UNIVERSITI PUTRA MALAYSIA

DOWN SYNDROME: DEVELOPMENT OF A NON-INVASIVE PRENATAL DNA SCREENING TEST USING SUPEROXIDE DISMUTASE 1 GENE IN MATERNAL BLOOD AND DETECTION OF CYSTATHIONINE p-SYNTHASE GENE MUTATIONS

THILAKAVATHY A/P KARUPPIAH

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By

THILAKAVATHY A/P KARUPPIAH

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

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DEDICATION

This thesis is dedicated to my spiritual master,
HIS DIVINE GRACE YOGA JNANA SITTHAR OM SRI RAJAYOGA GURU,
without whom none of this would have been even possible.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

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Down syndrome or Trisomy 21, is the most commonly occurring genetic disorder that stems from the failure of chromosome 21 to segregate normally during meiosis, resulting in an individual carrying an extra copy of chromosome 21. The main aims of this study were to develop a relatively non-invasive prenatal DNA screening method using maternal blood and to detect mutations on cystathionine β-synthase (CBS) gene, a folate pathway gene located on chromosome 21. As an initial step, the presence of foetal cells and DNA in the maternal blood was firstly determined by foetal haemoglobin (HbF) staining and polymerase chain reaction (PCR). It was found that the ratio of the nucleated foetal cell to maternal cell increased from 2 in 10^6 to 3 in 10^6 and 5 in 10^6 at the first, second and third trimester, respectively. By using Y chromosome specific primers, DNA from male foetuses could be detected as early as 6 weeks of gestation in 200 μl maternal blood obtained from fingertip. This is in line with the current technology in non-invasive screening methods of foetal aneuploidies which is focused on detecting Y chromosomal sequences which is impossible to be used

for female foetus pregnancies. Therefore, the superoxide dismutase 1 (SOD1) gene sequence, which is located on the Down Syndrome Critical Region, was used to overcome this situation by using real-time quantitative PCR. The level of SOD1 sequences in maternal blood was found to be significantly elevated in the third trimester normal pregnancies (mean = 11728 copies/μl) when compared to the second trimester (mean = 5705.6 copies/μl), $p<0.005$ and non-pregnant normal women (mean = 3580.2 copies/μl), $p<0.0001$. Down syndrome pregnancies have the greatest elevation compared to all the three trimesters of normal singleton pregnancies and twin pregnancies, $p<0.05$. The traditional approach of prenatal chromosomal diagnosis using amniotic fluid was found to be cumbersome and time-consuming compared to the newly developed method. The mutation detection on CBS gene was carried out using DNA sequencer and denaturing high performance liquid chromatography (DHPLC). This study revealed that the Down syndrome patients have four mutations, which are in intron 1 (A9231C), exon 10 (C20628T) and exon 17 (T27796C and C27817T). The Down syndrome children were found to have the same genotype as their mothers. The number of mothers and children having the substitutions in the CBS gene was twice the number of mothers and children with normal genotype, suggesting that the mothers who have these substitutions are at higher risk of having a child with Down syndrome. In conclusion, non-invasive prenatal diagnosis at first trimester using Y chromosomal sequence is feasible for diagnosis of foetal-derived paternally-inherited polymorphism/mutations or genes. Quantitative analysis using gene associated with a disorder has a potentially significant advantage over the invasive techniques currently used widely for prenatal diagnosis. Finally, the discovery of the mutations in the CBS

gene of Down syndrome patients and mothers will help contribute to new knowledge and the future studies on the folate pathway genes mutation and occurrence of Down syndrome. It may also suggest an opportunity to improve public health strategies for the primary prevention of Down syndrome.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

SINDROM DOWN: PENGHASILAN UJIAN PENYARINGAN PRENATAL DNA YANG TIDAK MERBAHAYA MENGGUNAKAN GEN SUPEROXIDA DISMUTASE 1 DALAM DARAH IBU MENGANDUNG DAN PENGESANAN MUTASI GEN SISTATHIONIN β-SINTASE

Oleh

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Sindrom Down atau trisomi 21, merupakan keabnormalan genetik yang paling biasa berlaku akibat kegagalan kromosom 21 untuk membahagi secara normal semasa meiosis, menyebabkan seseorang individu mempunyai tambahan satu kromosom 21. Objektif-objektif utama kajian ini adalah untuk menghasilkan sebuah teknik penyaringan prenatal DNA yang tidak merbahaya secara relatif dan juga mengesan mutasi-mutasi pada gene sistathionin β-sintase (CBS), satu gen yang terletak di tapak-jalan folat pada kromosom 21. Sebagai langkah pertama, kehadiran sel-sel dan DNA janin di dalam darah ibu hamil ditentukan dengan pewarnaan haemoglobin janin (HbF) dan tindakan rantai polymerase (PCR). Didapati bahawa, nisbah antara sel janin bernukleus dengan sel ibu hamil bertambah dari 2 dalam 10^6 kepada 3 dalam 10^6 dan 5 dalam 10^6 masing-masing pada trimester pertama, kedua dan ketiga. Primer-primer yang spesifik bagi kromosom Y dapat mengesan DNA dari janin-janin lelaki seawal enam minggu gestasi dengan menggunakan 200 μl darah ibu hamil yang diambil dari hujung jari. Ini adalah sejajar dengan teknologi terkini dalam penyaringan janin

aneuploidi yang tidak merbahaya yang difokus pada pengesanan jujukan kromosom Y di mana ianya tidak mungkin dapat digunakan untuk kandungan janin perempuan. Oleh itu, jujukan gen superoxida dismutase 1 (SOD1), yang berlokasi pada Kawasan Kritikal Sindrom Down, digunakan untuk mengatasi situasi ini melalui aplikasi kuantitatif masa-benar PCR. Tahap jujukan SOD1 dalam darah ibu hamil didapati meningkat secara ketara pada kehamilan normal trimester ketiga (min = 11728 salinan/μl) apabila dibandingkan dengan kehamilan normal trimester kedua (min = 5705.6 salinan/μl), $p<0.005$ dan wanita normal yang tidak hamil (min = 3580.2 salinan/μl), $p<0.0001$. Kehamilan sindrom Down mempunyai peningkatan yang paling tinggi berbanding dengan kehamilan tunggal kesemua trimester dan kehamilan kembar, $p<0.05$. Diagnosis prenatal kromosom tradisional yang menggunakan cecair amnion didapati sukar dan mengambil lebih masa berbanding dengan teknik baru yang telah dihasilkan. Penentuan mutasi pada gen CBS dilakukan dengan menggunakan penjujuk DNA dan "denaturing high performance liquid chromatography" (DHPLC). Kajian ini telah menunjukkan bahawa pesakit sindrom Down mempunyai empat mutasi, iaitu di dalam intron 1 (A9231C), exon 10 (C20628T) dan exon 17 (T27796C dan C27817T). Kanak-kanak sindrom Down didapati mempunyai genotaip yang sama dengan ibu mereka. Bilangan ibu dan anak yang mempunyai substitusi ini adalah dua kali ganda daripada ibu dan anak dengan genotaip normal, mencadangkan bahawa ibu yang mempunyai substitusi ini berisiko tinggi untuk mendapat anak sindrom Down. Sebagai kesimpulan, diagnosis prenatal yang tidak merbahaya pada trimester pertama menggunakan jujukan kromosom Y boleh digunakan untuk mendiagnosis janin yang membawa polimorfisma/mutasi-mutasi atau gen-gen warisan-bapa. Analisis kuantitatif

menggunakan gen yang berkaitan dengan penyakit mempunyai potensi yang tinggi berbanding dengan teknik-teknik merbahaya yang digunakan kini secara meluas untuk diagnosis prenatal. Penemuaan mutasi-mutasi pada gen CBS dalam pesakit sindrom Down dan ibu mereka akan membantu menyumbangkan pengetahuan baru dan kajian masa hadapan ke atas mutasi gen-gen di tapak-jalan folat dan kejadian sindrom Down. Ia juga mungkin memberi peluang untuk memperbaiki strategi kesihatan awam bagi pencegahan awal sindrom Down.

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LIST OF ABBREVIATIONS

AF	Amniotic fluid cell
AFP	Alphafetoprotein
bp	Base pair
CBS	Cystathionine beta-synthase
C_T	Threshold cycle
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanidine triphosphate
DHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
E-type	Epitheloid cell
F-type	Fibroblast like cell
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
HbF	Foetal haemoglobin
hCG	Human chorionic gonadotropin
ME-THF	5-methyltetrahydrofolate
$MgCl_2$	Magnesium chloride
MTHFR	5,10-methylenetetrahydrofolate reductase
MTRR	Methionine synthase reductase

NaCl	Sodium chloride
NF	Nuclear factor
PCR	Polymerase chain reaction
R_N	Fluorescence intensity
SAM	S-adenosylmethionine
SNPs	Single nucleotide polymorphisms
SOD1	Superoxide dismutase 1
SRY	Specific Region of Y
UE_3	Unconjugated estriol

CHAPTER I

INTRODUCTION

Chromosomal abnormalities are the most frequent genetic disorders seen in both live born babies and miscarriages. Down syndrome is a chromosomal abnormality, which manifests itself in a set of common physical and mental characteristics. This abnormality is due to the presence of an extra chromosome (chromosome 21). Hence, the scientific name, trisomy 21.

Most people are aware that the chance of having a baby with Down syndrome is greater in older women. But Down syndrome can occur at any maternal age. In fact, 75-80% of babies with Down syndrome are born in younger women simply because that age group has more babies (Benke et al., 1995). Studies have proven that inadequate folate status at the time of conception increases the risk of Down syndrome. Women with genetic mutations which interfere with the body's ability to absorb folic acid, are at higher risk for having children with Down syndrome (James et al., 1999).

Down syndrome is a major reason for prenatal diagnosis. Prenatal diagnosis employs a variety of techniques to determine the health and condition of an unborn foetus. Such diagnosis is usually performed by means of karyotyping and depends on analysis at 11-18 weeks of gestation. Karyotyping is not always possible, especially when the number of cells obtained is limited, where cell culture fails (in 1-2% of patients), or when the