UNIVERSITI PUTRA MALAYSIA

DETERMINATION OF IL- 10 GENE POLYMORPHISM AND CYTOKINE LEVEL MEASUREMENT IN DEVELOPMENT OF INHIBITORS AMONG SEVERE HAEMOPHILIA A PATIENTS IN MALAYSIA

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V S SELVAVAANI A/P K N VAIAPPURI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the Degree of Master of Science

May 2018

DEDICATION

I would like to dedicate this work especially to my supervisor, family members and friends. I couldn't have done it without them.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

DETERMINATION OF IL 10 GENE POLYMORPHISM AND CYTOKINE LEVEL MEASUREMENT IN DEVELOPMENT OF INHIBITORS AMONG SEVERE HAEMOPHILIA A PATIENTS IN MALAYSIA

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May 2018

Chairman : Sabariah Md Noor, MD, MPath
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Haemophilia A is a hereditary X-chromosomal recessive disorder which is characterised by a deficiency of functional factor VIII (FVIII) coagulant activity. Haemophilia can also be classified as severe, moderate or mild based on coagulation factor levels. Patients with severe haemophilia A (FVIII level of < 1%) possess a high risk of spontaneous bleeding. The FVIII concentrates infusion is the treatment of choice for haemophilia A sufferers. However, one of the most unwanted complications is the formation of neutralising antibodies known as inhibitors that will inhibit the clotting activity against the administered factor concentrates in some patients.

Patients who develop FVIII inhibitor shows an increase in the frequency of bleeding episodes, which cannot be adequately controlled by FVIII concentrates. They are also at increased risk of morbidity and mortality. There is several mechanisms involved in the formation of inhibitor, i.e. family history, ethnicity, FVIII gene mutations, major histocompatibility complex genotype and polymorphisms of immune-response genes. This study aims to characterise the polymorphism of -592C/A, - 819C/T and -1082G/A in the promoter region of interleukin- l0 (lL-10) and relate them with IL-10 plasma levels.

The patients' whole blood samples and some stored DNA were collected from the National Blood Centre, Kuala Lumpur. The whole blood samples were further processed to obtain the DNA and their serum subjected for IL-10 cytokines level by enzyme-linked immunosorbent assay (ELISA). A total of 64 severe haemophilia A respondents (32 with inhibitors (50%) and 32 without inhibitors (50%)) were involved in this study.

Among these respondents, half of the patients (50%) were with high titre inhibitor (≥ 5 BU) and another half (50 %) were low titre inhibitors (<5 BU). The median FVIII inhibitor titre of high and low titre were (Median=38.50, IQR=91 and (Median=1.35, IQR=2.18) respectively. Distribution of respondents according to the ethnicity of Malay, Chinese, and Indian were 65.6%, 26.6% and 7.8% respectively. The overall respondents mean age were 25.03±14.86 years old. The collected DNA that were analysed using the polymerase chain reaction-restriction fragment polymorphism showed that -592A and -819T alleles in the promoter region of IL-10 were observed more frequently in respondents with inhibitors (39 (60.60%) and 40 (66.67%) respectively).

However, there was no significant difference of allelic and genotype frequencies of -592C/A, - 819C/T and -1082G/A in the promoter region of interleukin- l0 (lL-10) among the respondents. These findings showed that there was no significant difference observed between the respondents with and without inhibitors. This suggests a lack of association between the promoter region of the IL-10 gene polymorphisms and the development of inhibitors among patients with severe haemophilia in Malaysia.

The -592CA heterozygous genotype were the most prevalent in both Malays and Indians, which had the highest and similar CA genotype percentage of 50% among the patients with inhibitors. The -819CC homozygous genotype was most prevalent among the Malay respondents with inhibitor with the percentage of 9.1%. The -1082 G/A heterozygous genotype were most prevalent among Malay respondents with inhibitor with percentage of 81.8%.

Moreover, the high level (Median=98.5 pg/mL, 203.91 pg/mL) of IL-10 concentration were observed among the severe haemophilia A respondents with inhibitors. Elevated level of IL-10 concentration in respondents with inhibitors may suggest an important role for IL-10 in an inhibitor formation. Therefore, larger scale prospective studies are required to confirm these findings.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains.

PENENTUAN GENE IL-10 POLIMORPHISME DAN UKURAN TAHAP SITOKIN DALAM PEMBENTUKAN PERENCAT DIKALANGAN PESAKIT HEMOFILIA A PARAH DI MALAYSIA

Oleh

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Hemofilia A adalah gangguan heresian X-chromosomal yang disifatkan oleh kekurangan faktor fungsi VIII aktiviti koagulan. Hemofilia juga boleh dikelaskan sebagai sedikit, sederhana atau teruk berdasarkan tahap faktor pembekuan. Pesakit hemofilia A yang parah (tahap faktor VIII <1%) mempunyai risiko berdarah spontan yang tinggi. FVIII menumpukan penyerapan adalah rawatan pilihan untuk hemofilia A. Walau bagaimanapun, salah satu komplikasi yang paling tidak diingini ialah pembentukan antibodi yang meneutralkan yang dikenali sebagai perencat akan menghalang aktiviti pembekuan terhadap faktor yang ditumpukan pada beberapa pesakit.

Pesakit yang ngalami perencat FVIII berhadapan dengan peningkatan dalam kekerapan episod pendarahan yang tidak dapat dikawal oleh rawatan kepekatan FVIII. Mereka juga berhadapan dengan peningkatkan risiko morbiditi dan kematian. Pelbagai mekanisme yang menyumbang kepada pembentukan perencat seperti sejarah keluarga, etnik, mutasi gen FVIII, genotip kompleks utama histokompatibiliti dan polimorfisme gen tindak balas imun. Kajian ini bertujuan untuk mengenal pasti polimorfisme gen pengekodan untuk -592C / A, - 819C/T dan -1082G / A di bahagian promoter interleukin-l0 (lL-10) dan menghubungkannya dengan tahap plasma IL-10.

Seramai 64 responden yang menghadapai hemofilia A parah (32 dengan perencat dan 32 tanpa perencat) terlibat dalam kajian ini. Sampel berupa darah pesakit dan DNA yang disimpan diperolehi dari Pusat Darah Negara. Sampel darah diproses untuk mendapatkan DNA dan serum (diunjurkan untuk ujian tahap sitokin IL-10 menggunakan asid imunosorben berkaitan enzim (ELISA)). Analisa data menunjukkan insiden perencat FVIII di kalangan responden yang kekurangan FVIII

parah adalah 50%. Separuh daripada pesakit (50%) adalah dengan perencat FVIII titre tinggi (≥5 BU) dan separuh lagi (50%) adalah perencat titre rendah (<5 BU).

Median titre FVIII perencat tinggi dan titre rendah adalah masing -masing (Median = 38.50, IQR = 91) dan median adalah (Median = 1.35, IQR = 2.18). Taburan responden menurut etnik Melayu, Cina dan India masing masing adalah 65.6%, 26.6% dan 7.8%. Purata umur respoden keseluruhan adalah 25.03 ± 14.86 tahun. Sampel DNA telah dianalisasi dengan menggunakan polimorfisme serpihan tindak balas rantaian polimerase menunjukkan bahawa alel-592A dan -819T di bahagian promoter IL-10 dilihat lebih kerap dikalangan responden dengan perencat (39 (60.60%) dan 40 (66.67%) masing-masing).

Walau bagaimanapun, tidak terdapat perbezaan yang signifikan antara kekerapan alel dan genotip -592C / A, - 819C / T dan -1082G / A di kawasan promoter interleukin-l0 (lL-10) di kalangan responden dengan perencat daripada yang diperhatikan dalam responden tanpa perencat. Oleh itu, tiada hubungkait di antara gen polimorfisme dan pembentukan perencat dikalangan pesakit hemofilia parah.

Genotip heterozigot -592CA adalah yang paling lazim di kalangan orang Melayu dan India mempunyai peratusan genotipe CA yang paling tinggi dan sama sebanyak 50% di kalangan pesakit dengan perencat. Genotip homozigot -819CC paling lazim di kalangan responden Melayu dengan perencat dengan peratusan 9.1%. Genotip heterozigot -1082GA adalah yang paling lazim di kalangan responden Melayu dengan perencat dengan peratusan 81.8%.

Selain itu, tahap tinggi (Median = 98.5 pg / mL, 203.91 pg / mL) kepekatan IL-10 diperhatikan di kalangan responden hemofilia A parah dengan perencat. Tahap peningkatan kepekatan IL-10 pada responden dengan perencat mungkin menunjukkan peranan penting IL-10 dalam pembentukan inhibitor. Oleh itu, kajian prospektif dengan jumlah responden yang lebih besar diperlukan untuk mengesahkan penemuan ini.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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I hereby confirm that:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

A/G	Adenine/Guanine
APC	Antigen-presenting cells
aPTT	prolonged activated partial thromboplastin time
BIA	Bethesda inhibitor assay
BU	Bethesda Unit
C/T	cytosine/ thymine
CI	Confidence interval
CSIF	Cytokine synthesis inhibitory factor
CTLA-4	Cytokine synthesis inhibitory factor
ddNTP	Dideoxynucleotides
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
dsDNA	Double-stranded DNA
ELISA	The enzyme-linked immunosorbent assay
FOXP3	forkhead box P3 also known as scurfin
FVIII	Factor FVII
HA	Haemophilia A
IL-10	Interleukin 10
IL-16	Interleukin 16
IRF5	interferon regulatory factor 5
IRG	Immune response genes
ITI	Immune tolerance induction
lFN-γ	Interferon gamma
MgCl2	Magnesium Chloride
MHC	Major histocompatibility complex
NK cell	Natural Killer cell
OD	Optical density
OR	Odd ratio
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase chain reaction–restriction fragment length
pdFVIII	plasma-derived FVIII
PT	Prothrombin time(PT)
RE	Restriction endonucleases
SLE	Systemic lupus erythematosus
SPSS	Statistical Package for the social Science
ssDNA	Single-stranded DNA
TAE buffer	Tris-acetate-EDTA
Taq	Thermus aquatics
TNF-a	tumour necrosis factor-alpha
VWF	von Willebrand factor

CHAPTER 1

INTRODUCTION

1.1 Introduction

Haemophilia is a hereditary X-chromosomal recessive disorder which is characterised by a deficiency of functional clotting factor (commonly factor VIII or factor IX) protein. Functional clotting factor proteins are an essential component in the intrinsic pathway of blood coagulation. It affects nearly 1 in 5000 male live births worldwide (Franchini & Mannucci, 2013). Haemophilia A is caused by mutations in factor VIII (FVIII) gene, whereas haemophilia B is caused by mutations in factor IX (IX) gene (Stachnik, 2015).

Haemophilia A (HA) also known as classical haemophilia is a deficiency or missing of factor VIII, whereas Haemophilia B (Christmas disease) is a deficiency of factor IX. Haemophilia can also be classified as severe (<1%), moderate (1–5%) or mild (>5% to <40%) depending on levels of affected clotting factors in the blood (Stachnik, 2015). According to the 2008 Global Survey of the World Federation of Haemophilia, almost 149,000 individuals around the world suffer from haemophilia. In the United States (Walsh, Soucie, Miller, & Network, 2015), it is estimated that approximately 16,000 individuals have haemophilia, with an annual incidence of 1 in 5,000 male births for haemophilia A and 1 in 25,000 male births in Haemophilia B (Stachnik, 2015).

Owaidah et al. (2017) reported that Haemophilia A was observed in 73.3% cases (148 cases of 237 patients) of which most patients with haemophilia A had a severe form of the disease (126 patients; 85.7%) in Saudi Arabia (Owaidah et al., 2017). The diagnosis of haemophilia A is considered when unusual bleeding occurs in a male. The laboratory screening tests done showed that isolated prolonged activated partial thromboplastin time (aPTT) contrasts with the normal platelet count, bleeding time, and Prothrombin time (PT). The confirmation of the disease is established by demonstrating a reduced level of specific FVIII assay in plasma (Franchini & Mannucci, 2013).

Patients with haemophilia A are at risk of developing spontaneous bleeding, according to the severity of their factor VIII level (Stachnik, 2015). Bleeding can occur spontaneously or post-traumatically in any organ or tissue. Patients with severe haemophilia have a high risk of spontaneous bleeding into muscles, joints and brain (Franchini & Mannucci, 2013). Over 90% of the bleeding events involve hemarthrosis, which is intra-articular bleeding. Recurrent and untreated hemarthrosis can result in permanent damage to the articular cartilage which causes deforming and crippling arthroplasty (Ferreira, Gonçalves, & Bustamante, 2014).

The bleeding problem in haemophilia A is treated by replacement of factor concentrates (FVIII) (Stachnik, 2015). The availability of purified plasma-derived and recombinant FVIII products have been led to health improvements and the well-being of affected haemophilia patients (Witmer & Young, 2013). However, some of the patients who received this treatment, especially patients with haemophilia may develop inhibitory IgG antibodies against the given FVIII (Witmer & Young, 2013) The registry of National BloodCentre Malaysia between 1st January 2014 and 31st December 2014 exhibited 112 cases which consisted of haemophilia A patients with inhibitors.

The antibodies which develop against FVIII are known as inhibitors (Witmer & Young, 2013). Inhibitors bind to FVIII and prevent its hemostatic function. The development of inhibitory alloantibodies against FVIII is the most challenging complication of replacement therapy for haemophilia A patients (Rocino, Franchini & Coppola, 2017). Thus, this will cause the treatment to become costlier and increases the morbidity. Inhibitor formation occurs almost 30% among the haemophilia A patients. This problem is one of the most significant complications among the severe haemophilia A patients (Christine, Kempton, White & Gilbert, 2015).

The mechanism of inhibitor formation among these groups of patients is complicated. Both genetic and environmental risk factors may play a role in inhibitor formation (Stachnik, 2015). The development rate of FVIII inhibitors among the haemophilia patients differs in different regions. Shirahata et al. (2011) studies reported that inhibitor formation occurs 30% among the Japanese population and 6.2% among the French (Shirahata et al., 2011). Moreover, Pinto et al. (2014) mentioned that the incidence of inhibitor development among the severe haemophilia A patients in India have been reported to be 8.2% (Pinto et al., 2014).

Lu et al. (2012) mentioned that immune-related genetic markers might have an influence on the immune response towards FVIII replacement therapy and thus, might contribute to the inhibitor formation (Lu et al., 2012). The Single-Nucleotide Polymorphism (SNPs) in the promoter regions of cytokine genes may affect cytokine transcription and impact the production of cytokine in a single individual (Lu et al., 2012).

Polymorphisms in cytokines-related genes might be a risk factor for inhibitor formation among the treated haemophilia A patients. In a previous study, Lu et al. (2012) found that significant associations between inhibitor risk and polymorphisms in interleukin-10 (IL-10) gene (Lu et al., 2012). The frequency difference in relation to cytokine gene polymorphism and haemophilia A patients with inhibitors may have clinical importance.

Therefore, analysing the frequencies of several polymorphisms in cytokine genes may attribute to discover the genetic profile of severe haemophilia A patients in regards to the risk of FVIII inhibitor development (Ryu, Park, Yoo, Lee & Choi, 2015). Interleukin -10 (IL-10) is an important anti-inflammatory cytokine (Pergantou &

Economou, 2013). Recently, the inhibitory antibodies among haemophilia A were reported to be more frequently encountered in patients with inhibitors of the IL-10G microsatellite in the promoter site (Astermark, Oldenburg, Pavlova, Berntorp & Lefvert, 2006c). Previous studies among the Chinese population in China had shown that the IL- 10G allele would increase the secretion of lL-10 (Lu et al., 2012).

In addition, Single Nucleotide Polymorphisms (SNPs) at position -592C/A, -819C/T and - 1082A/G in the promoter region of the lL-10 gene are likely to be associated with transcription of the IL-10 and thus, affecting the production of the IL-10 (Lu et al., 2012). The 1082G allele in the IL- 10 gene was confirmed to be related to a high expression of the IL-10 (Lu et al., 2012). Furthermore, it was reported that the frequency of -1082G allele distribution was higher among the Chinese Han haemophilia A patients with inhibitors in comparison to patients without inhibitors (Lu et al., 2012).

1.2 Problem Statement

One of the most unwanted complications in the treatment of patients with haemophilia is the formation of neutralising antibodies; an inhibitor that inhibits the clotting activity against the administered factor concentrates in some patients. Inhibitor development occurs approximately 25%–30% of the severely affected among the Haemophilia A patients worldwide. Clinical management of Haemophilia A with inhibitor complications is expensive and costs about 220 000 euros per year, per patient. Inhibitor formation in haemophilia is a complex process and is related to the severity of disease, family history, ethnicity, FVIII gene mutations, major histocompatibility complex genotype and polymorphisms of immune response genes.

Single polymorphisms (SNPs) at position -592C/A, -819C/T and - 1082A/G in the promoter region of the lL-10 gene is likely to be associated with transcription of the IL-10 and affecting of the IL-10 production. Lu et al. (2012) mentioned that the production of the IL-10 could contribute to the inhibitor development among the haemophilia A patients (Lu et al., 2012). We aimed to determine the association of the polymorphism in the immune-related gene (IL-10) and inhibitor development among the severe haemophilia A patients in Malaysia.

Variants of the Immune Response Genes (IRG) are considered a potential source of individual differences in both innate and adaptive immune responses. IRG polymorphisms may also affect the individual predisposition to complex diseases or cause theirclinical course to be modified. The outcomes from this study may contribute to a better understanding of the genetic role on the risk of inhibitor development. The findings from this study aims to provide sufficient data for future research that would be looking into the potentials and alternative treatment approach in this group of patients. This will be the first local case-control study of severe haemophilia A with and without inhibitor. This focuses on the association of polymorphism of the immune-related gene at position -592C/A, -819C/T and -1082A/G, in the promoter region of lL-10 gene and cytokine level measurement in inhibitor development among the severe haemophilia A patients amid the different ethnic groups in Malaysia.

1.3 Significance of the Study

There is evidence that shows that polymorphisms in genes coding in the promoter regions of lL-10 gene could contribute to the inhibitor development among the haemophilia A patients. In this study, we will determine the genotype and allele frequency of the IL-10 gene in the promoter region. The studies of single nucleotide polymorphisms (SNPs) at the position of -592C/A, -819C/T and - 1082A/G in the promoter region of lL-10 gene have been done in other populations such as Brazil, India, and China among the severe haemophilia A patients with and without inhibitor. Therefore, this study was performed in order to provide a better understanding of the genotype and allele frequency distribution of -592C/A, -819C/T and - 1082A/G in the promoter region of the lL-10 gene among the severe haemophilia A patients with and without inhibitor in Malaysian.

1.4 Study Objectives

1.4.1 General Objective

To determine the polymorphism on the site of promoter region of the IL-10 gene in the development of inhibitor among the Malaysian patients with severe haemophilia A.

1.4.2 Specific Objectives

1. To identify the distribution and frequency (age, race, inhibitor status, inhibitor level) of patients with severe haemophilia A.

2. To analyse the distribution and frequency of -1082A/G, -819C/T and -592 C/A in the promoter region of the IL-10 gene in the development of inhibitor among severe haemophilia A.

3. To describe the association of patients' socio-demographic factors (age, race, inhibitor, inhibitor level) with polymorphism of the promoter region of the IL10 gene (-1082A/G, -819 C/T and -592C/A) among the Malaysian severe haemophilia patients as compared to control.

4. To evaluate the distribution and frequency of cytokine level of IL-10 according to patients' socio-demographic factors (age, race, inhibitors status and inhibitor level) with polymorphism of the promoter region of IL-10 (-1082A/G, -819C/T and -592C/A) among the Malaysian severe haemophilia A patients as compared to control.

5. To determine the association of cytokine level of IL-10 according to patients' socio-demographic factors (age, race, inhibitor level, inhibitors status) with polymorphism of the promoter region of IL-10 (-1082A/G, -819C/T and -592C/A) among the Malaysian severe haemophilia A patients as compared to control.

1.5 Hypothesis

- There is an association between single nucleotide polymorphisms (SNPs) at position -592C/A, -819C/T and 1082A/G in the promoter region of lL-10 genepatients with inhibitors among the severe haemophilia A patients in Malaysia.
- There is a significant relationship between single nucleotide polymorphisms (SNPs) at position - 592C/A, -819C/T and -1082A/G in the promoter region of the lL-10 gene among the severe haemophilia A patients with inhibitor in Malaysia.
- There is a significantly elevated serum levels of IL-10 concentration observedamong the Malaysian severe haemophilia A patients with inhibitors.
- There is a significant relationship between elevated cytokine level of IL-10 and polymorphism of the.promoter region of IL-10 (-1082A/G, -819C/T and -592C/A) among the Malaysian with severe haemophilia A patients

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