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PUTATIVE ROLE OF BACH1 GENE IN HBE/BETA-THALASSAEMIA PATIENTS

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PUTATIVE ROLE OF BACH1 GENE IN HBE/BETA-THALASSAEMIA PATIENTS

By

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PUTATIVE ROLE OF BACH1 GENE IN HBE/ BETA THALASSAEMIA PATIENTS

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Beta thalassaemia is an autosomal blood disorder due to a quantitative reduction or total absence of beta globin chain synthesis caused by beta globin gene mutations. Haemoglobin E/ beta thalassaemia individuals have a diverse clinical severity due to globin chain imbalance and the effects of other modifiers. Basic leucine zipper transcription factor 1 (Bach1) is known to be among the crucial molecules that has the ability to regulate gene expression when faced with oxidative stress. This study was done to investigate the role of Bach1 gene in HbE/ beta thalassaemia patients. Peripheral blood samples from 62 patients were collected. Full blood count analysis and HPLC were performed on the peripheral blood. Patients with blood transfusions of less than three months were excluded. A total of 47 selected samples without underlying iron deficiency or alpha thalassaemia co-inheritance were genotyped with ARMS PCR for

common beta globin mutations and detection of uncommon beta globin mutations were done via direct gene sequencing. TaqMan® quantitative RT-PCR was employed to correlate Bach1 expression to severity, HO-1 and globin expression levels of HbE/ beta thalassaemia. Bach1 expression among 47 HbE/ beta thalassaemia patients varied up to 2-log differences which was positively correlated to age (p=0.006), alpha globin expression level (p=0.002), beta globin expression level (p=0.001), gamma globin expression level (p=0.001) and HO-1 expression level (p=0.001). Apart from that, Bach1 expression was also negatively correlated to reticulocytes level (p=0.005). The positive correlation of Bach1 expression with age could be due to the increasing oxidative stress caused by natural cellular ageing process. Excess precipitated alpha chains and unstable beta globin chains cause Bach1 expression to increase whereas Bach1 is correlated indirectly with gamma globin chains to ameliorate the clinical severity of HbE/ beta thalassaemia. The compensatory mechanism of Bach1 trying to counter-react with the increased reticulocyte counts and the indirect link of Bach1 with HO-1 as a cytoprotective vehicle further substantiate the role of Bach1 in modifying the phenotypic severity in HbE/ beta thalassaemia. Taken together, these results suggest that Bach1 could be a potential modifier of HbE/ beta thalasssaemia individuals.

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

PERANAN GEN BACH1 DALAM PESAKIT HBE/BETA TALASEMIA

Oleh

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Beta talassemia adalah penyakit darah autosomal yang disebabkan oleh pengurangan kuantitatif atau tidak adanya sintesis rantai β-globin akibat daripada mutasi gen beta globin. Pesakit HbE/ beta talasemia mempunyai tahap keparahan klinikal yang luas kerana ketidakseimbangan rantai globin dan kesan pengubah lain. ‘Basic leucine zipper transcription factor 1’ (Bach1) dikenalpasti antara molekul penting yang berupaya mengawal ekspresi gen semasa menghadapi tekanan oksidasi. Kajian ini dilakukan untuk mengkaji peranan gen Bach1 dalam pesakit HbE/ beta talasemia. Sebanyak 62 sampel darah periferi dikumpulkan. Analisis hitung darah lengkap dan HPLC dilakukan pada darah periferi. Pesakit dengan pemindahan darah kurang daripada tiga bulan telah dikecualikan. Pada akhirnya, 47 sampel tanpa defisiensi besi dipilih atau ko-warisi daripada α-thalassemia atau tambahan α-gen. Sample berkenaan telah digenotip dengan ARMS PCR untuk mutasi β-globin biasa dan β-globin mutasi yang luar biasa dikesan

melalui teknik jujukan. TaqMan® RT-PCR kuantitatif digunakan untuk mengaitkan ekspresi Bach1 dengan tahap keparahan dan ekspresi globin pada HbE/β-talasemia. Ekspresi Bach1 antara 47 HbE/β-talasemia pesakit berubah-ubah dengan perbezaan 2-log yang berkorelasi positif dengan umur (p=0.006), ekspresi alpha globin (p=0.002), ekspresi beta globin (p=0.001), ekspresi gamma globin (p=0.001) dan ekspresi HO-1 (p=0.001). Selain itu, ekspresi Bach1 berkorelasi negatif dengan tahap reticulosit (p=0.005). Kolerasi positif Bach1 dengan umur berkemungkinan disebabkan oleh peningkatan stres oksidatif daripada proses penuaan sel semulajadi. Presipitasi berlebihan rantai alpha serta ketidakstabilan rantai beta globin mengakibatkan ekspresi Bach1 meningkat, manakala Bach1 mempunyai kolerasi secara tidak langsung dengan rantai gamma globin untuk menurunkan keparahan klinikal HbE/β-talasemia. Mekanisme pampasan oleh Bach1 yang cuba untuk bertindak balas dengan peningkatan hitungan reticulosit dan kaitan tidak langsung Bach1 dengan HO-1 sebagai sito-perlindungan terus menyokong peranan Bach1 dalam mengubah suai keparahan fenotipik dalam HbE/β-talasemia. Secara keseluruhannya, daripada kajian ini dicadangkan bahawa Bach1 berkemungkinan merupakan salah satu pengubahsuai yang berpotensi bagi individu-individu HbE/ β-talasemia.

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I certify that a Thesis Examination Committee has met on (2013) to conduct the final examination of Lee Tze Yan on his thesis entitled "**The putative role of Bach1 gene in HbE/beta thalassaemia patients**" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

LEE TZE YAN

Date: 11 July 2013

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LIST OF ABBREVIATIONS

A	adenine
ARMS	amplification refractory mutation system
Bach1	Basic Leucine Zipper Transcription Factor 1
Bp	base pairs
C	cytosine
CD	Codon
cDNA	complementary DNA
ddNTP	Dideoxynucleotide triphosphate
DMSO	Deoxynucleotide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide solution mix
EDTA	Ethylenediaminetetraacetic acid
FAM	Carboxyfluorescein
FBC	full blood count
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G6PD	Glucose-6-phosphate dehydrogenase deficiency
Hb	Haemoglobin
HbA	adult haemoglobin
HBA	Alpha globin gene
HBB	Beta globin gene
HbF	foetal haemoglobin
HBG	Gamma globin gene
HO-1	Heme oxygenase-1
HPFH	hereditary persistence of foetal haemoglobin
HPLC	High Performance Liquid Chromatography
IVS	intervening sequence
KCl	Potassium chloride

LCR	locus control region
LIP	labile iron pool
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MED	Mediterranean
Mg_2Cl	Magnesium chloride
OH	hydroxyl group
PCR	polymerase chain reaction
PBS	phosphate buffered saline
qPCR	quantitative real-time PCR
RBC	red blood cell
RDW	red cell distribution width
RFLP	restriction fragment length polymorphism
RNA	Ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcription
SEA	Southeast Asia
T	Thymine
TAE	Tris-Acetate-EDTA
Taq	Taq DNA polymerase
TRI	Trizol

CHAPTER 1

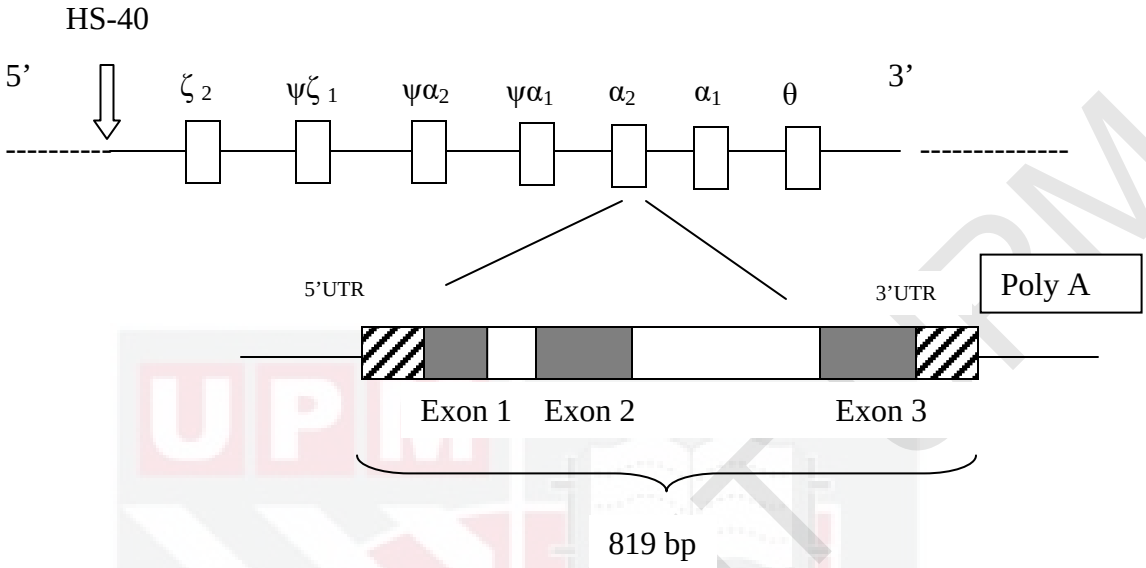
INTRODUCTION

1.1 Haemoglobin Structure

Haemoglobin is the core protein involved in oxygen transportation in the human body. All functional haemoglobins consist of two complementary pairs of globin chains and packed with an iron-containing haem molecule in each globin chains (Bain *et al.,* 2010). HbA, a major haemoglobin in normal adults constitutes about 97% of the total haemoglobin and another component, HbA_2 accounts for a mere 2-3% of the total. Foetal haemoglobin (HbF) is the major haemoglobin in foetus and in most of the normal adults, a minute amount of HbF can still be present (Weatherall & Clegg, 2001).

The globin genes which encode the globin chains can be found in two clusters (**Figure 1.1**). They are located in chromosome 16 (alpha globin gene cluster) and chromosome 11 (beta globin gene cluster). Alpha globin gene cluster is made up of a few pseudogenes, a zeta (ζ) gene and two alpha (α) genes. On the other hand, beta globin gene cluster consists of an epsilon (ε) gene, two gamma (γ) genes, a sigma (δ) gene and a beta (β) gene (Bain *et al.*, 2010). Both alpha and beta globin gene cluster are arranged according to their developmental expression (Thein, 2004).

a) Alpha gene, chromosome 16p13.3



b) Beta gene, chromosome 11p15.4

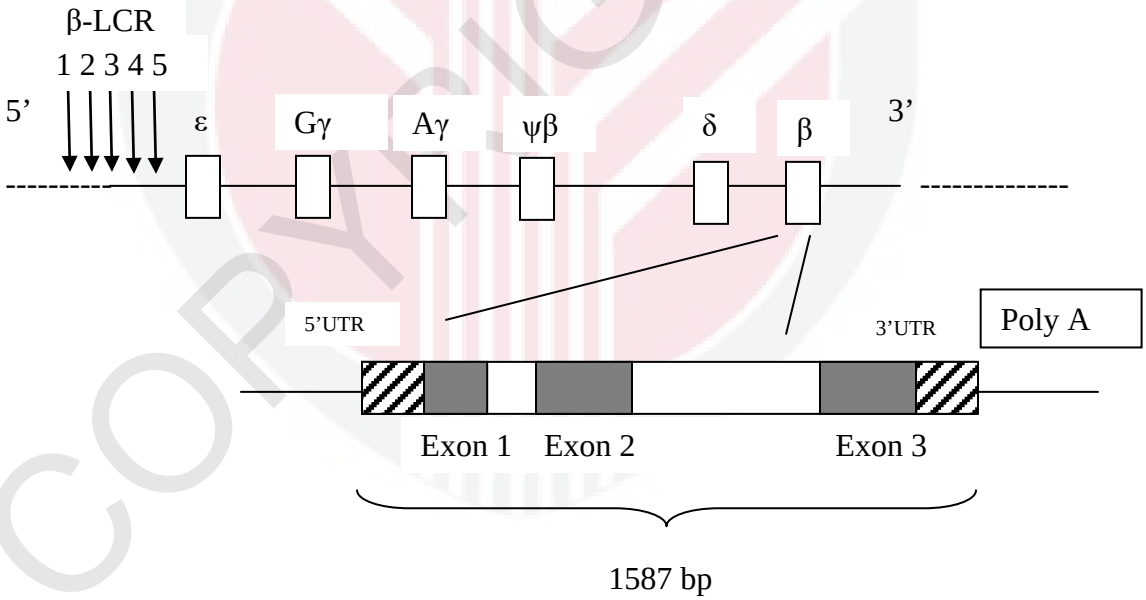


Figure 1.1 Maps of the α-like and β-like globin gene clusters. (a) Alpha gene locus on chromosome 16p13.3. Down-point vertical arrow represents the important gene expression locus, HS-40 (upstream hypersensitive site). **(b) Beta locus on chromosome 11p15.4.** Five hypersensitive sites are indicated by 5 vertical down point arrows, representing the gene regulatory sequences in the β-globin cluster, also known as locus control region (LCR) (Modified from Clark and Thein, 2004).

1.2 Haemoglobinopathies

There are basically two types of haemoglobin abnormalities. One is due to the synthesis of an abnormal haemoglobin (structural haemoglobinopathy) while the other is the reduction or absence of normal alpha (α) and/or beta (β) chains production (Hoffbrand *et al.*, 2006). Structural haemoglobinopathies is the term used to describe structurally abnormal haemoglobins which includes haemoglobin E, haemoglobin C and haemoglobin S (Bain, 2006; Hoffbrand, 2006). Thalassaemia is classically defined as an inherited autosomal blood disorder which results in a reduced rate or absence of one of the globin chains which makes up the haemoglobin (Old, 2003; Hoffbrand *et al.*, 2006).

1.3 Beta thalassaemia

β- thalassaemia is indicated by a quantitative lack of β-globin chains (Thein, 2004). The main causes of β-thalassaemia are point mutations or small deletions within the β-globin gene. The high β-thalassaemia carrier frequency has been translated into a high prevalence of β-thalassaemia major patients especially in countries like Malaysia, Singapore and Thailand (Quek *et al.*, 2007). Beta thalassaemia can be categorized into three main forms based on clinical phenotypes which include thalassaemia minor, thalassaemia intermedia and thalassaemia major although overlaps among the groups tend to occur (Thein, 1998). The clinical phenotypes range from asymptomatic to those who require medical attention such as regular blood transfusions (Galanello & Origa, 2010). For beta thalassaemia major, the clinical manisfestations include hypersplenism,

chronic hepatitis, iron overload and growth retardation due to a combination of excessive peripheral erythrocyte haemolysis and ineffective erythropoiesis. As for beta thalassaemia intermedia, jaundice, spleen and liver enlargement, leg ulcers and girth expansions are some of the usual clinical features (Cao & Galanello, 2003).

Beta thalassaemia intermedia such as HbE/ beta thalassaemia is common in South-East Asia and the Indian subcontinent. There is also evidence that the clinical heterogeneity may be affected by factors such as co-inheritance of alpha-thalassaemia, an increase of HbF synthesis and other factors (Weatherall & Clegg, 2001).

Currently, there are over 200 different mutations which have been identified to produce a β-thalassaemia phenotype. Each country has its own unique spectrum of abnormal haemoglobins and thalassaemia mutations in which 4-5 common mutations contribute about 90% of the thalassaemia population and a few rare mutant alleles making up the rest (Bhardwaj *et al.*, 2005).

In Malaysia, thalassaemia is a public health problem among the ethnic Malay and Chinese populations whereas ethnic Indians form a minority percentage of those with thalassaemia (Weatherall & Clegg, 2001). Fortunately, the mutations have been well classified according to ethnicity in Malaysia. For the Malay population, the common mutations are IVS 1-5, IVS 1-1, codon 19 and HbE. Mutations such as codon 41/42, IVS II-654, codon 17, -28 are prevalent in the ethnic Chinese group. In a study conducted by George *et al.* in 1992, it was discovered that the ratio of HbE/ beta

thalassaemia to beta thalassaemia homozygotes was 1 to 3 in the Chinese population, however in the Malay descents the ratio was 4 to 1 (George *et al.*, 1992). Generally, there are three forms of beta thalassaemia modifiers that can modulate the phenotypic severity of the disorder namely primary, secondary and tertiary modifiers. Primary modifiers involve the mutations occurring in the beta globin gene cluster, secondary modifiers are correlated with the imbalance in the alpha and beta globin chains ratio and tertiary modifiers are phenotypes of certain thalassaemic complications which might play a role in determining the severity of the phenotypes involved (Thein, 2004).

1.4 Bach1 gene

Bach1 is a transcription regulator gene that targets beta globin, HO-1 and alpha globin genes (Igarashi & Sun, 2006; Tahara *et al.*, 2004). This transcriptional factor inhibits the activity of the beta globin gene locus control region (LCR) in erythroid cells and cause a repression in the β-globin expression (Igarashi *et al.*, 1998). Bach1 also has the ability to bind to the HO-1 enhancers to inhibit HO-1 activity (Igarashi & Sun, 2006). A study by Tahara *et al.* in 2004 indicated that Bach1, being a heme-binding protein gene, is also involved in the down-regulation of the alpha-globin gene expression which is heme dependent in human and mouse erythroid cells. It was reported that heme-depleted cells show a low expression of alpha globin genes whereas up-regulation of heme elevates the alpha globin gene expression (Tahara *et al.* 2004).

1.5 Hypothesis and objectives

Therefore in this study, we hypothesised that our gene of interest Bach1 gene is a potential modifier for HbE/ beta thalassaemia patients as the severity of alpha and beta thalassaemia is correlated to the alpha/beta globin chain imbalance. We chose HbE/ beta thalassaemia patients as our study cohort as any changes of phenotypes are mostly seen in this group of patients. Therefore, the effect of modifiers can be clearly seen in this group of patients.

The general objective of this study is:

1. To study the expression of Bach1 gene in HbE/ beta thalassaemia patients.

The specific objectives of this study are:

1. To investigate the influence of red blood cell parameters on Bach1 expression in HbE/ beta thalassaemia.
2. To associate the expression of Bach1 gene with other potential modifier (HO-1 gene) of HbE/ beta thalassaemia.
3. To determine the role of Bach1 gene with globin genes expression of HbE/ beta thalassaemia.

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