ORIGINAL ARTICLE

CTLA-4 +49A/G And -318 C/T Polymorphisms and Cytokines Level on Inhibitors Development of Haemophilia A among Different Ethnicity in Malaysia

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ABSTRACT

Introduction: Haemophilia A (HA) is an inherited X-chromosome recessive disorder characterized by the deficiency of factor VIII (FVIII). About 25-30% of HA patients which received FVIII concentrate developed inhibitors towards FVIII. Ethnicity has been associated as a predisposing factor for the formation of FVIII inhibitors. This study characterizes the polymorphism of +49A/G and -318C/T of Cytotoxic T-Lymphocytes Antigen 4 (CTLA-4) which relates to the development of inhibitors among Malaysian HA patients of Malay, Chinese and Indian ethnicity. Cytokines level of tumour necrosis factor alpha (TNFα), interleukin 4 (IL-4) and interleukin 10 (IL-10) were also measured to assess the link to inhibitors development. Method: Severe HA patients with and without inhibitor who were being treated at the National Blood Centre, Kuala Lumpur were recruited and consented where their collected blood was genotyped for both polymorphisms using the Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) and their cytokines levels were measured using the Enzyme-linked Immunosorbent Assay (ELISA). Results: Analysis of the total 64 respondents who fulfilled the study criteria found polymorphisms of CTLA-4 +49A/G and CTLA-4 -318C/T were not significantly different among patients with and without inhibitors. However, HA patients without inhibitors revealed that the Chinese population exhibited a higher +49G allele which is protective towards inhibitors development. Indian patients expressed a higher level of inhibitors titre. Patients without inhibitors showed statistical differences between ethnicity for both genotypes and allele frequencies of CTLA-4 +49A/G ($P < 0.05$). Conclusion: The mixed pattern of polymorphisms and cytokine profiles were observed in multi-ethnicity. This finding requires further verification with a larger sample size involving multicenter for further confirmation.
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INTRODUCTION

Haemophilia A (HA) is an inherited disorder resulting from an X-linked recessive trait that occurs in males and homozygous females (1). Current epidemiology estimates that 1 in every 5000 males has a prevalence that varies among the different parts of the world, with a range of 5.4 - 14.5 cases per 100,000 males (2). The prevalence of HA cases in Malaysia is about 6.6 per 100,000 males (3). The World Federation of Haemophilia (WFH) reported that Malaysia has 950 registered HA patients out of the 31.9 million population (4). Patients with HA develop spontaneous bleeding resulting from abnormal thrombin generation due to the deficiency of coagulation Factor VIII (FVIII) which causes the formation of weak and unstable clots (5). The symptoms of intramuscular and recurrent bleeding into a joint cavity or haemarthrosis are among the medical emergency of HA (6). Coagulation profile with the FVIII assay measurement is the diagnostic approach for HA (1). The severity of the disease depends on the FVIII concentration where plasma FVIII levels of 0.01 IU/mL (<1%) is classified as severe HA. (7, 8).

Replacement therapy using FVIII concentrate derived from either human blood plasma, recombinant forms, or a hybrid form of both is the gold standard (1, 9).

Unfortunately, about 25-30% of severe HA patients who received the replacement therapy developed neutralizing antibodies called inhibitors (10, 11). This complication ultimately prevents the patient from receiving safe and effective care which leads to a higher risk of morbidity and mortality rate (12).
Both genetic and non-genetic factors have been identified in stimulating the development of FVIII inhibitors in HA patients (13). Genetic factors such as the mutation of FVIII gene, MHC genes, immune response genes and non-genetic factors such as ethnicity variation, family history, age of treatment onset, type of treatment and mode of treatment were discussed in various studies (14).

CTLA-4 is a protein expressed on an activated T cells which is a down regulator by binding with the B7 molecules on the antigen-presenting cells (APC) (15). Studies suggested that CTLA-4 antigen blockade by antibodies enhances T cell proliferation and B cell activation (16, 17). The secretion of anti-FVIII is a T cell-dependent process which involves a series of immunological action initiating with antigen presentation, T-lymphocytes activation, cytokines secretion (pro-inflammatory cytokines: IL-2, TNFα, IFNɣ and down-regulatory cytokines: IL-4, IL-5, IL-10) and antibody secretion by plasma cell (18). CD28 of T-lymphocytes binds with CD80 and CD86 in antigen presenting cells as co-stimulatory signals which fully activates the Th-lymphocytes and subsequently releases cytokines to signal and induce B-cell proliferation, differentiation and finally antibody secretion. As a down regulatory event, the CTLA-4 antigen of T-cell competitively binds with the CD80 and CD86 to suppress the activation and eventually inhibits the antibody production (19). Several studies on single nucleotide polymorphisms (SNPs) of CTLA-4 gene at position -318C/T and +49A/G have been found to modulate the immune response in autoimmune disease such as Grave's Disease, Systemic Lupus Erythematosus, Hashimoto's Thyroiditis, Multiple Sclerosis and Wegener's Granulomatosis (20-24).

Inhibitors development involving the interaction of pro-inflammatory and down regulatory immune cytokines is the complex series which is not clearly understood. A study conducted in the past revealed mixed immune response profiles (25-27). The present study was executed to further understand the involvement of CTLA-4 gene polymorphisms and cytokines level of tumour necrosis factors (TNFα), Interleukin 4 (IL-4) and Interleukin 10 (IL-10) in association with inhibitors among severe HA patients in the multi-ethnic population of Malaysian.

MATERIALS AND METHODS

Study Population

This study was approved by the Medical Research Ethics Committee of the Ministry of Health Malaysia (NMRR) [Ref No: NMRR-15-666-24000 (IIR)]. Severe haemophilia A patients who attended National Blood Centre (NBC) for various medical reasons during the study period were enrolled. Some of the patients were contacted via phone call and recruited to join the study. Severe HA patients who fulfilled the study criteria were consented and included in this study. The inclusion criteria includes Malaysian, male patients with parents of the same ethnicity, severe HA patients with or without inhibitors receiving treatment in NBC. Non-Malaysian patients with moderate or mild HA, haemophilia B patients and patients with incomplete data were excluded. During the study period, most of the patients were receiving factor FVIII concentrate. Generally, a minority of the patients might be receiving recombinant factor concentrate depending on their inhibitor level and clinical response to the given treatment. However, the type of factor concentrate which the patients had received during the study period was not considered in the current study.

Sample Size Calculation

The sample size for this study was calculated based on the CTLA-4 gene polymorphisms and the formula was adapted from Designing and conducting health surveys: a comprehensive guide (2006). The initial calculated sample size was 266 with 133 with and 133 without inhibitors respectively. Potential respondents were contacted from NBC registered list but some are uncontactable and the majority of the respondents unable to join as they out of Klang Valley. Eventually 47 blood samples were collected. However, due to the low sample number of respondents, an additional 17 stored DNA samples from NBC were included in the study.

Sample Collection and Processing

Three (3) ml whole blood was collected in the ethylenediaminetetraacetic acid (EDTA) tubes from 47 patients who were subjected for the DNA extraction using QIAamp DNA blood kit manufactured by Qiagen, Germany. The remaining blood samples after the DNA extraction was spun at 3000rpm for 10 minutes and stored at -80°C for cytokines measurement.

Genotyping

The PCR-RFLP method was used to detect single nucleotide polymorphism (SNP). PCR was first performed to obtain the expected band of 162bp and 247bp for +49A/G and -318C/T respectively. The total volume of master mixture for PCR was 20ul where the cocktail consisted of forward and reverse primers respectively and DNA sample (10ng). Primers for +49A/G were F: 5′ GCTCTACTTCCTGAAGACCT 3′, R: 5′ AGTCTCACTCACCTTTGCAG 3′ and for -318C/T were F: 5′ AAATGAATTGGACTGGATGGT 3′, R: 5′ TTACGAGAAAGGAAGCCGTG 3′. The PCR test was performed to quantitatively amplify the DNA sample by following the PCR protocol of pre-denaturation (94°C) for 5 minutes, denaturation (94°C) for 45 seconds, annealing (60°C) for 45 seconds, extension (72°C) for

1 minute and final extension (72°C) for 10 minutes where the PCR was run for 35 cycles. Then, all the PCR products were further digested with the restriction enzyme. The restriction enzyme was obtained from New England Biolabs (NEB), England. The cocktails consist of restriction enzyme (10,000 units/ml), buffer and 5ul of PCR product. The mixture was incubated for 90 minutes at 37°C for digestion to take place. Fnu4HI enzyme digests 162bp of +49A/G into 89bp and 72bp while the *Mse I* enzyme were used to digest 247bp of -318C/T into 115bp and 131bp (15, 28, 29). The samples were then processed through gel electrophoresis in 4% agarose gel at 50 volts to observe for the presence of the expected bands.

Cytokines Measurement

Interleukin 4 (IL-4), Interleukin 10 (IL-10) and tumour necrosis factor alpha (TNFα) were measured using the Fine Test Elisa kit manufactured by the Wuhan Fine Biological Technology Co., Ltd, China. The procedure of ELISA testing was carried out in accordance with the protocol provided by the manufacturer. The cytokines concentration was measured in picograms per millilitre, pg/mL. All values below the detection levels were considered to be undetectable and were valued as 0.

Statistical Analysis

The analysis of data was carried out using the Statistical Package for the Social Sciences (SPSS) version 23.0. Statistical measurement tools were selected based on the distribution of data. The statistical tests such as Pearson Chi-Square test, Independent T test, Kruskal-Wallis and Mann-Whitney U tests were used for analysis. Values were presented as mean± SD, median (IQR) or percentage as appropriate. A p-value of less than 0.05 was considered to be statistically significant.

RESULTS

Demographic Details

A total of 64 severe HA patients; 32 with inhibitors and 32 without inhibitors who have sought treatment at Haemophilia Clinic, NBC, Kuala Lumpur were studied. The mean age of HA patients with inhibitors and without inhibitors was 25.33 and 28.02 years respectively. The majority of the HA patients with inhibitors (40.6%) were in the age range of 0 to 14 years old. Whereas the majority of the HA patients without inhibitors (46.9%) were aged between 25 to 44 years. The ethnicity distribution among HA patients with inhibitors and without inhibitors found that the majority of HA patients were Malays (68.8% vs 62.5%) followed by Chinese (18.8% vs 34.4%) and Indians (12.5% vs 3.1%). Among the HA patients with inhibitor, almost half (46.9%) had high titre inhibitors (≥5 BU) and the remaining respondents (53.1%) had low titre inhibitors. Ethnicity comparison with inhibitors titre showed that Indian patients expressed a higher level of inhibitors titre followed by Malays and Chinese (IQR: 127.55BU/mL vs 47.20BU/mL vs 11.45BU/mL).

Genotyping of CTLA-4 +49 A/G and CTLA-4 -318 C/T

Genotype, allele frequencies and ethnicity distribution of CTLA-4 +49 A/G polymorphism are shown in Table I & II. Heterozygous genotypes A/G were present in 59.4% of patients with inhibitors and 62.5% of patients without inhibitors. Homozygous G/G was found in 40.6% of those with inhibitors and 37.5% in those without inhibitors. Patients with inhibitors had the highest frequency of the G allele (70.3%) compared to patients without inhibitors (68.7%). Heterozygous A/G genotype was observed in 54.5% of Malays, 66.7% of Chinese, and 75.0% of Indians patients with inhibitors. Meanwhile, among severe HA patients without inhibitors, Malay patients had the highest frequency of heterozygous A/G genotype (85.0%) in contrast to Chinese patients who had the highest frequency for homozygous G/G genotype (81.8%). Patients without inhibitors showed statistical differences among ethnicity for both genotypes and allele frequencies ($P < 0.05$). Among patients with inhibitors, homozygous G/G expressed higher inhibitors titre compared to heterozygous A/G (51.00BU/mL vs 25.20BU/mL) but the difference was not statistically significant ($P = 0.176$). For the -318 C/T, all patients with inhibitors had homozygous C/C and 96.9% of patients without inhibitors had C/C. One patient without inhibitors had heterozygous C/T of 3.1%. There were no statistical differences found in the genotype and allele frequencies between patients with and without inhibitors ($P > 0.05$). Genotype prevalence among HA patients with and without inhibitors showed that all three ethnicities had homozygous C/C suggesting -318 C/T is conserved.

Cytokines

TNFα and IL-4 cytokines were successfully analyzed in 46 samples. Meanwhile, IL-10 cytokine was only measured on 44 samples due to insufficient plasma. The cytokines results were statistically analyzed using different variables such as inhibitors status, age group, inhibitors level, overall ethnicity among patients with

Table I: Genotype and Allele distributions of CTLA-4 +49A/G gene polymorphism in severe HA patients with and without inhibitors

	With Inhibitors (N=32) n (%)	Without Inhibitors (N=32) n (%)	Odd Ratio (95% Confidence Interval)	P Value
Genotype:				
A/G	19 (59.4)	20 (62.5)	1.140 (0.417±3.115)	0.798
G/G	13 (40.6)	12 (37.5)		
Alleles:				
A	19 (29.7)	20 (31.3)	1.077 (0.507±2.286)	0.848
G	45 (70.3)	44 (68.7)		

P value were evaluated by Pearson Chi-Square Test

Table II: Genotype and allele distributions of CTLA-4 +49A/G gene polymorphism in severe HA patients with and without inhibitors based on ethnicity

Inhibitors Status	Ethnicity	Genotypes		P value	Alleles		P value
		A/G	G/G		A	G	
With Inhibitors	Malay	12 (54.5)	10 (45.5)	0.687	12 (27.3)	32 (72.7)	0.817
	Chinese	4 (66.7)	2 (28.6)		4 (33.3)	8 (66.7)	
	Indian	3 (75.0)	1 (33.3)		3 (37.5)	5 (62.5)	
Without Inhibitors	Malay	17 (85.0)	3 (15.0)	**0.001***	17 (42.5)	23 (57.5)	**0.021***
	Chinese	2 (18.2)	9 (81.8)		2 (10.0)	20 (90.0)	
	Indian	1 (100.0)	0 (0.0)		1 (50.0)	1 (50.0)	

Data were evaluated by Pearson Chi-Square Test
Note: * P value<0.05

and without inhibitors.

Patients with low inhibitors level of <5 BU/mL portrayed a higher level of TNFα with an average of 263.32 pg/mL compared to patients with inhibitors level of ≥5 BU/mL of 177.58 pg/mL (Figure 1) where there was no significant association (P = 0.053). Similar findings were observed when comparing among the three ethnicities even though the Indian patients expressed higher levels of TNFα followed by Chinese and Malay patients (Mean: 353.80 pg/ml vs 249.00 pg/ml vs 211.47 pg/ml) (Figure 2).

Contrast to patients without inhibitors, patients with inhibitors had a higher IL-4 concentration (IQR: 439.30 pg/ml vs. IQR 256.20 pg/ml). The highest level of IL-4 was found among patients in the age group of 0 - 14, with an average of 495.60 pg/ml. Patients with inhibitor levels of <5BU/mL (Figure 1) had the highest IL-4 levels (IQR of 1678.65 pg/mL). Ethnicity comparison among patients with inhibitors (Figure 2) showed that Malay patients had the highest levels of IL-4 followed by Chinese and Indian patients of (IQR: 974.45 pg/ml vs 282.00 pg/ml vs 0.00 pg/ml). The ethnicity comparison was statistically significant (P < 0.035).

Severe haemophilia A patients with inhibitor levels of ≥5 BU/mL had higher levels of IL-10, with an average of 414.5 pg/mL compared to 125.41 pg/mL in patients with inhibitor levels of <5 BU/mL (Figure 1). Ethnicity comparison among these patients with inhibitors showed higher levels of IL-10 found in Indian followed by Malay and Chinese patients (592.00 pg/mL vs 157.33 pg/mL vs 109.53 pg/mL) but yet these were statistically not

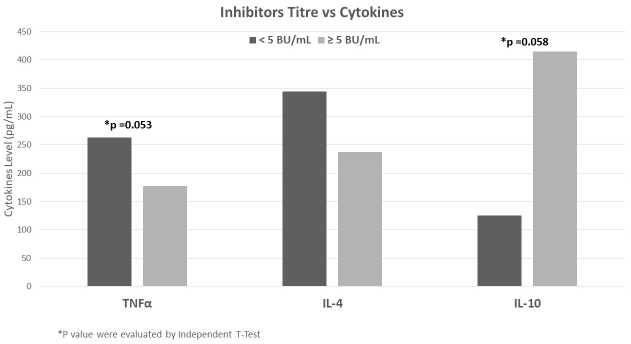

Figure 1: Patients with inhibitors titre against cytokines level of TNFα, IL-4 and IL-10

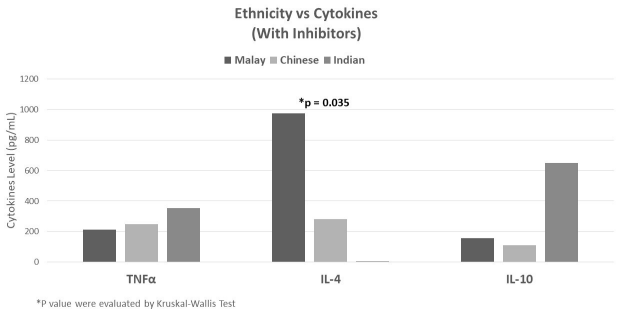

Figure 2: Ethnicity of patients with inhibitors against cytokines level of TNFα, IL-4 and IL-10

significant (P = 0.068). Although patients with inhibitors expressed a higher level of IL-10 compared to patients without inhibitors (IQR 203.90 pg/mL vs IQR 37.10 pg/mL), there was no significant association found (Figure 3).

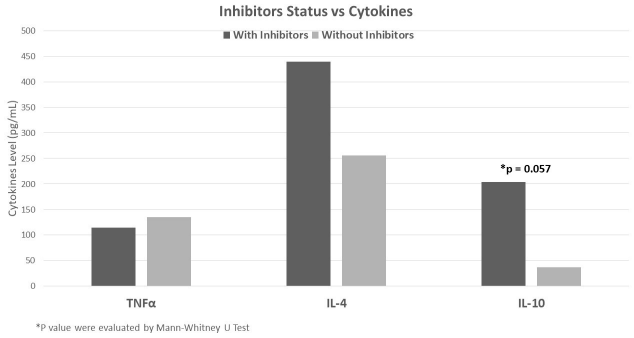

Figure 3: Patient's inhibitors status against cytokines level of TNFα, IL-4 and IL-10

DISCUSSION

Malaysia is one of the countries which is known to have a multi-ethnic Asian population. The total population in Malaysia is estimated to be 32.7 million and is represented by the major ethnic groups of Bumiputra; 69.6% (Malays and indigenous people), Chinese; 22.6%, Indians; 6.8% and other minor ethnic groups; 1.0% (30). In addition, the population in the state of Selangor alone is estimated to be 6.3 million (31). During the recruitment phase the National Reference Centre for haemophilia was positioned at the NBC which is located at the centre of the federal territory of Kuala Lumpur. Registered HA patients from all over the country were referred to the NBC for registry and as the main treatment site besides being managed by various state hospitals. This study was aimed to characterize the association of CTLA-4 gene polymorphisms at +49A/G and -318C/T and cytokines profile of IL-4, IL-10 and TNFα with inhibitors development among Malaysian with severe HA. Limited studies were reported in the past on CTLA-4 gene polymorphisms which created limitations for comparison.

This study demonstrated a higher frequency of +49A/G genotypes and G alleles among both the patients with and without inhibitors respectively (59.4% vs 62.5% & 70.3% vs 68.7%). A significant finding ($P < 0.021$) was observed in the ethnicity comparison among patients without inhibitors, where the Chinese ethnic group expressed a higher frequency of +49G/G genotype and G allele values of 81.8% and 90.0% respectively. This may suggest that +49G allele among the Chinese ethnic is a protective mechanism to inhibitors development. In agreement with the current finding, a study among the Chinese population showed that A/G and G/G genotypes were more frequently observed in both groups and the G allele being higher in occurrence (32). An increase in the frequency of +49A/G genotype is also in agreement with the study conducted by J.Astermark (15). His study reported a higher level of G allele for the inhibitors group while A allele was greater among the group without inhibitors. However, no association was linked with inhibitors development.

A study conducted among Iraqi Kurdish involving 126 patients showed significant increase inhibitors risk for the +49A/G G allele among inhibitors (33). Other studies with a greater sample size showed a lower trend of +49G/G and higher frequency of A allele in both groups (34-36). However, the current study failed to prove that +49A/G gene polymorphisms influenced the inhibitor's development, but on the ethnicity it suggests that +49G allele could be a protective polymorphism among the Chinese patients which prevents inhibitors development.

CTLA-4 -318 C/T polymorphism in this study expressed the highest frequency of C/C among patients with and without inhibitors group (100.0% vs 96.9%) with the C allele of 100.0% and 98.4% respectively. Occurrence of the C/C genotype indicates that there were no genetic mutations that took place in this study group. A few studies however did report the presence of C/T polymorphism among patients with and without inhibitors with low frequency of T allele but the study did not prove the association of polymorphism and inhibitors formation (32, 33, 34, 36, 37). Interestingly J. Astermark reported the presence of C/T polymorphisms among Swedish patients and significantly ($P < 0.012$) the T allele promotes inhibitors development among HA patients (15).

Pro-inflammatory cytokines of TNFα and down regulatory cytokines of IL-4 and IL-10 were also measured in this study. TNFα had a similar value among both groups of severe HA patients but IL-4 and IL-10 cytokines expressed a higher concentration among severe HA with inhibitors, particularly the increase of IL-10. Further comparison with inhibitors levels showed an increase in IL-4 and IL-10 among those patients with inhibitors level of ≥5BU/ml but in contrast, TNFα concentration was increased in patients with inhibitors level of <5BU/ml ($P = 0.053$). This finding seemed to be associated with the presence of inhibitors and the titre level. Several studies on cytokines measurement indicated that mixed cytokines profile patterns of IL-4, IL-10 and TNFα eventually led to a poor understanding of cytokines involvement (25-27). In fundamentals of immunology, immune cytokines act as a mediator in the immune response but there are many other risk factors from genetic and non-genetic factors including stress, pregnancy, malignancy and drugs used for other illness which interacts with inhibitors production among HA patients that requires further investigation (18, 26, 36, 38).

CONCLUSION

This study found that gene polymorphisms of CTLA-4 +49A/G and CTLA-4 -318C/T were not significantly different among severe HA with inhibitors and without inhibitors. However, among the severe HA patients without inhibitors, it was suggested that the

Chinese population exhibited a higher +49G allele which is probably being preventive towards inhibitors development. Findings of the mixed pattern of cytokine profiles of TNFα, IL-4 and IL-10 and in severe HA patients seemed to be associated with the presence of FVIII inhibitors. Further study using a larger sample size that represents the severe HA population may help to further verify the involvement of CTLA-4 gene and cytokines profile.

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