

## Review Article

# Immunogenetic Analysis of Severe Forms of Parasitic Diseases

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Malaria and Schistosomiasis are the major endemic parasitic diseases in the world. The former is killing over one million children in Africa per year, and the latter suffers several millions in the endemic areas in Asia, Africa and South America.

Collaborative studies were performed to identify some genetic factors contributing to the development of fatal forms of these infectious diseases. In Thailand, TNF- $\alpha$  5'-flanking region showed biallelic polymorphic sites at -238, -308, -857, -863, -1031, and there were 7 alleles found in the patients from Myanmar. We found that the TNFP-D allele was significantly associated with cerebral malaria in the populations from Karen ( $P_c < 0.0001$ , OR=124.86) and Burma ( $P_c < 0.0001$ , OR = 34.50). In China, we have identified two major genes related to severity of liver fibrosis, one was HLA-class II and the other was IL-13 gene. The allele frequencies of HLA-DRB5\*0101 and IL-13 promoter A/A homozygote were both increased in the fibrotic group although the two genes are located in the different chromosomes, Chromosome 6p and 5q. The person who had both genotypes showed much higher Odds Ratio (OR=24.5) compared with sum of either genotype positive persons (OR=5.1 for HLA-DRB5\*0101, OR=3.7 for IL-13P A/A). The observation that the effect of the two susceptible markers were synergistic rather than additive, strongly suggested that the pathogenic Th2 response directly influence the prognosis of post-Schistosomal liver fibrosis.

ACTA MEDICA NAGASAKIENSIA 46 : 1-5, 2001

**Key Words:** *Plasmodium falciparum*, *Schistosoma japonicum*, genetic susceptibility, HLA, cerebral malaria, schistosomal liver fibrosis.

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## Introduction

Here I will introduce our recent findings that clearly indicated the presence of genetic factors that predict the prognosis of two major parasitic infectious diseases, Falciparum malaria and Schistosomiasis japonica. The reason why we were interested in those diseases was that those two diseases provoke serious sequelae namely cerebral malaria and post-schistosomal liver cirrhosis. People in the endemic areas are suffering those two forms of complications even now. The identification of susceptible or resistant genes to the severe forms is not only useful for the prediction of the prognosis but also for understanding the pathogenesis and the new treatment.

To identify the responsible genes, generally two different approaches are undertaken. One is targeted genes analysis and the other is genome wide survey. We adopted the former one, especially focused on the immune related genes such as HLA, cytokines, and adhesion molecules genes. HLA genes are well known to be highly polymorphic and their alleles are well identified by the DNA sequence levels. For example, HLA-DRB1 gene has over 100 alleles in the human population. Especially, HLA-DR, DQ, DP, A, B and C are believed to be functioning immune response genes to exogenous pathogens. Cytokines are also believed to play important roles to control the intensity and duration of the immune response. Recently, single nucleotide polymorphisms (SNPs) are much more frequently observed in the promoter regions of some cytokines such as TNF, IL-4, IL-13, Interferon-gamma, etc. Several studies indicated those polymorphisms directly affect its promoter activity.

We had serious and enthusiastic collaborators in the endemic areas and initiated those difficult but very interesting studies several years ago. Finally, we found surprisingly strong associations between those markers and the diseases.

### TNF- $\alpha$ Promoter region polymorphism and cerebral malaria

The most severe complication of Plasmodium falciparum infection is cerebral malaria (CM) (1). Little is known about the pathogenesis of this condition (2). Postmortem examination of the affected brain shows a typical microembolism, commonly called a sequestration, which consists of infected red blood cells in capillaries in the brain mesenchyma (3). Therefore, sequestration is considered to be one of the major sequelae causing the typical neurological disturbances that occur in CM. Because the serum level of TNF- $\alpha$  is reported to be elevated in severe malaria (4), excessive production of TNF- $\alpha$  is suspected to produce conditions optimal for sequestration in capillaries (5, 6).

We performed a case control study of CM patients in Myanmar to analyze their HLA and TNF- $\alpha$  promoter polymorphisms. A total of 245 unrelated malaria patients, consisting of 145 Karen and 100 Burmese ethnic groups, were randomly selected. The patients were assessed by WHO criteria (7) to be uncomplicated or to have CM at the Mae Sot Malaria Clinic or at the Mae Sot General Hospital.

The 5'-flanking region of TNF- $\alpha$ , 1,042-bp DNA fragment spanning the 5'-flanking region of the TNF- $\alpha$  gene from position -66 to -1,107 was amplified by PCR. After hybridization, 7 possible combinations of polymorphic sites in the TNF- $\alpha$  promoter (TNFP) were determined in the present study population, giving TNFP alleles A, B, C, D, M1, M4, and M7. PCR products from each tentative genotype were cloned and were sequenced to confirm the alleles. The DNA typing of HLA-DRB1 gene was performed by the PCR-SSOP method used in the XIth HLA workshop (8). HLA-B DNA typing was performed as previously described (9).

**Table 1.** TNF- $\alpha$  5'-flanking region alleles detected in the Myanmar patients with malaria.

	Polymorphic sites of the promoter region of TNF- $\alpha$ gene				
	-238	-308	-857	-863	-1031
TNFP-A	G	G	C	C	T
TNFP-B	G	G	C	A	C
TNFP-C	A	G	C	C	C
TNFP-D	G	G	T	C	T

Footnote for Table 1.

The TNFP-A, B, C, D types are reported by Higuchi et al. (10).

**Table 2.** Genotype frequency of the 5'-flanking region of TNF- $\alpha$  gene in Myanmar CM and uncomplicated patients.

Genotype	Burmese		Karen	
	Uncomplicated	CM	Uncomplicated	CM
	n=94 (%)	n=21 (%)	n=106 (%)	n=22 (%)
TNFP-D -/-	92 (97.9)	12 (57.1)	106 (100)	14 (63.6)
+/+ and +/-	2 (2.1)	9 (42.9)*	0 (0.0)	8 (36.4)**

Footnote for Table 2.

\*  $P_c < 0.001$ , OR=34.50, 95%CI: 6.65 - 178.98.

\*\*  $P_c < 0.001$ , OR=124.86, 95%CI: 6.84 - 2279.51,

Table 1 shows 7 alleles detected in the present study. The TNFP-A, B, C, D alleles were exactly the same types as were reported in the previous study from the Japanese population (10,11). The remaining three alleles, TNFP-M1, M4, and M7 were novel types that have not been reported elsewhere. Table 2 shows the allele frequencies of TNF- $\alpha$  promoter region in patients with CM and in uncomplicated patients from Karen and Burma. The occurrence of the TNFP-D +/+ and +/- genotypes were significantly increased in patients with CM in both ethnic groups.

This is the first study that reports the positive association of the TNFP-D allele with CM. The first report that there was a relationship between TNF- $\alpha$  promoter polymorphism and CM was published by McGuire, et al. restriction enzyme NcoI polymorphism (-308A/G) was used to show that the TNF2 (-308A) allele was associated with CM in Gambian school children. The position -576 polymorphic site where the nuclear factor OCT-1 binds, was reported to be associated with CM in the same Gambian population (13). We have also checked this region using specific probes and sequencing but have found no polymorphisms in the Myanmar subjects.

The TNFP-D allele that we found to be strongly associated with CM in Myanmar was reported to be a high expresser of TNF- $\alpha$  compared with the TNFP-B or C alleles in studies of peripheral blood mononuclear cells (PBMCs) stimulated with Concanavalin A in vitro, and by luciferase assays with the Raji cell transfection system (10). Skoog, et. al. (11) reported that in the TNFP-B allele the Nf- $\kappa$ B binding motif is broken by the substitution of -863C to A. This allele is common in Myanmar but is rare in Sweden (11) and the mutation results in reduced promoter activity. Serum level of TNF- $\alpha$  was lower in the healthy volunteers who were homozygous for this mutant type in Sweden (11). Though there is little information

available, when we compared the gene frequencies of the TNFP alleles in different ethnic groups, we found that the gene frequencies of the -863C or -1031T alleles, both typical for TNFP-D, were much higher in Sweden (0.94 for -863C, 0.80 for -1031T) (19) and in Japan (0.82 for -863C, 0.84 for -1031T) (9) than in the Myanmar patients (0.08 for -863C, 0.15 for -1031T). It would be of interest to see the frequencies of those alleles in African populations. It is possible to generate such a difference by natural selection.

### HLA-class II genes and IL-13 promoter region polymorphism and Schistosomal liver fibrosis

Schistosomiasis japonica (*S. japonica*) is a chronic helminthic infectious disease that affected at least 860,000 individuals in China in 1995. Morbidity and mortality are dependent on its chronic sequelae, post-schistosomal hepatosplenic disease, which is characterized by liver fibrosis, portal hypertension, ascites accumulation, esophageal varices, and eventual death (Figure 1). The liver fibrosis seen in these patients is provoked by a granulomatous immune response against the eggs that are deposited in the periportal area (14). Schistosomal egg antigen-specific CD4<sup>+</sup> T cells play a major role in the formation of granuloma through Th2 type cytokine production in experimental schistosomiasis mansoni (15,16). However, in humans, little is known about immunology on the chronic phase of hepatosplenic disease (17). Because only 5-10 percent of the chronic patients with *S. japonica* develop hepatosplenic disease and because granulomatous response is derived from CD4<sup>+</sup> T cells reactive to Schistosomal antigen, HLA-class II polymorphism, which controls the reactivity of the CD4<sup>+</sup> T cells, possibly relates to susceptibility to hepatosplenic disease. Indeed, associations of schistosomal hepatosplenic disease with HLA alleles have been reported in *S. mansoni* (18) and in *S. japonica* (19,20).

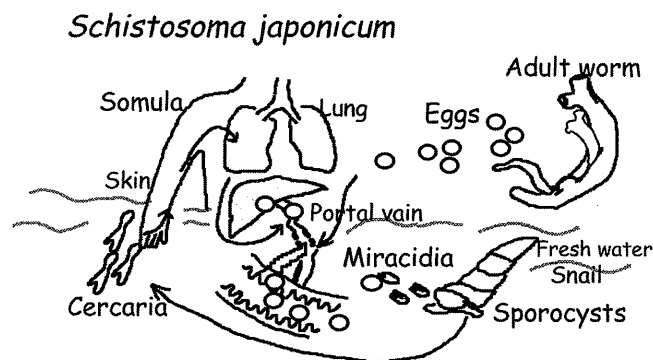


Figure 1. Life cycle of *Schistosoma japonicum*.

Recently, more objective diagnostic methods using ultrasonography have become popular and standardized to measure the changes in liver morphology (21). Therefore, we used this method to categorize the patients into fibrotic group and no fibrotic group and observed their genetic features by the analyses of polymorphism of candidate genes encoding HLA-class II, class I, TNF, and cytokines.

A total of 230 current or former patients with chronic schistosomiasis japonica were examined for liver changes. All patients were from the agricultural village, Beishan, in Yushan county, China, and had their first episode of infection and treatment at least 10 years before this study began. The mean age of the subjects was  $52.6 \pm 10.5$  years and the mean duration after their initial treatment was  $27.4 \pm 8.8$  years. The ultrasonographic diagnosis was carried out according to the World Health Organization standard for the diagnosis of liver fibrosis due to schistosomiasis japonica (21, 22, 23) (Figure 2). Ultrasonographic diagnosis determined that there were 44 persons with grade 0, 81 with grade I, 99 with grade II, and 6 with grade III fibrosis. The presence of Hepatitis B virus (HBV) was not examined in these patients, but the prevalence of HBV positives is about 15 % in the Jiangxi province (24). Most of the men in the village smoke tobacco and drink alcoholic beverages, but the women generally do not. The patients had all been

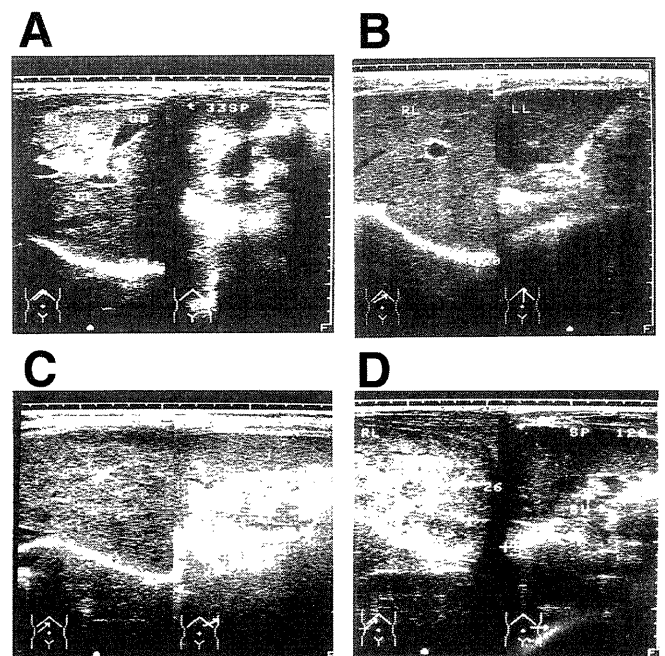
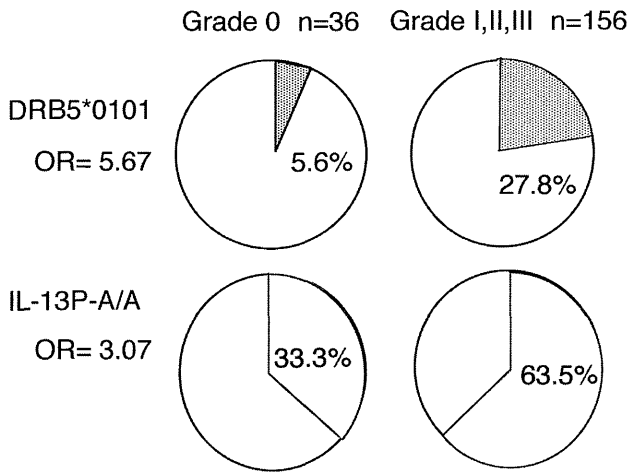


Figure 2. Dignosis of post-schistosomal liver fibrosis and cirrhosis by Ultrasonography.

A; Grade 0. B; Grade I. C; Grade II.

D; Grade III. Showing the fish scale pattern.



**Figure 3.** Associations of HLA-DRB5\*0101 and IL-13P\*A/A with fibrotic group of chronic Schistosomiasis.

treated with praziquantel for each positive fecal examination throughout their lives, but the precise total worm burden of each patient during their clinical course was not available. Therefore, we tentatively defined a person who had a record of repeated treatments for *S. japonica* over a ten-year period as an appropriately exposed person (22).

In total, 29 alleles for HLA-DRB1, 3 for HLA-DRB3, 2 for HLA-DRB5, 13 for HLA-DQA1, 11 for HLA-DQB1, 4 for HLA-DPA1, 18 for HLA-DPB1, and 24 alleles for HLA-B were detected in this population. No significant change occurred in the frequency of the HLA-B types in the two different severities of fibrosis. On the contrary, the frequencies of several HLA-class II alleles significantly increased or decreased in the fibrotic groups. When we compared the frequencies of alleles between grade 0 and grades I, II, and III, we found that HLA-DRB1\*1101 ( $P_c < 0.001$ ), DQA1\*0501 ( $P_c < 0.02$ ), and DQB1\*0301 ( $P_c < 0.03$ ), which are closely linked, significantly increased in grade 0 and that HLA-DRB5\*0101 significantly increased in grade I, II, and III fibrotic patients ( $P_c < 0.03$ ) (Figure 3). This suggests that the HLA-DRB1\*1101-DQB1\*0301-DQA1\*0501 haplotype ( $P_c < 0.02$ ) decreases while the HLA-DRB1\*1501-DRB5\*0101 haplotype ( $P_c < 0.02$ ) increases the susceptibility to grades I, II, and III fibrosis. If we assume that these gene associations are derived from the function of HLA molecules themselves, then the critical question is how do these molecules present the antigens to CD4<sup>+</sup> T cells for initiation of the immunological process leading to fibrosis. So far we have not yet identified the responsible antigen(s) for the stimulation of pathogenic or protective T cells through such HLA-molecules.

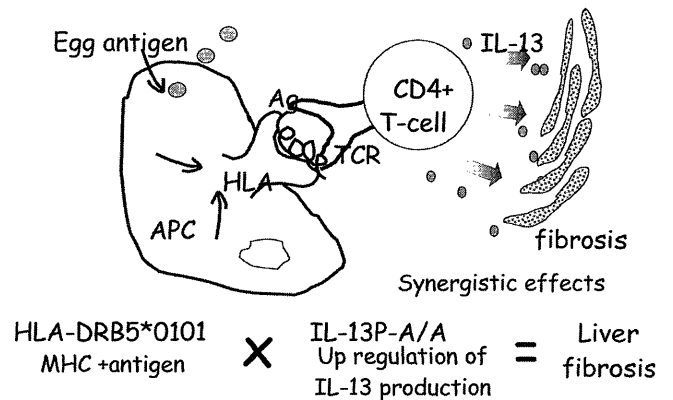
For the same subjects, we have analysed their

**Table 3.** Synergistic effect of the two susceptible markers, HLA-DRB5\*0101 and IL-13P\*A/A.

		Grade 0		Grade I,II,III		P value*	OR*	(95%CI)*
DRB5*	IL-13P	n=36 (%)	n=156 (%)	n=36 (%)	n=156 (%)			
0101	A/A	0 (0.0)	21 (13.4)	p<0.01	24.49	(1.41 – 424.0)		
0101	–	2 (5.6)	18 (11.5)	p<0.05	5.08	(1.08 – 23.9)		
–	A/A	12 (33.3)	78 (49.7)	p<0.05	3.67	(1.64 – 8.17)		
–	–	22 (61.1)	39 (25.0)					

Footnote for Table 3.

\* P value, OR and 95%CI were calculated against individuals negative for both DRB5\*0101 and IL-13P\* A/A



**Figure 4.** Synergistic effect of two susceptible markers suggests the presence of pathogenic CD4 T cells in the liver.

polymorphism of Th2 cytokine genes. Within those, significant association was observed between IL-13 promoter SNPs allele homozygote and liver fibrosis group as shown in Figure 3. Because the IL-13 gene is localized on the long arm of Chromosome 5, IL-13 promoter allele must be independent from HLA-class II allele that is on the short arm of chromosome 6. So the next question was whether there is any interaction between those two genetic markers, HLA and IL-13. As shown in Table 3, both HLA-DRB5\*0101 and IL-13P\*A/A positive persons showed much higher Odds Ratio (24.5) than either positive groups (5.1 for HLA, 3.7 for IL-13P\*A/A) indicating those two genetic markers synergistically enhanced the development of fibrosis after infection. This synergy is well explained by the cartoon of Figure 4 showing that the pathogenic IL-13 producing CD4 T cells are preferentially stimulated by the antigen presenting cells bearing HLA-DRB5\*0101. Further study will be needed to show that this cartoon is not just the cartoon.

## Conclusion

TNF- $\alpha$  promoter allele profoundly influenced the prognosis of cerebral malaria in the adult patients in Myanmar. Synergistic effect of two susceptible markers, HLA-DR and IL-13 promoter alleles, was clearly shown in the development of liver fibrosis of the chronic patients with *Schistosoma japonicum* in China.

## Acknowledgements

Ratawan Ubalee (Nagasaki Univ.), Fumi Suzuki (Saitama Medical), Mihoko Kikuchi (Nagasaki Univ.), Oumaporn Tasanor (Mahidol Univ.), Yupaporn Wattanagoon (Mahidol Univ.), Ronnatrai Ruangweerayut (Mae Sot General Hosp), Kesara Na-Bangchang (Thammasat Univ.), Juntra Karbwang (WHO/TDR), Akinori Kimura (Tokyo Med. Dent. Univ.), Kyogo Itoh (Kurume Univ.), Tozo Kanda (St.Marianna Med. Col.), contributed to malaria study. Chen Honggen, Yin Tong, Gu Xiaonan, Zhang Shaoji, (Jiangxi Provinc. Inst. Parasitic Dis.), Mihoko Kikuchi (Nagasaki Univ.), Liu Jianxiang and Yuan Hongchang (Shanghai Medical University) contributed to the Schistosomiasis study.

This work was supported in part by the Grants-in-aid for Scientific Research from the Ministry of Education, Sports, Science and Culture, Japan, (08281104, 08044316, 09470071, 10044317), from Ministry of Health and Welfare (H12-SHINKO-18), and by the Maruki Memorial Research fund (A) of 1998 from Saitama Medical School.

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