphism; Liver fibrosis

Introduction

Schistosomiasis is the most prevalent water-borne parasitic disease worldwide and remains a major public health problem in many developing countries. Among the pathogenic *Schistosoma* species, *S. japonicum* is responsible for endemic infection in China, Indonesia, and the Philippines (1). Recorded cases of schistosomiasis in China date back more than 2100 years (2). Not surprisingly, one of the first and most substantial human health campaigns of the newly founded People's Republic of China was to control this parasitic disease. By the beginning of the 2000's, schistosomiasis had been eradicated in five of the twelve previously endemic provinces of China, accompanied by

dramatic reductions in morbidity and mortality (3,4).

Currently, the geographic distribution of schistosomiasis in China is primarily centered around the Yangtze River, with the worst situations in the Dongting Lake and Poyang Lake regions (3,4). The Dongting Lake, located in the north of Hunan Province, is the second largest freshwater lake in China and provides an ideal habitat for *Oncomelania* snails, the intermediate host for *S. japonicum*. Sporocysts are generated in the snail and produce infective cercariae that are released from the snail into the surrounding aqueous environment. These free-swimming parasitic larvae secrete a proteolytic enzyme that facilitates penetration of

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the human epidermis. Inside the human host, the parasites mature in the hepatic portal vein and adult paired sex worms migrate to the mesenteric veins, depositing eggs that are carried by the circulation to the host's liver, intestine, urinary bladder or other organs. These eggs trigger the host immune response, including a localized inflammatory reaction; however, as the simple immune response is unable to clear these eggs, granuloma formation is induced to wall off the foreign substances. The granulomatous reaction is accompanied by fibrosis, which is particularly devastating to the function of liver tissues (5). Thus, the pathogenesis of schistosomiasis involves the dynamic coordination of many tissue- and process-specific genes. Undoubtedly, the parasite has evolved to exploit genes that vary in expression among different populations to establish infection.

The human major histocompatibility complex (MHC) class I chain-related (MIC) gene family consists of seven members, *MICA* to *MICG*; however, only *MICA* and *MICB* are functional genes and all other members represent pseudogenes (6,7). The human *MICA* gene contains 6 exons encoding a cell-surface glycoprotein with three extracellular domains (encoded by exons 2, 3, and 4, respectively), a transmembrane fragment (encoded by exon 5), and a carboxy-terminal cytoplasmic tail (encoded by exon 6) (6,8). In response to cellular stress, *MICA* expression is induced in many cell types, including epithelium, fibroblasts, keratinocytes, endothelial cells, and monocytes (7,9,10), whereby it regulates the autoimmune response through binding to its cognate receptor (5), the natural killer (NK) cell receptor D (NKG2D) (11-16). The *MICA* gene is highly polymorphic, and the exons encoding the extracellular domains present the highest frequency of polymorphisms (8).

The two different types of alleles of the gene represent 76 (<http://hla.alleles.org/nomenclature/stats.html>) sequence alleles in the mature protein-coding region (*MICA**001 to *064N, <http://www.ebi.ac.uk/imgt/hla/allele.html>) and seven microsatellite alleles. Also known as short tandem repeats (STR), these microsatellite alleles (*MICA**A4, *A5, *A5.1, *A6, *A7, *A9, and *A10) were formed by insertion/deletion of variable numbers of trinucleotide GCT repeats within exon 5.

MICA has been identified as a candidate disease gene by several linkage disequilibrium mapping studies of common autoimmune-related diseases, including Behcet's disease, insulin-dependent diabetes mellitus, and Addison's disease (17). In our ongoing attempts to characterize the pathogenesis of schistosomiasis in the Hunan Han population, we became intrigued by the underlying mechanistic features involving autoimmune processes and hypothesized that ethnicity-related *MICA* polymorphisms may confer susceptibility to *S. japonicum* infection and affect the outcome of severe liver fibrosis. To this end, we performed polymerase chain reaction-sequence specific

priming (PCR-SSP) and sequencing-based typing (SBT) to analyze the *MICA* polymorphisms in schistosomiasis patients from the Dongting Lake region and compared the distribution of *MICA* alleles with data for other ethnic groups around the world.

Material and Methods

Subjects

A cohort of 103 patients of Han nationality attending the Schistosomiasis Outpatient Clinic of the Teaching Hospital, School of Medical Science, Central South University (Changsha, China) between 2008 and 2010 were recruited into this study. All patients resided in Changde or Yueyang city in the Dongting Lake region, Hunan Province, China (Figure 1). All patients had a recorded clinical diagnosis of *S. japonicum* infection between the ages of 10 and 24 years. Diagnosis of liver fibrosis in these patients was made by B-scan ultrasonography findings fitting World Health Organization (WHO) criteria. Patients with grade 0 and grade 1 fibrosis were classified into a mild fibrotic group, and those with grade 2 (moderate) and grade 3 (severe) fibrosis into an advanced fibrotic group. For healthy controls, 141 unrelated Han individuals were recruited from the Central Blood Bank (Yueyang, China) according to the following criteria: residing in Changde or Yueyang city at the time of blood collection, no previous diagnosis of schistosomiasis, no family history of schistosomiasis, and a gender ratio match to the patient cohort. The clinicopathological features of the patients and controls are listed in Table 1. Written informed consent was obtained from each study participant, and the study was approved by the Ethics Review Board of the Central South University.

High-resolution allele typing of the *MICA* gene by PCR-SSP and SBT

Genomic DNA was isolated from EDTA-treated peripheral venous blood using a conventional proteinase K digestion/salting-out extraction method (18). The PCR-SSP method was carried out as previously described with the following minor modifications (19). The human growth hormone gene was used as an internal control and amplified (834-bp fragment) by gene-specific primers (sense 5'-GCCTTCCAACCATTCCTTA-3' and anti-sense 5'-GAGAAAGGCCTGGAGGATTC-3') (19). Ninety-five primers targeting *MICA* exons 2-4 were designed according to sequences identified in a previous study (19). PCR mixtures contained 100 ng genomic DNA template, 1X PCR buffer, 200 μ M each deoxy-nucleoside triphosphate, 1.75 mM $MgCl_2$, 0.25 U Taq polymerase (all from MBI Fermentas, Lithuania), 1.5-2.0 μ M allele or group-specific primers, and 0.115 μ M internal control primers. PCR was carried out in an Eppendorf Mastercycler 5333 thermocycler (Germany) programmed with the following series of thermal cycling conditions: initial denaturation at

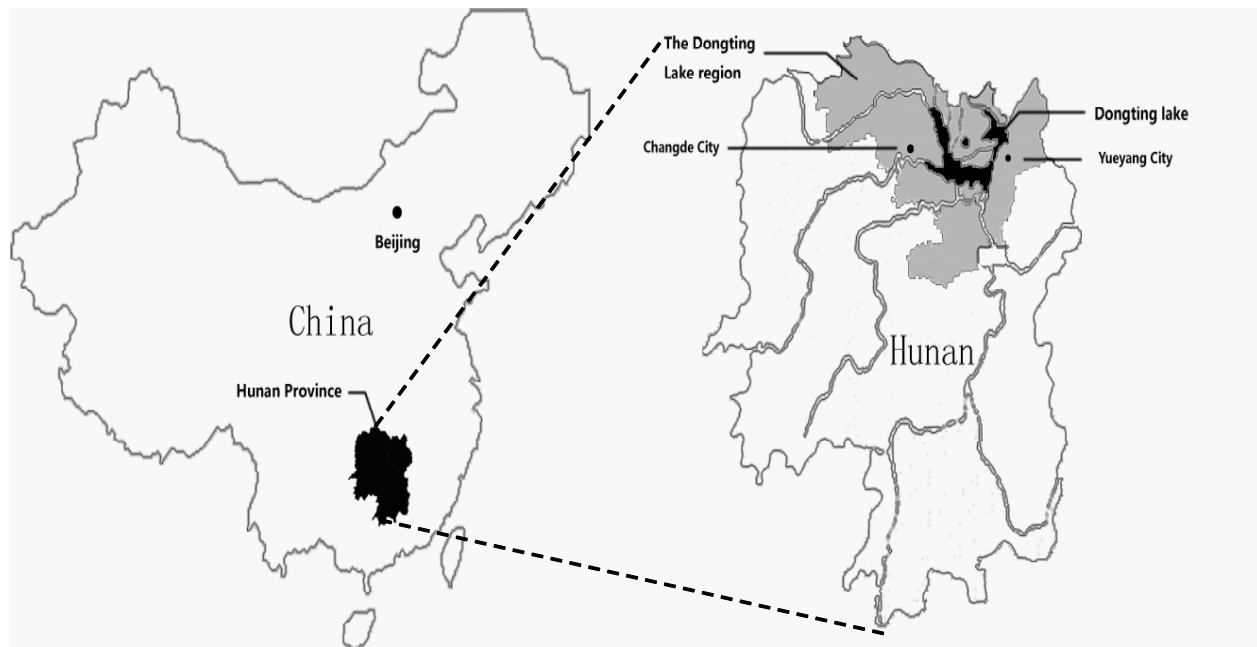


Figure 1. Map showing the relative locations of Hunan Province, the Dongting Lake region, Changde City, and Yueyang City in China.

94°C for 4 min; 10 cycles of denaturation at 94°C for 30 s, annealing at 64.5°C for 50 s, and elongation at 72°C for 20 s; 10 cycles of denaturation at 94°C for 30 s, annealing at 61.5°C for 50 s, and elongation at 72°C for 30 s; 10 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 50 s, and elongation at 72°C for 40 s, and a final rapid cooling to 4°C. Amplification products (8 µL) were verified by electrophoretic resolution through a 2% agarose gel stained with ethidium bromide and visualized under ultraviolet (UV) illumination. Fifty-five *MICA* sequence alleles, including *MICA*001* to *MICA*050*, were detectable and distinguished by the PCR-SSP analysis employed. Two notable exceptions were that *MICA*007:01* could not be distinguished from *MICA*026*, and *MICA*002:01* could not be distinguished from *MICA*020*.

Since the structure of most *MICA*-STR alleles can be obtained from the Anthony Nolan Trust (HLA Informatics Group website: <http://www.ebi.ac.uk/imgt/hla/allele.html>), the sequences of the alleles genotyped above were used to determine the corresponding STR genotypes. Considering that there could be some unreported STR from known sequence alleles and that some rare alleles may not have been genotyped by the PCR-SSP method, we applied the SBT method as previously described to further differentiate *MICA* alleles and validate the results from PCR-SSP (20,21). A total of 45 samples were genotyped by PCR-SBT, including some randomly selected samples and all of the *MICA*007:01/MICA*026* and *MICA*002:01/MICA*020* alleles.

Table 1. Clinicopathological features of schistosomiasis patients and healthy controls.

Characteristics	Patients (N = 103)	Controls (N = 141)
Gender, N (%)		
Male	64 (62.1%)	73 (51.8%)
Female	39 (37.9%)	68 (48.2%)
Age (years)		
Range	36.2-80.5	28.6-73.3
Average	55.6	50.3
Liver fibrosis, N (%)		
Mild	52 (50.5%)	
Advanced	51 (49.5%)	

Statistical analysis

MICA allele distributions were tested for Hardy-Weinberg equilibrium to assess Mendelian inheritance. Statistical analysis was performed using the SPSS 11.5 statistical software (SPSS, Inc., USA). Allelic frequencies were calculated by direct counting, and statistical comparisons between groups were performed by the χ^2 method, Yates' correction, or the Fisher exact test. Odds ratios (OR) and 95% confidence intervals (95%CI) were calculated according to Woolf's method to determine disease risk in carriers of specific alleles. Bonferroni's multiple correction was used for the corrected P (P_c) by multiplying the P value by the number of statistical tests.

A two-sided $P < 0.05$ value was considered to be statistically significant.

Results

Association between *MICA* polymorphisms and susceptibility to *S. japonicum* infection in the Hunan Han population

To determine the correlation between *MICA* polymorphisms and susceptibility to *S. japonicum* infection, we genotyped *MICA* and analyzed the frequencies of distinct *MICA* alleles in 103 schistosomiasis patients and 141 healthy controls. As shown in Table 2, a total of 14 *MICA* sequence alleles and five STR alleles were identified from the two groups. *MICA**018 and *MICA**023 were only present in the patient group and occurred at a low frequency (0.97 and 0.49%, respectively). *MICA**017 and *MICA**049 were only present in the healthy controls and occurred at a low frequency (2.13 and 0.71%, respectively). The 10 remaining alleles were found in both groups. *MICA**A5 had the highest frequency in both groups (32.04% for patients and 37.23% for healthy controls). *MICA**012:01/02 allele ($\chi^2 = 3.95637$, $P = 0.04669$, $OR = 0.501$, $95\%CI = 0.251-$

1.001), *MICA**017 allele ($\chi^2 = 4.43754$, $P = 0.03516$, $OR = 1.022$, $95\%CI = 1.004-1.039$) and *MICA**027 allele ($\chi^2 = 3.94081$, $P = 0.04713$, $OR = 0.242$, $95\%CI = 0.053-1.102$) showed dramatically higher frequencies in healthy controls; however, the difference from the patient groups was not statistically significant after correcting for multiple tests ($P_c > 0.05$). Thus, none of the *MICA* alleles identified by our analysis correlated with susceptibility to *S. japonicum* infection in this population.

Association between *MICA* polymorphisms and liver fibrosis severity in Hunan Han schistosomiasis patients

Further analysis was carried out on the 12 *MICA* sequence alleles and 5 STR alleles identified in the patient group to determine the potential association between the *MICA* polymorphisms and liver fibrosis severity (Table 3). Only the *MICA**A5 allele showed a significantly higher frequency in schistosomiasis patients with advanced fibrosis compared to patients with mild fibrosis (45.10 vs 26.92%, $P = 0.00656$, $P_c = 0.03279$, $OR = 2.230$, $95\%CI = 1.245-3.994$). Although the *MICA**010 allele also tended to occur more frequently in patients with advanced fibrosis ($P = 0.03123$),

Table 2. *MICA* allele frequencies in schistosomiasis patients and healthy controls.

	Patients		Controls		χ^2	P	P_c
	No. of alleles (N = 206)	Allele frequency (%)	No. of alleles (N = 282)	Allele frequency (%)			
<i>MICA</i> alleles							
<i>MICA</i> *002:01	35	16.99	36	12.77	1.70867	0.19116	NS
<i>MICA</i> *004	3	1.46	5	1.77	0.07406	0.78551	NS
<i>MICA</i> *007:01/02	2	0.97	2	0.71	0.10025	0.75153	NS
<i>MICA</i> *008:01/02	64	31.07	70	24.82	2.33093	0.12683	NS
<i>MICA</i> *009:01/02	12	5.83	11	3.90	0.98176	0.32176	NS
<i>MICA</i> *010	43	20.87	76	26.95	2.38385	0.12259	NS
<i>MICA</i> *012:01/02	12	5.83	31	10.99	3.95637	0.04669*	NS
<i>MICA</i> *017	0	0.00	6	2.13	4.43754	0.03516*	NS
<i>MICA</i> *018	2	0.97	0	0.00	2.74913	0.09731	NS
<i>MICA</i> *019	20	9.71	18	6.38	1.83367	0.17569	NS
<i>MICA</i> *023	1	0.49	0	0.00	1.37174	0.24151	NS
<i>MICA</i> *027	2	0.97	11	3.90	3.94081	0.04713*	NS
<i>MICA</i> *045	10	4.85	14	4.96	0.00309	0.95567	NS
<i>MICA</i> *049	0	0.00	2	0.71	1.46701	0.22582	NS
STR alleles							
<i>MICA</i> *A4	26	12.62	47	16.67	1.53133	0.21591	NS
<i>MICA</i> *A5	66	32.04	105	37.23	1.41152	0.2348	NS
<i>MICA</i> *A5.1	64	31.07	70	24.82	2.33093	0.12683	NS
<i>MICA</i> *A6	18	8.74	18	6.38	0.96613	0.32565	NS
<i>MICA</i> *A9	32	15.53	42	14.89	0.03795	0.84555	NS

NS = nonsignificant. * $P < 0.05$ patients compared with controls (chi-square test).

Table 3. *MICA* allele frequencies in schistosomiasis patients with liver fibrosis.

	Advanced fibrotic group		Mild fibrotic group		χ^2	P	Pc
	No. of alleles (N = 102)	Allele frequency (%)	No. of alleles (N = 104)	Allele frequency (%)			
<i>MICA</i> alleles							
MICA*002:01	16	15.69	21	20.19	0.70958	0.39958	NS
MICA*004	0	0.00	3	2.88	2.98579	0.0840	NS
MICA*007:01/02	1	0.98	1	0.96	0.00019	0.98899	NS
MICA*008:01/02	25	24.51	33	31.73	1.3274	0.24927	NS
MICA*009:01/02	5	4.90	7	6.73	0.31395	0.57527	NS
MICA*010	33	32.35	20	19.23	4.64029	0.03123*	NS
MICA*012:01/02	4	3.92	7	6.73	0.80397	0.36991	NS
MICA*018	0	0.00	1	0.96	0.98555	0.32083	NS
MICA*019	11	10.78	8	7.69	0.58801	0.44319	NS
MICA*023	0	0.00	1	0.96	0.98555	0.32083	NS
MICA*027	2	1.96	0	0.00	2.05921	0.15129	NS
MICA*045	5	4.90	2	1.92	1.39206	0.23806	NS
STR alleles							
MICA*A4	10	9.80	11	10.58	0.03361	0.85454	NS
MICA*A5	46	45.10	28	26.92	7.38996	0.00656*	0.03279*
MICA*A5.1	25	24.51	34	32.69	1.68696	0.1940	NS
MICA*A6	5	4.90	10	9.62	1.69453	0.19301	NS
MICA*A9	16	15.69	21	20.19	0.70958	0.39958	NS

NS = nonsignificant. *P < 0.05 advanced fibrotic group compared with mild fibrotic group (chi-square test).

Table 4. Comparison of the *MICA* allelic distributions of the Dongting Lake Han healthy population with other ethnic groups.

	Dongting Lake	Northern Chinese	Southern Chinese	Korean	Thai	American-Caucasian	Afro-American
χ^2	-	22.234	14.367	38.980	38.680	43.161	73.575
Degrees of freedom	-	15	12	11	16	16	17
P	-	0.102	0.278	0.000*	0.001*	0.000*	0.000*

*P < 0.05 compared to Dongting Lake Han healthy population (chi-square test).

this trend was not statistically significant following multiple testing corrections (Pc > 0.05).

Comparison of *MICA* distribution between Chinese Han and other ethnic groups

To characterize the differences in *MICA* gene polymorphisms between the distinct ethnic groups, which may modify the susceptibility of different groups to schistosomiasis or affect associated pathologies, we compared the distribution of *MICA* alleles from the healthy controls in this study to those reported for Southern and Northern Han

populations (22,23), as well as for Korean (24), Thai (25), American-Caucasian (23), and Afro-American populations (23). As shown in Table 4, no dramatic differences were observed in the *MICA* allelic distribution between the Han populations from different areas of China (P > 0.05). In contrast, significant differences were detected between the Han population from the Dongting Lake region and all other non-Chinese ethnic groups examined, including Koreans ($\chi^2 = 38.980$, P = 0.000), Thais ($\chi^2 = 38.680$, P = 0.001), American-Caucasians ($\chi^2 = 43.161$, P = 0.000), and Afro-Americans ($\chi^2 = 73.575$, P = 0.000).

Discussion

Identification of candidate genes and/or specific alleles associated with susceptibility to or the progression of a disease will benefit clinical assessment of disease predisposition, early diagnosis and preventive therapeutic intervention. In this study, we report for the first time that a higher frequency of the *MICA* allele**A5* correlated significantly with the development of advanced liver fibrosis in schistosomiasis patients from the Dongting Lake region in Hunan Province, China. In addition, allelic distribution of *MICA* in the Chinese Han population is significantly different from that of many other ethnic groups in the world.

Epidemiologic studies on schistosomiasis indicate that this disease has a genetic basis. Alleles of HLA, in particular those within the HLA-DR and -DQ loci, have been correlated with advanced schistosomiasis and severe liver fibrosis outcome (26,27). The human *MICA* gene is localized within the HLA class I region of chromosome 6, between *MICB* and *HLA-B*, and is known to be highly polymorphic (17). Functionally, *MICA* binding to NKG2D stimulates the release of interferon- γ (IFN- γ) from NK cells (28,29). *MICA* polymorphisms are associated with a number of NK-involved diseases, such as viral infections and tumor development (8). In schistosomiasis patients infected with *S. mansoni*, IFN- γ has been shown to confer a protective immune response against liver fibrosis (30). In addition, non-infected individuals over the age of 70 years from a *Schistosoma* endemic area have a significantly higher number of IFN- γ -producing NK cells than their infected counterparts (31). The importance of NK cells and IFN- γ in schistosomiasis prompted us to examine whether *MICA*, with its polymorphic alleles, modifies propensity to schistosomiasis.

To address this question, we analyzed the *MICA* alleles of schistosomiasis patients and compared them with healthy controls of Han nationality from one of the most severe endemic areas in China, the Dongting Lake region. To our knowledge, this is the first study addressing the importance of *MICA* polymorphisms in schistosomiasis. Our analysis identified a total of 14 sequence alleles and five STR alleles among both populations, but none of these *MICA* alleles correlated significantly with the presence of schistosomiasis. Thus, these specific polymorphisms do not appear to pre-condition an individual to *Schistosoma* infection. However, several alleles, including *MICA**012:01/02, *MICA**017, and *MICA**027, occurred at higher, yet nonsignificant, frequencies in healthy controls compared to patients. In view of the relatively small number of subjects in this study (103 subjects), it is important to determine whether these findings would be statistically significant if the cohorts were larger.

Although no statistically significant association was observed between the *MICA* alleles identified in both groups and susceptibility to schistosomiasis, the *MICA***A5* allele did

correlate with infected patients with advanced liver fibrosis. Likewise, the *MICA**010 allele also showed a similar trend, but no statistically significant for multiple testing.

Liver fibrosis is a chronic change caused by the granulomatous immune response against eggs lodged in the periportal area (5). Both hepatic stellate cells (HSCs) and NK cells play important roles in the development of liver fibrosis. Upon liver damage, HSCs become "activated", proliferate and produce excessive extracellular matrix leading to the formation of the fibrotic scar (32). A recent study has shown that senescent activated HSCs, which can act to limit fibrosis progression, express significantly higher levels of immune modulators, such as *MICA*, than the proliferating activated HSCs; moreover, the senescent cells are selectively targeted by NK cells, potentially through interaction of the *MICA* ligand and NKG2D receptor (33). Several studies have demonstrated that various *MICA* family members are elevated in the sera of patients with autoimmune and cholestatic liver diseases (34-36). In the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-induced liver fibrosis model, NK cells ameliorate liver fibrosis by killing activated HSCs in an NKG2D-dependent manner (37).

The Hunan Han schistosomiasis patients with advanced liver fibrosis examined in our study had a higher frequency of the *MICA***A5* allele. The *MICA***A5* allele codes for a valine (V) amino acid at position 129 (IMGT/HLA Database), while the *MICA***A5.1* allele, also identified in our subjects, may possess either a methionine (M) or V at position 129. It has been shown that *MICA* M129 confers a higher affinity for the NKG2D receptor, whereas *MICA* V129 has reduced affinity (38). Therefore, a higher frequency of *MICA***A5* may reflect more V129 products. In this situation, reduced engagement with the NKG2D receptor might lead to less activation of NK cells, and, subsequently, to less cytotoxicity against activated HSCs and more aggressive development of liver fibrosis. In contrast, the *MICA***A5.1* allele, producing variant numbers of M129 and V129 products, would not be expected to push the NK-induced cytotoxicity to an extent that would favor either severe or mild liver fibrosis.

So far, few studies have compared *MICA* polymorphisms among distinct ethnic groups. We compared our genotyping result with those from other studies of different ethnic populations and showed that the distribution of *MICA* alleles does not differ dramatically among Han populations in different areas of China. However, the distribution is significantly different from that of all non-Chinese ethnic groups examined, implying that a differential susceptibility and progression of *MICA*-involved diseases exist among distinct ethnic groups.

Our data show that the *MICA* gene exhibits great variability among different ethnic groups. In the schistosomiasis-endemic Dongting Lake region, *MICA***A5* positively correlates with the more severe phenotype of liver fibrosis in infected patients. Our findings will serve as a foundation

for future *in vitro* and *in vivo* studies to elucidate the underlying immunopathological mechanisms associated with *MICA*-regulated liver fibrosis in *S. japonicum* infection.

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