Study on the association of the polymorphism of HLA-II gene with leukemia

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Abstract. We explored the association between the HLA-II gene polymorphisms and the occurrence of leukemia. For this study, we selected 53 patients with leukemia treated at Zhongnan Hospital of Wuhan University from February 2014 to September 2015 and 46 healthy patients as the control group. We used polymerase chain reaction with sequence specific primers for DNA typing which was carried out to analyze the patients HLA-A/B gene polymorphism. We also used enzyme-linked immunosorbent assay and western blotting method to measure the protein expression of different genotypes and activity. Compared to the control group, HLA-A04, B08 gene frequencies were significantly lower than those of HLA-A04, B08 gene frequencies of the observation group; results were statistically significant (χ^2=16.28, P<0.05; χ^2=16.47, P<0.05). However, in the control group, the frequency of HLA-A09 gene was significantly higher than that of the observation group; there was a significant difference between the two groups (χ^2=15.28, P<0.05). Through the measurement of the protein expression levels of the different genotypes in the control group and the observation group, it was found that in the observation group, HLA-A04, B08 protein contents (4.6 and 3.2 μg/l) were significantly higher than those of the control group (0.13 and 0.1 μg/l). While the control group HLA-A09 genotype protein content (3.7 μg/l) was significantly higher than that of the observation group (0.2 μg/l); there were significant differences between both (P<0.05). Therefore, there is a significant correlation between HLA-II gene polymorphism and leukemia that is higher than HLA-A04 and B08 gene frequency and can help promote the occurrence of leukemia. The higher frequency of HLA-A09 gene can help to suppress the occurrence of leukemia.

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Introduction

As one of the tumors with the highest incidence in China, the related research results show that (1) at present, the incidence of leukemia is increasing year by year (2). Currently, there is a high incidence of leukemia, especially acute leukemia, which is a cancer that progresses rapidly (3). The current mechanism and cause of the pathogenesis of leukemia is not clear, which is the reason why there are no specific drugs available for leukemia (4). In recent years, with the development of molecular biology techniques, increasing research shows that abnormal expression of some human genes (e.g., HLA and other immune regulation related genes) are correlated to the incidence of leukemia (5). Results of this study show that the HLA gene located on human chromosome 6 is related to many diseases of the human body (6). For example, in children with acute lymphoblastic leukemia (7), HLA-DQA*101 and *014 in children increased significantly compared to normal. At the same time, a study on the correlation between the chronic myelogenous leukemia (CML) and HLA showed that (8) there was a correlation between the incidence of CML and the abnormal increase and the decline of HLA antigen frequency (9). The abnormal changes of HLA-B8, -B14, -DR3 and other antigen frequencies greatly promotes the occurrence and progression of CMNL (10). Currently, the research on the HLA gene polymorphism is mainly focused on HLA-1. There are few studies on the polymorphism of HLA-II gene, especially the association between HLA-II gene polymorphism and leukemia. In this study, we investigated the correlation between HLA class II gene polymorphism and leukemia for the first time in order to reveal the correlation between HLA class II gene polymorphism and acute leukemia that may exist in order to provide certain theoretical and experimental basis for leukemia diagnosis and treatment.

Materials and methods

Sample selection. For this study, we selected 53 patients with leukemia from February 2014 to September 2015 at Zhongnan Hospital of Wuhan University as the research subjects. This cohort included 26 males and 27 females with an average age of 24.3±18.2 years. We also selected 46 healthy persons as the control group, including 23 males and 23 females, with

an average age of 22.6±16.7 years. This study was approved by the Ethics Committee of Wuhan University. Signed written informed consents were obtained from all participants before the study. Inclusion criteria: Patients must be diagnosed with leukemia based on cell morphology and immune cell typing. Exclusion criteria: Patients with other related diseases such as other types of cancer and patients suffering from other immune deficiency diseases such as AIDS.

Main instruments and reagents. The main instruments used were PCR, refrigerated centrifuge and gel imaging instrument (all from Bio-Rad Laboratories, Inc., Hercules, CA, USA). The main reagents were DNA extraction and enzyme-linked immunosorbent assay (ELISA) kits (both from Qiagen, Hilden, Germany), and HLA goat anti-mouse first antibody (cat. no. ab6789; dilution, 1:1,250; Abcam, Cambridge, MA, USA) and PCR reagents (Takara Bio, Dalian, China), SSO-HLA gene type kit (Dynal, Wirral, UK).

Method

Genome extraction. We extracted 5ml of peripheral blood from both patients in the observation and control group, centrifuged at 2,750 x g for 10 min, and added EDTA to the supernatant and stored at -80˚C. Using Qiagen's DNA extraction kit extract, and in accordance with the instructions, we extracted cell genome from the patients' blood. Genomic DNA concentration was measured using micro-UV points spectrophotometric gauge (Biosharp, Hefei, China). The DNA purity was A260/280=1.8-2.0, then the extraction of genomic DNA was used in the following experiments.

Genotyping. In this study, different genotypes were classified using polymerase chain reaction with sequence specific primers (PCR-SSP) kit (KeyGen, Nanjing, China). The primers were synthesized by the Shanghai Biological Engineering Co. Ltd. (Shanghai, China). Primer sequences are shown in Table I. HLA-II genotypes with high polymorphism were obtained by different primers amplification, and then the PCR product was heated to 95˚C in the PCR instrument. The single stranded DNA was obtained by unzipping double-stranded DNA, and then adding it to the nylon membrane labeled with a specific nucleotide fragment. The single stranded DNA and specific nucleotide fragments fused for double stranded DNA through complementary pairing between base groups. Then, streptomycin labeled HRP was added, and reacted at room temperature for 2 min. Nylon membrane after hybridization was then treated with color in the gel imaging instrument, and the HLA-II poly-morphism was identified by the hybridization bands.

ELISA. Total protein extracted from different genotypic samples and control samples were studied. The expression of HLA-II protein in different samples was determined according to the specification of ELISA kit (Qiagen). In the study, protein samples for the ELISA standard curve were diluted by the elution buffer at a ratio of 1:50. After diluted solution of different concentrations were obtained and according to the specification of the operation, the standard curve was produced. In samples of the observation and the control group, PBS (pH 7.2), through the sterilization, was diluted with a scale of 1:200. A 100 μl of solution was taken and added into 96-well plates. Fifty microliters of test solution was added to each well, and after incubation for 2 h at room temperature, the TMB color substrate was added. The absorbance at 495 nm was measured and then the protein expression and concentration of HLA-II in each sample were calculated according to the standard curve.

Table I. Primer sequences.

Primer	Sequences
HLA-A-F	GTGATCGTAGTGCTAGCTAGC
HLA-A-R	CGTCGTAGCTGATCGTAGCTAG
HLA-B-F	CTGCTAGTCGATCGGCATATACG
HLA-B-R	CTGATCGTAGCTAGCCTAGATCG

F, forward; R, reverse.

Western blotting experiment. The total protein extraction from each subject was the experimental material. Approximately 150 mg of the frozen (-80˚C) sample was rapidly ground in an EP tube in a mortar containing liquid nitrogen. Then, we added 300 μg protein extraction and 10 μl protease inhibitors, and then incubated in ice water for 30 min. We centrifuged the sample at 10,000 x g for 150 min, collected the supernatant and analyzed total protein extraction. Secondly, the expression of protein was detected by western blotting, a mix of 10 μl supernatant was obtained and the sample buffer for conventional SDS-PAGE and electrophoresis were prepared. The membrane was incubated at room temperature for 2 h with the HLA-II protein antibody (1:250, 4˚C overnight). Then, it was washed with TBST 5 times, each time for 10 min, and incubated with the horseradish peroxidase-labeled secondary antibody (cat. no. ab6789; dilution, 1:1,250; Abcam; 60 rpm incubated with shaker for 1 h at room temperature). The membrane was washed 3 times, each time for 10 min, and then the color was shown by diaminobenzidine, and the image was obtained by the Fluorchem 9900 imaging system (Alpha Innotech, San Leandro, CA, USA). We determined the integral optical density of color belt of the protein, and the relative content of HLA-II protein was calculated.

Statistical analysis. SPSS 20.0 software (IBM SPSS, Armonk, NY, USA) was used for data statistical analysis. The experimental data were expressed by mean ± standard error. The single factor analysis method was used to analyze the data between different groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HLA-A gene polymorphism and genotype frequency detection in the observation group and the control group. Based on the HLA-A alleles and gene frequency detection in the observation and the control group, a total of 12 loci were found. Results are shown in Table II. Compared to the control group, 53 cases of leukemia patients had higher gene frequency of HLA-A04;

Table II. HLA-A allele and gene frequency statistics of the observation group and the control group.

	Observation group (53)		Control group (46)		
HLA-A	No.	Frequency (%)	No.	Frequency (%)	χ^2
A01	2	3.8	1	2.2	6.04
A02	2	3.8	3	6.5	0.12
A03	3	5.7	1	2.2	0.18
A04	26	49	3	6.5	0.24
A05	3	5.7	4	8.7	0.64
A06	3	5.7	1	2.2	0.116
A07	1	1.8	1	2.2	0.19
A08	3	5.7	2	4.3	0.13
A09	2	3.8	26	57	4.07
A10	2	3.8	1	2.2	0.26
A11	3	5.7	1	2.2	0.32
A12	3	5.7	2	4.3	0.48

P<0.05 indicates significant difference.

Table III. HLA-B allele and gene frequency statistics of the observation group and the control group.

	Observation group (53)		Control group (46)		
HLA-B	No.	Frequency (%)	No.	Frequency (%)	χ^2
B01	1	1.9	2	4.3	0.14
B02	2	3.8	2	4.3	0.116
B03	6	11.3	3	6.5	0.32
B04	3	5.7	2	4.3	0.21
B05	8	15.1	4	8.7	0.204
B06	6	11.3	5	10.9	0.165
B07	2	3.8	4	8.7	0.186
B08	7	13.2	13	28.3	6.05
B09	5	9.4	3	6.5	0.214
B10	3	5.7	2	4.3	0.24
B11	4	7.5	3	6.5	0.253
B12	2	3.8	1	2.2	0.32
B13	2	3.8	1	2.2	0.36
B14	2	3.8	1	2.2	0.42

P<0.05 indicates significant difference.

differences were significant (P<0.05). The frequency of the HLA-A09 gene was lower, and the frequency of the gene had a significant difference from the control group (P<0.05). A06, A12, A08 loci gene frequencies were slightly higher than those of the control group, but results were not significantly different (P>0.05). A02 and A05 were slightly lower than those of the control group, however, there was no significant difference between the two proteins (P>0.05).

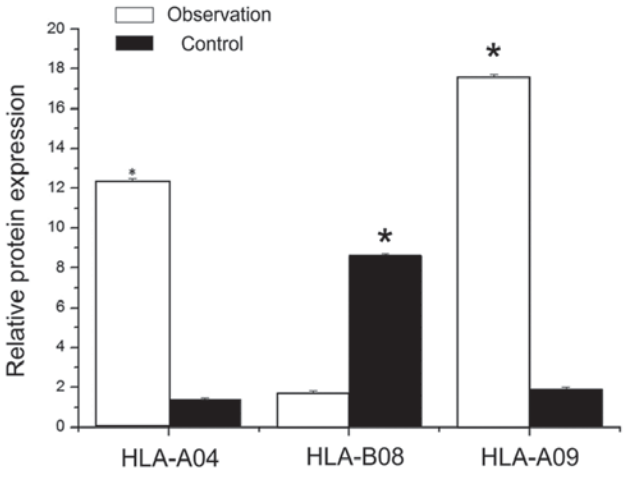

Figure 1. ELISA to detect the expression of HLA-II protein in different genotypes. Asterisk shows significant difference.

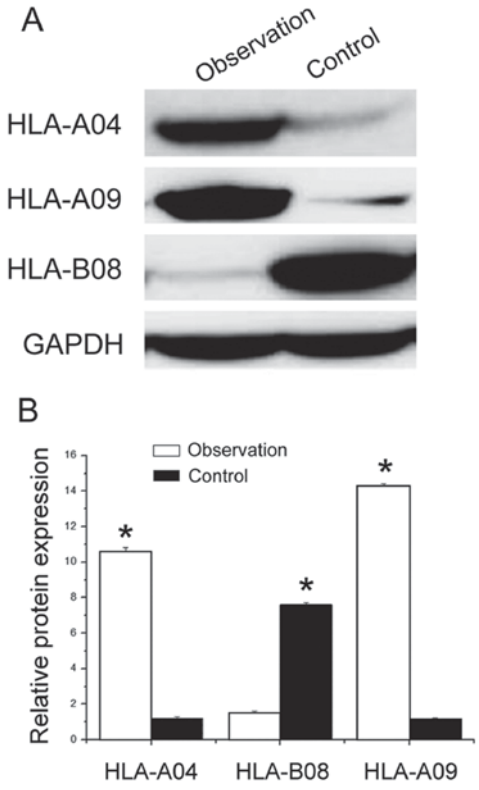

Figure 2. Western blotting to detect the expression of HLA-II protein in different genotypes (A/B). (A) Western blot lanes for two groups; (B) semi-quantitative analysis for western blot results. Asterisk shows significant difference.

HLA-B gene polymorphism and genotype frequency detection in the observation group and the control group. Based on the HLA-A alleles and gene frequency detection in the observation and the control group, a total of 14 loci was found. Results are shown in Table III. Compared to the control group, in 53 cases of leukemia patients, the HLA-B08 gene frequency was lower; results were found to be significantly different from the control group (P<0.05). B03, B05 and B11 loci gene frequencies were slightly higher than those of the control group, but there was no significant difference (P>0.05). B01 and B07 were slightly

lower than those of the control group; there was no significant difference between the two proteins (P>0.05).

ELISA to detect the expression of HLA-II protein in different genotypes. The total protein extraction of patients in the control group as well as the observation group were analyzed. The expression of HLA-II protein in different genotypes was determined by ELISA method, and the results are shown in Fig. 1. Compared to the control group, the HLA-A04 genotype protein content (12.3±0.2 μg/l) in the observation group was significantly higher than HLA-A04 protein content in the control group (1.4-0.1 μg/l); there were no significant differences between the two groups (P<0.05). In the observation group, the HLA protein content (1.7±0.13 μg/l) was significantly lower than HLA-B08 protein content in the control group (8.6±0.12); there were significant differences between the two (P<0.05). The content of HLA-09 protein in the observation group (17.6±0.11 μg/l) was significantly higher than HLA-09 protein of the control group (1.9±0.12 μg/l), and there was a significant difference between the two groups (P<0.05).

Western blotting to detect the expression of HLA-II protein in different genotypes. The total protein extracted from patients in the control group as well as the observation group was investigated. The expression of the HLA-II protein in different genotypes was determined by western blotting; the results are shown in Fig. 2. The content of the HLA-04 protein in the observation group was significantly higher than that in the control group; there were significant differences between the two (P<0.05). In the observation group, the content of HLA protein in HLA-A09 was significantly lower than that in the control group, and there was a significant difference between the two (P<0.05). Whereas, the content of HLA protein in the HLA-B08 genotype in the observation group was significantly higher than that in the control group, and there were significant differences between the two (P<0.05). These results are in agreement with the ELISA results.

Discussion

In recent years, research has shown that (11) the HLA gene is located on the human sixth chromosome. It is related to a complex gene regulation and control system in the human body (12). Since the 1990s, research on (13) HLA alleles in the human body (14) and HLA related diseases (15,16) in chronic myeloid leukemia has shown that the frequencies of HLA-A32, B27, B45 and B44 were significantly higher than those in the control group (17). The HLA-A32 gene frequency was lower compared to the control group, and with further study of the HLA-II genotype, it was found that HLA-II genotype in the human body is not only involved in an external immune response of the body, but also has an important role in the promotion of the maturation of lymphocytes and other immune cells *in vivo* (18).

As a type of genetic disease that is very harmful, the diagnosis and treatment of leukemia has been paid increasing attention, but because of the complexity of leukemia (19), its incidence is often affected by the external environment and the genetic factors. Therefore, there is no cure for leukemia. Early diagnosis and treatment of leukemia has become the key to the treatment and prevention of leukemia (20). In this study, we found associations between different polymorphisms of the HLA-II gene polymorphism and leukemia. Particularly, the polymorphisms of HLA-A04, A09 and B08 are associated with the occurrence and suppression of leukemia to a large extent; HLA-A04 and HLA-A09 can promote the occurrence and progression of leukemia, and HLA-B08, to a certain extent, can inhibit the occurrence and progression of leukemia through the detection of different genotypes of HLA protein activity. The HLA activity of HLA-A04 and HLA-A09 significantly increased, which showed that the above mutations promote the deterioration of leukemia by promotion of the activity of HLA. However, due to the experimental conditions and the time limit, this study did not analyze the spatial structure of protein of different genotypes from the aspects of protein structure, or reveal the potential relevance between different genotypes and protein spatial structure.

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