DETERMINATION OF PATHOGENICITY NOVEL MUTATIONS AND THEIR EFFECTS ON G6Pase PROTEIN FUNCTION IN MALAYSIAN GSD1a PATIENTS

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DETERMINATION OF PATHOGENECITY NOVEL MUTATION AND THEIR EFFECT ON G6PASE PROTEIN FUNCTION IN MALAYSIAN GSD1A PATIENTS

ABSTRACT

Mutation in the glucose-6-phosphate catalytic subunit (*G6PC*) gene causes Glycogen Storage Disease (GSD) type Ia – a disease associated with glycogen metabolism deficiency. This condition is majorly characterized by hepatomegaly (enlargement of the liver), hepatosplenomegaly (enlargement of the spleen) and growth retardation which may advance to death if not treated early. The *G6PC* gene encodes for glucose-6-phosphatase (G6Pase) which is a key enzyme for the maintenance of glucose homeostasis in humans. GSDs are 100% penetrant and segregate in an autosomal recessive manner. Screening for mutations in the *G6PC* gene represents pre-emptive genetic screening and risk assessment efforts for individuals with problems of glycogen metabolism. Knowledge of the mutation spectrum that is prevalent in specific populations is essential to ensure precise diagnosis as well as the ensuing treatment strategies. This study aims to screen for pathogenic *G6PC* mutations in a set of Malaysian GSD Ia patients and to establish the local *G6PC* mutation spectrum. A total of 21 GSD Ia patients' DNA were purified and each of the exon of the *G6PC* gene were amplified by Polymerase Chain Reaction (PCR). These were then subjected to DNA sequencing. A total of nine different mutations were detected: five mutations (c.648 G>T, c.248 G>A, c.706 T>A, c.664 G>A,c.518 T>C) have been reported previously while four are potentially novel mutations (c.155 A>T, c.226 A>T, c.337 C>T and c.1036 G>C). One single nucleotide polymorphism (SNP) was also detected from the screening. Three of the potentially novel mutations detected (c.155 A>T, c.337 C>T and c.1036 G>C) are missense mutation that change the original nucleotide to another while another mutation (c.226 A>T) is a nonsense mutation that creates a premature stop

codon. The SNP detected in this study (c.547 A>G) has been previously reported in SNP databases. In order to determine the pathogenicity of the potentially novel mutations, restriction enzyme and TaqMan probe-based assays were designed to investigate its presence and allele frequency in a representative cohort of healthy individuals (n=50 Malays, n=50 Chinese, n=50 Indians). Restriction enzymes *Mse*I and *Mbo*1 were used to screen forthe c.226 A>T mutation and c.337 C>T mutation respectively. For the other two mutations, c.155 A>T and c.337 C>T mutations, the TaqMan probe-based assayswere applied due to the inavailability of a restriction enzyme cutting site. Results obtained showed that no mutant allele was found in all 150 healthy individuals. In order to further confirm the pathogenicity of these potentially novel mutations, mutant alleles were constructed using site-directed mutagenesis (SDM) and transfected into mammalian cells for functional studies. However, functional studies were halted as satisfactory expression of the mutated protein was not achieved.

Keywords: Glycogen Storage Disease Type 1a (GSD1a), glucose-6-phosphatase enzyme (G6Pase), *Glucose-6-Phosphate catalytic subunit (G6PC)*

PENENTUAN KEPATOGENAN MUTASI BARU DAN KESAN TERHADAP FUNGSI PROTEIN G6PASE PADA PESAKIT GSD1A DI MALAYSIA

ABSTRAK

Mutasi di dalam gen "glucose-6-phosphate catalytic subunit" (*G6PC*) menyebabkan penyakit "Penyakit Penyimpanan Glykogen" (GSD) jenis Ia, yang merujuk kepada masalah metabolisma glikogen. Penyakit ini dapat dikenalpasti melalui simptom seperti hepatomegali (pembesaran hati), hepatosplenomegali (pembesaran hati dan limpa) dan perencatan pertumbuhan yang boleh berlaku hingga menyebabkan kematian jika tidak dirawat lebih awal. Gen *G6PC* adalah gen yang mengekod glukosa-6-phosphatase (G6Pase) yang merupakan enzim utama untuk penyelenggaraan homeostasis glukosa pada manusia. Penyakit menunjukan penembusan 100% dan ianya diwarisi secara autosomal resesif. Kaedah penyaringan untuk mutasi dalam gen *G6PC* dijalankan melalui pemeriksaan genetik awalan dan penilaian risiko bagi individu yang mengalami masalah metabolisma glikogen. Pengetahuan tentang jenis-jenis mutasi yang terdapat dalam gen *G6PC* bagi populasi masyarakat di Malaysia adalah sangat penting bagi memastikan prognosis terhadap penyakit ini secara ujian genetik dapat dilakukan dengan tepat. Sehubungan itu, kajian ini telah dijalankan bagi mengenalpasti jenis mutasi gen *G6PC* yang terdapat dalam pesakit-pesakit GSD Ia di Malaysia. Sejumlah 21 DNA pesakit GSD Ia ditulenkan dan setiap ekson gen *G6PC* telah diamplifikasikan bilangannya secara Tindakbalas rantaian polimerasa (PCR). Sample kemudiannya dianalisis secara penjujukan DNA. Sebanyak sembilan mutasi yang berbeza telah dikesan: lima mutasi (c.648 G> T, c.248 G> A, c.706 T> A, c.664 G> A, c.518 T> C) telah dilaporkan sebelum ini manakala empat adalah mutasi yang berpotensi mutasi baru (c.155 A> T, c.226 A> T, c.337 C> T dan c.1036 G> C). Satu polimorfisma nukleotida tunggal (SNP) juga dikesan dari pemeriksaan yang dijalankan. Tiga daripada mutasi berpotensi baharu yang dikesan (c.155 A> T, c.337 C> T dan c.1036 G> C)

adalah mutasi salah erti “missense” yang mengubah nukleotida asal kepada yang lain, manakala mutasi yang satu lagi (c.226 A> T) adalah mutasi tanpa erti “nonsense” yang mewujudkan kodon berhenti yang pramatang. SNP yang dikesan dalam kajian ini (c.547 A> G) merupakan SNP yang telah dilaporkan sebelum ini dalam pangkalan data SNP. Untuk memastikan bahawa mutasi baharu adalah berpatogen, assai-assai berasaskan enzim pembatasan penyaringan berasaskan prob TaqMan direka untuk menentukan kehadiran dan frekuensi alel dalam kohort kawalan yang terdir dari individu tak bepenyakit (n = 50 Melayu, n = 50 Cina, n = 50 India). Enzim pembatasan *Mse*I dan *Mbo*I digunakan untuk menyaring mutasi c.226 A> T dan c.337 C> T mutasi. Bagi dua mutasi yang lain iaitu mutasi c.155 A> T dan c.337 C> T, assai berasaskan prob TaqMan telah digunakan kerana ketidakhadiran enzim pembatasan yang sesuai untuk digunakan. Hasil yang diperoleh menunjukkan bahawa tiada alel mutan didapati dalam kesemua 150 individu yang sihat tersebut. Untuk mengesahkan lagi bahawa mutasi baharu ini adalah berpatogen, alel mutan telah dihasil menggunakan teknik mutagenesis terarah (SDM) dan ditransfeksikan kedalam kultur sel-sel mamalia untuk kajian fungsi protein. Bagaimanapun, kajian fungsi dihentikan kerana ekspresi protein mutan tidak tercapai.

Kata kunci: Penyakit Penyimpanan Glykogen Jenis 1a (GSD1a), glucose-6-phosphatase enzyme (G6Pase), *Glucose-6-Phosphate catalytic subunit (G6PC)*

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%	:	percentage
xg	:	Gravity
&	:	and
°C	:	degree Celsius
µl	:	microlitre
µM	:	micromolar
α	:	alpha
β	:	beta
A	:	Adenine
AGE	:	Agarose Gel Electrophoresis
Ala	:	alanine
Arg	:	arginine
BLAST	:	Basic Local Alignment Search Tool
bp	:	base pair
BSA	:	bovine serum albumin
C	:	Cytosine
$CaCl_2$	:	calcium chloride
CO_2	:	carbon dioxide
Del	:	deletion
dH_2O	:	distilled water
DMEM	:	Dulbecco's modified Eagle medium
DMSO	:	dimethyl sulfoxide
DNA	:	deoxyribonucleic acid

dNTP	:	deoxyribonycleoside triphosphate
E.coli	:	*Escherichia coli*
EDTA	:	ethylenediaminetetraacetic acid
et al.	:	*et alii* (and other people)
EtBr	:	Ethidium Bromide
FBS	:	fetal bovie serum
g	:	gram
G	:	Guanine
GC	:	guanine-cytosine
Glu	:	glutamic acid
GSD	:	Glycogen Storage Disease
HGMD	:	Human Gene Mutation Database
Ile	:	isoleucine
kb	:	kilobase pair
kDa	:	kilo Dalton
kg	:	kilogram
LB	:	Luria Bertani
Leu	:	leucine
Lys	:	lysine
M	:	Molar
$MgCl_2$	:	magnesium chloride
Min	:	minute
ml	:	millilitre
mM	:	milli Molar
mRNA	:	messenger RNA

NaOH	:	sodium hydroxide
ng	:	nanogram
OD	:	optical density
PAGE	:	polyacrylamide gel electrophoresis
PBS	:	phosphate buffered saline
PCR	:	polymerase chain reaction
pH	:	power Hydrogen
Phe	:	phenylalanine
pmol	:	picomole
Pro	:	proline
RE	:	restriction enzyme
RNA	:	ribonucleic acid
RNase	:	ribonuclease
rpm	:	rotation per minute
sdH_2O	:	sterilised distilled water
SDM	:	site-directed mutagenesis
SDM-F	:	site-directed mutagenesis-forward
SDM-F	:	site-directed mutagenesis-reverse
SDS	:	sodium dodecyl sulphate
Ser	:	serine
SNP	:	single nucleotide polymorphism
T	:	Thymine
Taq	:	*Thermus aquaticus*
TEMED	:	tetramethyl-ethylenediamine
Thr	:	threonine

Tris	:	tris(hydroxymethyl) amino methane
U	:	unit
UTR	:	untranslated region
V	:	volt
Val	:	valine

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CHAPTER 1: INTRODUCTION

Glycogen Storage Diseases (GSDs) is a rare group of inherited disorders due to defects in glycogen metabolism. This innate metabolic disease can usually be detected during early infancy, or some time at later stage of life. There are several types of GSDs which is categorized by the effects brought upon by different enzymes involved in the process of glycogen synthesis (Berhman *et al*., 1987). GSD Ia is one of the most common types of GSDs, which is prevalent in world populations at approximately 1 in 20,000 to 40,000 cases per birth (Zheng *et al*., 2014). The prevalence of carrier is estimated to be approximately 1 in 150 individuals (Chen, 2001). GSD Ia was first described by von Gierke in 1929, where it was characterized by hepatomegaly, short stature, hypoglycaemia, lactatemia and hyperlipidemia as its main clinical symptoms in the patients (Rake *et al.,* 2002)

GSD Ia is caused by defects in the Glucose-6-phosphatase (G6Pase) enzyme, which results in an excessive accumulation of glycogen in the liver, kidney and intestinal mucosa. This primal enzyme plays important roles for homeostatic blood regulation (Beaudet, 1991), especially for the final step of gluconeogenesis where the process of breaking down the glucose-6-phosphate (G6P) to phosphate group (Pi) and glucose (Lei *et al.,* 1994) takes place. The defective protein is caused by loss-of-function mutation in the *Glucose-6-phosphatase Catalytic subunits* (*G6PC*) gene (Lei *et al*., 1993). This disease has an autosomal recessive mode of inheritance, where the defects in both copies of *G6PC* allele will result in disease manifestation. Therefore, GSD Ia patients will either carry a homozygous mutation in both alleles or be compound heterozygotes where two different mutations are present in different loci (Shieh *et al*., 2002).

GSD Ia are usually diagnosed during infancy. However, some patients do not show the relevant phenotypes of GSD Ia in their early stage of life and they develop the

disease only during adulthood. The diagnosis of the disease is achieved through different methods which includes liver biopsy assay, where this assay will determine the G6Pase activity in the liver tissue. Another method involves molecular genetic studies in which *G6PC* gene was screened for mutations responsible for the development of GSD Ia. The mutation screening of *G6PC* gene is a more definitive method and it is often used to confirm the enzyme assay results (Rake *et al*., 2000). Furthermore, this method is accepted to be less invasive especially for screening mutation in infants. The follow-up treatment usually involves controlling their diet and reducing glucose intake in order to prevent the progression of the disease.

Information about GSD Ia incidence in Malaysia has not been officially established and the mutation frequency is still unknown. The annual incidence of this disease occurred approximately 1/100,000 birth (Froissart et al., 2011) worldwide. More than 111 different germline mutations have been reported and recorded in the Human Gene Mutation Database (HGMD) to date. Over 550 unrelated patients affected with GSD 1a have been studied worldwide and most of the mutations found in the G6PC gene thus far are small gene alterations (Froissart et al., 2011).

1.1 Objectives of the study

This study has three main objectives. The first objective is to identify the mutations in the*G6PC* gene in Malaysian GSD Ia patients using standard molecular genetic methods. DNA-base analysis will be used to screen the mutations in GSD Ia patients and their parents' (and/or siblings, when available) DNA samples. The second objective is to determine the pathogenicity of putatively novel mutations (c.115 A>T, H52L; c.226 A>T, K76X; c.337 C>T, P113S; c.1036 C>G, A346P). For most cases, the pathogenicity of mutations found in this study will be confirmed by comparing data with other published reports. If data obtained are not found in the literature, further supporting evidence will be sought by studying the mutant allele's frequency in the

population. The final objective is to study the effects of these mutations on normal G6Pase function. Mutant construct will be created, and the expression of mutant protein will be analysed. Specific steps include creation of mutant gene constructs, cloning, verification and expression in mammalian cell systems, and finally the analyses of G6Pase activity.

CHAPTER 2:LITERATURE REVIEW

2.1 Glycogen as a source of energy

All living organism need energy to grow and reproduce, maintain their structure and respond to their environment. As for humans, they need energy to perform heavy labour exercises. For every action that requires energy, many chemical reactions take place to provide chemical energy to the systems of the body, including muscles, nerves, heart, lungs and brain. One of the important sources of energy in humans is glycogen.

Glycogen is a large energy source for the body and also a multi-branched polymer of glucose that contains a minor amount of phosphate and glucosamine (Maria *et al*., 2016). Glycogen is the common form of energy storage synthesized by animal, fungi and eukaryotic microorganism (Yong & Todd, 2014). These glycogens are structurally analogous to amylopectin, which is a component storage of energy in plant, that is composed of the α-1, 4 linked glucose units interconnected by every 10^{th} residue comparatively more extensive than α-1, 6 linked glucose branches (Figure 2.1) (Berg *et al.*, 2002).

Branches of glycogen with normal glucose are distributed at even intervals that result in a structure with spherical shape (Maria *et al*., 2016). Glycogen molecules are composed of over 120,000 glucosyl units which are linked together in a "tree-like" appearance with comparatively long inner chain (Mahler, 1969). In 2016, Maria and her colleague stated that glycogen particles are made up of up to 55 000 of glucose residues. In the liver, the size of glycogen particles are approximately 110–290nm in diameter while in the skeletal, glycogen particles are much smaller compared to the ones in the liver, having a size range of 10-44nm (Maria *et al*., 2016).

At present, the size of the glycogen molecule can be identified by electron microscopy inside the cells (Maria *et al*., 2016) apart from using localized CNMR

spectrometry which is noninvasive (Grutter, 2012). Other that checking the size of glycogen, the metabolism of the glucose can also be traced using tapping of radio-active phosphorylated deoxyglucose (Grutter, 2012).

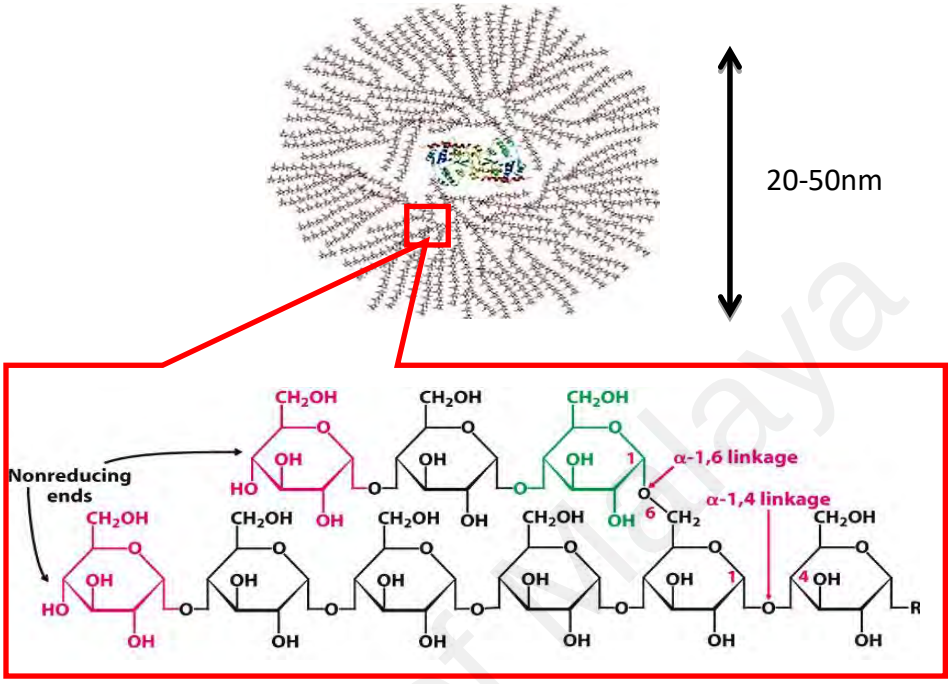


Figure 2.1: Structural of glycogen polymer showing the position of α-1,4 linked glucose units interconnected with α-1,6 linked glucose branches. In this structure of the outer branches of a glycogen molecule, the residues at the non-reducing ends are shown in purple and residue that starts a branch is shown in green. (*Source:* edited from Berg *et al*., 2002)

2.1.1 Function of glycogen

Glycogen is a branched polymer of glucose that permits glucose storage in humans which is responsible as a secondary long-term energy storage. Indeed, the primary function of glycogen is to be the reservoir of glucose which acts as energy in many species, in various tissue types and in mammalian cells (Peter and Anna, 2012). When our bodies need energy, glycogen will be converted into glucose in the liver or muscles cells and transported to the energy-requiring cells (Charles *et al.*, 2001). This glucose will be used throughout the body including the central nervous system.

In humans, there are two sites which have glycogen in abundance, namely the liver and skeletal muscles. However, the total concentrations of glycogen in both sites are different. The total concentration of glycogen in the liver is higher compared to skeletal muscles in ratio of 10% versus 2% of weight (Berg *et al.*, 2002). Nevertheless, Berg and colleagues argued that in terms of mass, the overall glycogen storage is mainly found in the skeletal muscles. It is interesting to note that different sites of glycogen storage feature different functions. In the liver, the glycogens are synthesised and degraded in order to maintain the blood glucose level regulation as required to meet the needs of whole organisms (Agius, 2015). Meanwhile, the glycogens in skeletal muscles are used mainly to regulate the energy needed by the muscles themselves.

Other than the functions mentioned above, glycogen also functions as buffer to sustain the blood glucose levels especially when glycogen is virtually the fuel used in the brain during prolonged starvation (Berg *et al.*,2002). As described by Grutter (2012), glycogen represents the greatest glucose depot equivalents in the brain. The presence of glycogen concentration in the human and rodent are about 5μmol glucosyl units per gram for every 10 minutes.

Although glycogens are predominantly found in the liver and skeletal muscles, it also has been identified in other human tissues, for example the heart, kidney, adipose tissue and erythrocytes. However, the functions of these tissues are mostly unknown yet (Maria *et al*., 2016).

2.1.2 Diseases involving glycogen metabolism

As mentioned by Chen and Burchell, there are several categories of problems involving glycogen metabolism that have been identified since 1995, and one of the main contributing factors that leads to abnormal glycogen metabolism diseases are heritable genetic lesions. Glycogen Storage Diseases (GSDs) are the most common

group of disorders that is caused by problems in glycogen metabolism. Different types of GSDs are caused by different malfunctions of glycogen. For instance, GSD type 2 is caused by deficiencies of theα-1, 4 glucosidase (acid maltase) link and this GSD type has also been called the lysosomal storage disease which is defined by neuromuscular disease, metabolic myopathy and cardiac disorder (Barbara, 1961).

Defects in glycogen metabolism can also leads to Human Type 2 Diabetes Mellitus, which is caused by a post binding defect in action of insulin due to an intrinsic property of peripheral cells or caused by a change in total concentration of humoral component in plasma, or both (Cooper *et al*., 1988). Cooper and his colleagues further explained that this disease is the result of large decreases in the rates of glucose uptake in peripheral tissue. However, uncompleted glucose-induced suppression of hepatic glucose production is also probably a factor.

Peter and Anna (2012) showed that polysaccharide storage myopathy (PSSM) is associated with the formation of polyglucosan. The formation of polyglucosan is due to the activation of glycogen synthase that increased the level of precursors and indirectly developed over-accumulation of glycogen. In humans, formation of polyglucosan altered the branching pattern of the abnormal glycogen and caused the imbalance between glycogen elongation through glycogen synthase and branching mediated by branching enzyme (Peter & Anna, 2012).

2.2 Glycogen Storage Diseases (GSDs)

Glycogen storage disease (GSD) is a group of disorders that is caused either by abnormalities of glycogen synthesis (glycogenesis) or glycogen degradation (glycogenolysis). In addition, other causes of this category of disorders are errors in the process of converting glucose to energy (glycolysis), and the process of glucose formation from non-carbohydrate source (gluconeogenesis). Killman and Oldfors

(2014) mentioned that any genetic defects of glycogen metabolism can cause GSDs that can be diagnosed histologically by abnormal quantity and quality of glycogen in the cells involved. As a result, a gradual abdominal protuberance from early childhood and common hepatomegaly symptoms pointed to be a glycogen metabolic disorder (Berhman *et al.,* 1987).

Different types of GSDs may have different prenatal and neonatal onset, which some of GSDs may lead to fatal, which demises or death within first year of life, whereas other types of GSDs may only present with exercise intolerance or with no clinical symptoms until adulthood (Wang *et al*., 2013). So far, Wang and colleague (2013) discovered that the prevalence rates of GSDs have been underestimated because of their clinical presentation and rare occurrence, and especially lack of efficient molecular diagnostic methods. The overall occurrence of these diseases, as estimated by Zheng and friends in 2014 is about 1: 20 000 to 40 000 cases per live birth.

2.2.1 A brief account of different types of GSDs

GSDs are classified according to the type of enzymatic lesions and on the basis of distinctive clinical defects (Kannouraskis, 2002). Each of GSDs' designation are named after its specific enzyme defect and organ impairment (Marcelo *et al*., 2013; Zheng *et al*., 2014). To date, there are over 12 different types of GSDs, in which some of them are also divided into subgroups. Since glycogen is abundant in the liver and muscles tissues, GSDs can be subdivided into those affecting the liver and also those affecting muscles (Smit *et al*., 2006). Table 2.1 shows the types of GSD and figure 2.2 shows the scheme of glycogen metabolism stating all types of GSDs involved in glycogenolysis and glycolysis.

Table 2.1: List of types of GSD and its description

GSDs type	Description	References
GSD type I- von Gierke's disease	Caused by a defective glucose-6-phosphatase enzyme which will forbid the hydrolysis of Glucose-6-phosphate (G6P) into phosphate and glucose.	(Von Gierke, 1929)
GSD type II- Pompe's disease	Caused by generalized deficiency of the lysosomal enzyme, acid α-glucosidase or acid maltase.	(Makos *et al*., 1987)
GSD type III- Cori's disease	Caused by the deficiency in glycogen debranching enzyme, the amylo-alpha-1, 6-glucosidase,4-alpha-lucanotransferase (*AGL)*	(Lucchiari *et al*., 2003).
GSD type IV- Andersen's disease	Caused by the lack of glycogen branching enzyme (*GBE*).	(Moses & Parvari, 2002)
GSD type V- McArdle' deficiency	Cause by the complete inability of those affected to convert glycogen into glucose in the muscle due to a complete lack of activity of the enzyme phosphorylase in the muscles	(Servidei *et al*., 1988)
GSD type VI- Hers disease	Due to the deficiency of the liver isoform of glycogen phosphorylase.	(Hendrickx & Willems, 1996)
GSD type VII- Tarui disease	Because of enzyme phosphofructokinase (*PFK*) deficiency and affecting the muscles.	(Smit *et al*., 2006)
GSD type IX	Due to the deficiency of enzyme phosphorylase β kinase (*PHK*) which is reflected by the mode of inheritance such as X-linked	(Huijing & Fernandes, 1969)
GSD type X	Characterized by enzyme phosphoglycerate mutase deficiency	(DiMauro *et al*., 2004)
GSD type XI	Defect in the gene encoding lactate dehydrogenase A (*LDHA*) gene	(Tsujibo *et al*., 1985)
GSD type XII	Known as the disorder of red blood cell aldolase A deficiency.	(Kreuder *et al*., 1996)
GSD type O	Deficiency of liver glycogen synthase.	(Holme *et al*., 2005).

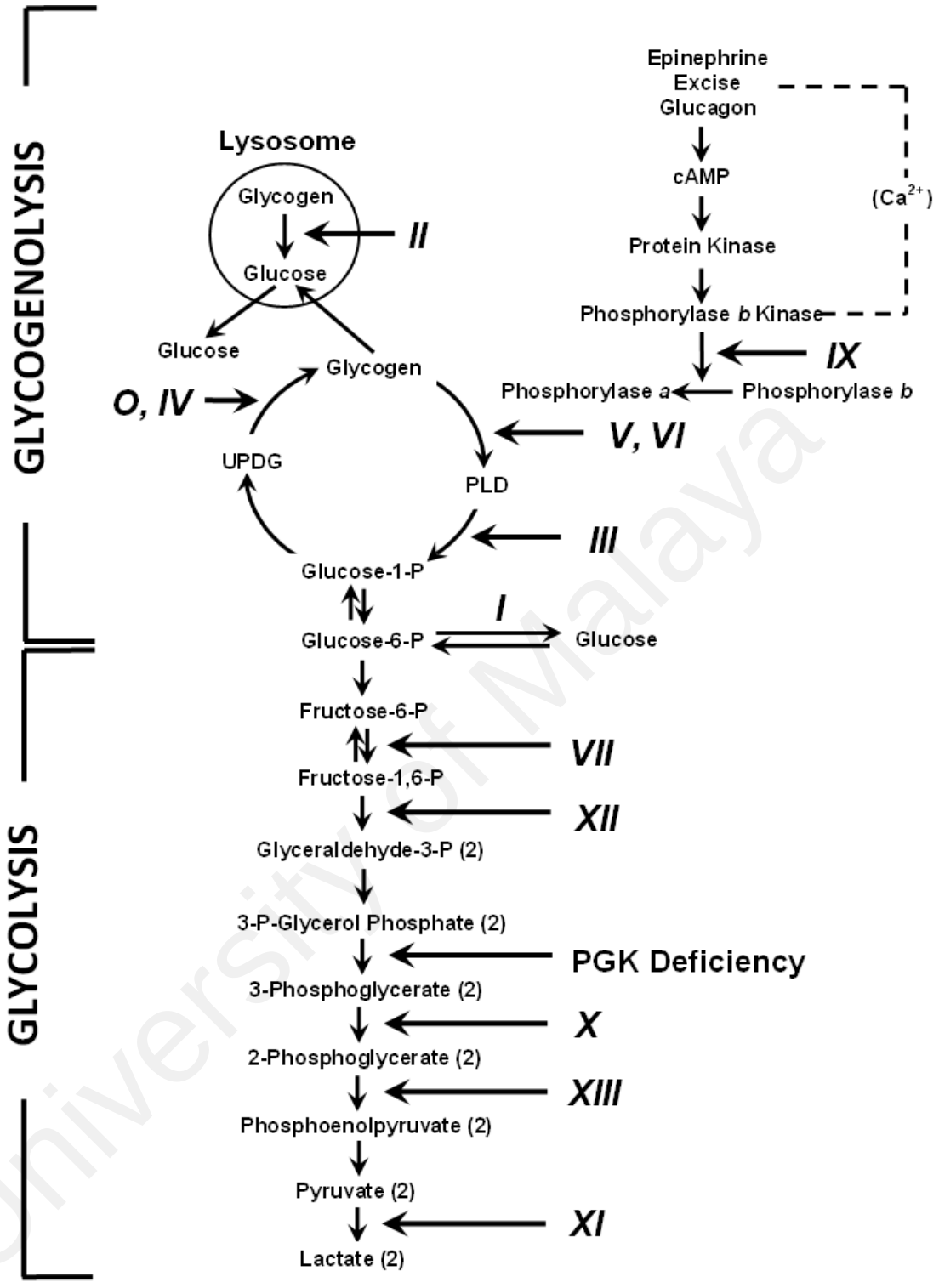


Figure 2.2: Scheme of glycogen metabolism pathway. The roman numerals indicate the location of enzyme defects for each types off GSDs. P, phosphate; PLD, phosphorylase limit dextrin; UDPG, uridine diphosphate glucose. (*Source*: Smit *et al.,* 2006*)*

2.3 Glycogen Storage Disease type I (GSD type I)

As previously mentioned, Glycogen Storage Disease Type I is the first type of GSDs described clinically and also the first type to be explicable in biochemical basis. Chronologically, this GSD type was first proposed in 1928, where Van Crevald found a symptom of hepatomegaly in an 8-year-old boy as well as showing features of carbohydrate metabolism syndrome. Later, in 1929, Von Gierke published a description of the clinical and pathological finding of the enlarged liver and kidney in two children, where at autopsy were shown to have accumulated huge amounts of glycogen. Since then, GSD Type I is also known as Von Gierke's disease.

GSD Type I occurs as an autosomal recessive inherited disorder and is composed of 4 subtypes depending upon the deficient enzyme involved and also based on biochemical and chemical heterogeneities (Arion *et al.,* 1980;Lange *et al.,* 1980; Nordlie *et al.,* 1983; Burchell *et al.,* 1985; Hers *et al.,* 1989; Burchell and Waddel, 1990; Beaudet, 1991). GSD Ia and 1b are the major subgroups that have been defined based on clinical and biochemical criteria. As reviewed by Shieh *et al.* (2002), more than 80% of GSD type I cases are represented by GSD Ia, that are caused by a defective of glucose-6-phosphatase (G6Pase) enzyme which catalysed the hydrolysis of glucose-6-phosphate (G6P) in terminal steps in gluconeogenesis and glycogenolysis.

The second most common GSD type I is GSD type Ib (MIM232200), and this type of GSD is caused by deficiencies in the transport of G6P into the microsome by involvement of the glucose-6-phosphate translocase (*G6PT*) gene (Shieh *et al*., 2002). It is also associated with normal G6Pase catalytic activity (Weston *et al*., 2000). Both GSDIa and Ib share a common metabolic phenotype which is characterised by fasting hypoglycaemia, hepatic glycogen accumulation and hepatomegaly (Martin *et al*., 2002; Chou *et al*., 2014)

GSD Ic and Id are much less common compared to GSD Ia and Ib, so much so that there are insufficient information for a general description of its clinical symptoms (Lei *et al*., 1994). As highlighted by Jenecke and colleagues (2001), patients with GSD Ic have been diagnosed with increased latency in hepatic microsomal inorganic pyrophosphate activity.

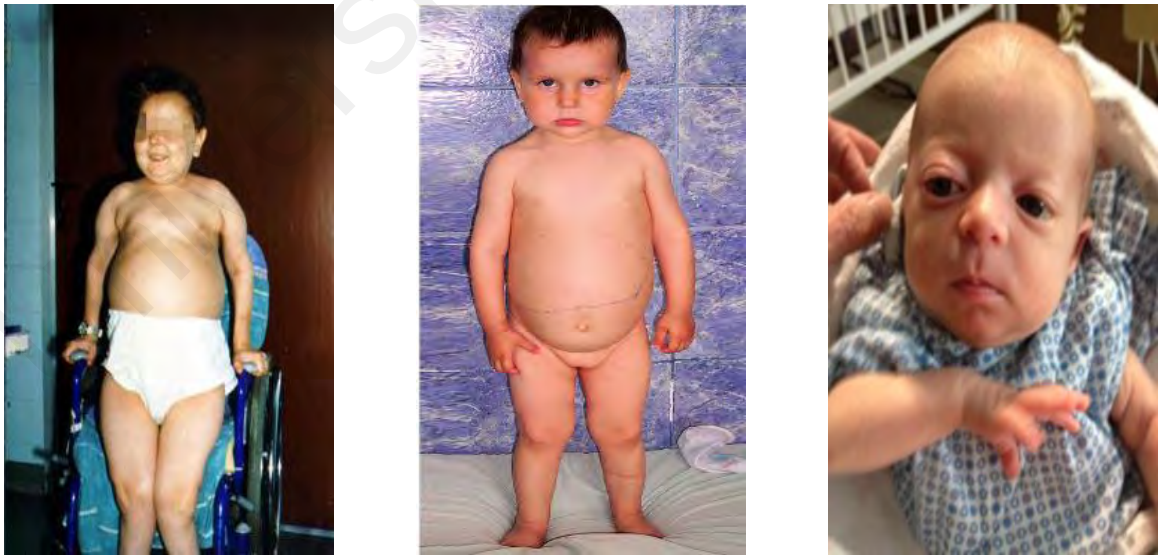
2.3.1 Clinical manifestation of GSD Ia

GSD Ia is the most clinically severe form of GSDs where it represents approximately one-fourth of the hepatic glycogenosis (Froissart, 2002). This fact was also supported by Marcelo *et al.,* (2013) who stated that GSD Ia is responsible for more than 90% of cases affecting mainly the kidney and liver. The prevalence of this autosomal recessive inheritance is still unknown (Froissart *et a.l*, 2011) and the annual incidence estimated is about 1 in 100 000 up to 1 in 400 000 live birth (Marcelo *et al*., 2013). Carvalho *et al.,* (2013) depicted that the carrier frequency of GSD Ia is expected to be 1 in 150 worldwide.

Generally, the symptoms and clinical phenotypes of GSD Ia patients are usually present at an early stage of life. Infants at 6 to 8 months of age typically present seizures and hepatomegaly (Figure 2.3) (enlargement of the liver due to accumulation of glycogen and fat) as common symptoms. As described by Muller (Muller *et al*., 1961), the infants are clinically manifested with growth retardation which is displayed by a small body size with "doll-like" features (Figure 2.3) and a sticking enlarged liver. Other than that, the kidneys are also enlarged at times. In some cases stated by Froissart (Froissart *et al*., 2011) infant at earlier age of 3 months shows the first symptoms of hepatomegaly which means the liver may have already be enlarged at birth.

Since GSD Ia patients are unable to synthesis glucose from glycogen sources, clinical manifestation are usually in relation with the level of glucose or reserve compound in the body. The accumulation of the abnormal glycogen mainly occurs in the liver, kidney and intestinal mucosa (Mahler, 1969). Deficiencies of G6Pase enzyme have been known to cause life-threatening, hypoglycaemia (low blood sugar) and growth retardation (Figure 2.3) (Chen, 2001).Studies by Miller and friends (1977) mentioned that those who survived in the early labile period will suffer incomplete sexual maturation, pathologic bone fractures and have slow psychomotor development (Burchell *et al*., 2000).

Other metabolic consequences associated with GSD1a include hyperuricemia (elevation of blood uric acid), hyperlipidemia (high cholesterol level in blood) and hyperlacticacidemia (build-up of lactic acid in the bloodstream) (Chou & Mansfield, 1999).For adults patients, they will experience long term complications which include hepatic adenomas that can develop into hepatocellular carcinoma (HCC)(Chen, 2001; Franco *et al*., 2005a), gout, renal disease and growth failure (Wang *et al*., 2011).



(a) (b) (c)

Figure 2.3: Examples of the symptoms of the GSD1a disease resulting from **(a)** growth retardation. Retrieved from http:emedicine.medscape.com/article/1116574 ; **(b)**hepatosplenomagaly (Adapted from: Moruru *et al*., 2007) and **(c)** "doll-like" face. Retrieved from http:emedicine.medscape.com/article/1116574

2.3.2 Diagnosis and treatment of GSD1a

The diagnosis of this disease is usually made on basis of clinical symptoms and biochemical abnormalities, histologic analysis and measurement of G6Pase activity in the liver by biopsy (Lei *et al*., 2014). Lei and colleague (1993) reported that the current treatments are solely focus on controlling symptomatic effect. In 1998, Lam and colleagues depicted that GSD Ia patients are to be diagnosed by liver biopsy. The liver tissue of the patients was taken and subjected to a G6Pase enzyme assay. This assay will ensure the G6Pase protein activity in the liver (Lei *et al*., 1995).

However, liver biopsy analysis was not well accepted by patients and family because the procedure was invasive and included high-risk procedure to patients (Lam *et al*., 1998). Since the biopsy is invasive, the disease can be detected by prenatal diagnosis and DNA-based diagnosis analysis by analysing the nucleotide level of patients' and parents' blood (Kajihara *et al*., 1995; Lei *et al*., 1995).

Nowadays, due to increased knowledge of genetic basis and high mutation detection rate of mutations, another type of diagnosis was designed by other studies. In 2000, Kozak and colleagues (Kozak *et al*., 2000) using denatured gradient gel electrophoresis (DGGE) analysis to analyse a huge amount of patients at a time. Another screening and detection assays was established in 2002 by Matern and colleague (Matern *et al*., 2002). DNA sequencing analysis and Single-strand conformation polymorphism (SSCP) analysis were done in order to check the spectrum and frequency of the mutation in a population. It is the easiest way to do a parental testing and carrier detection (Ki *et al*., 2004).

So far, there is no cure for GSD Ia patients. However, the symptoms of the disease can be dealt and improved using diet therapies and therapeutic adjuvant drugs in order to maintain normoglycemia and to limit secondary metabolic disorder (Yiu *et al*., 2010).

GSD Ia patients are therefore unable to maintain the concentration of blood glucose outside the time meal, so, they need to manage their meal by continues feeding to prevent them from suffering from hypoglycemic episode (Mutel *et al.,*2011)

In controlling their diet, meticulous dietary guidelines have been made in any available format with strict adherence and interprandial supplementation (Salganik *et al*., 2009). This is so that, GSD Ia patients can have a pretty good diagnosis and are able to maintain their healthy lifestyle. This task could be an incredible challenge since the severities of symptoms are often found in childhood. By age, glucose requirement of patients will decrease. Here, patients' theoretical glucose production rate are calculated and are only supplied with required amount needed to prevent excessive amount of exogenous glucose and the increase of peripheral body fat storage (Smit *et al*., 2006).

In terms of pharmacological treatment, several types of drug are supplied in order to decrease the severity of the symptoms. For example, sodium bicarbonate is recommended to reduce hyperlactatemia. This drug is used to induce alkalisation of the urine and can diminish the risk of urolithiasis and nephrocalcinosis (Smit *et al*., 2006). Intake of uric acid also helps against the development of atherosclerosis since uric acid is a potent radical scavenger (Smit *et al*., 2006).

2.3.3 Epidemiology and prevalence of GSD1a

To date, more than 111 different germline mutations have been reported and recorded in Human Gene Mutation Database (HGMD). Over 550 unrelated patients affected with GSD 1a have been studied worldwide (Zheng *et al*., 2014) and all of the mutations found in the *G6PC* gene thus far are small gene alterations (Froissart *et al.,* 2011). Small gene alterations include missense mutations, small base deletions and insertions, and splice site mutation (Zheng *et al*., 2014). The most frequently found mutations are missense mutations at 64% (Rake *et al.*, 2000; Janecke *et al.,* 2001).

GSD Ia is not only predominantly restricted to any racial or ethnic group. Nowadays, the allelic homogeneity have been defined in some geographical region (Eminoglu *et al*., 2013). The mutation in *G6PC* gene is seemingly unique to Caucasian, Hispanic, Chinese, Japanese, Korean and Jewish, which suggests separated ethnic founder effects are for some mutations (Chou *et al*., 2014).

Some of the mutations are found at a higher frequency such as the R83C and Q347X mutations that were found at 48% and 21% respectively in the Caucasian population especially in the United States and Europe (Eminoglu *et* al., 2013; Marcelo *et al*., 2013). An even higher frequency of R83C mutation was found in Jewish patients (98%) (Froissart *et al.,* 2011). Other common population-specific mutations include R83C (93%) in Jewish, R83H (70%) in the Chinese population (Eminoglu *et al*., 2013) and R170Q in the Tunisian population (Barkaoui *et al.,* 2007). L216L mutation was reported to present mild clinical finding and most common mutations in East Asia region including in Japan (88%), Chinese (54%) (Froissart *et al*., 2011) and Korean (75%) (Lei *et al*., 2014).

2.4 Glucose-6-phosphate, catalytic subunit (*G6PC*) gene

Glucose-6-phosphate catalytic subunit (*G6PC*) gene is the gene that is responsible for GSD Ia. Any mutation that is found in the gene will contribute to the development of the disease and directly lead to the defective of G6Pase protein. The sequence of the *G6PC* gene has been established for more than 10 years now (Clottes *et al*., 2002).

2.4.1 Background of *G6PC* gene

In humans, the *G6PC* gene (GeneBank accession number: NG_011808.1) is located in cytogenetic band q21 of chromosome 17 (17q21) (Figure 2.4). The gene spans approximately 12.5kb and consists of 5 exons (Figure 2.5) (Shu *et al*., 2000). As reviewed by Rake *et al*., 2000 this gene encodes a protein of 357 amino acids with a

total molecular mass of 35kD. This gene is located mainly with its catalytic site oriented towards the luminal side of the endoplasmic reticulum (ER) membrane in liver and kidney cells as a part of a multi-component system in the liver (Burchell *et* al., 2000; Guionie *et al*., 2003).

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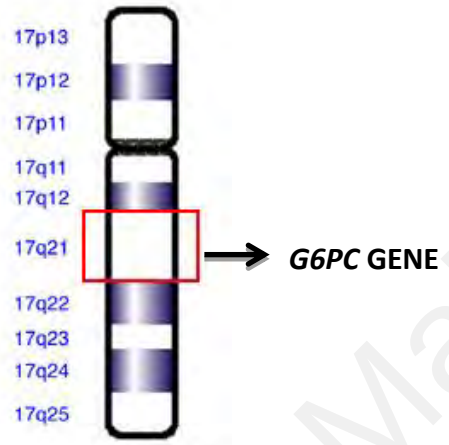


Figure 2.4: Human chromosome 17. Arrow and red box indicate the location of *G6PC* gene on human chromosome 17 (*Source*: edited from Shieh *et al*., 2002)

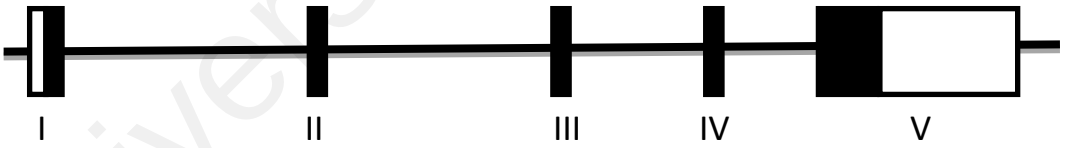


Figure 2.5: The gene structure of human *G6PC* transcription unit. The exonic regions are indicated by filled box (black box) and the untranslated regions by open boxes (White box). The Roman numerals represent the number of exons (*Source*: edited from Shieh *et al*., 2002)

2.5 Glucose-6-phosphatase G6Pase enzyme

The *G6PC* gene encodes a protein named Glucose-6-phosphatase (G6Pase) enzyme. G6Pase is a catalytic enzyme in G6Pase complex which also consists of glucose-6-phosphate translocase (*G6PT*) enzyme. As reviewed by Barbara (Barbara *et al*., 1961) this enzyme is important in the maintenance of the blood glucose level in the normal

liver and other parts of the body. Defective of the enzyme accounts for the clinically observed tendency to hypoglycaemia and other main symptoms of GSD Iaand also indirectly disrupts the G6Pase activity (Hume & Burchell., 1993; Kajihara *et al*., 1995). Among the enzymes involved in the glucogenic pathway, G6Pase enzyme is unique by virtue of its intimate association with the membranous element of the ER (Arion *et al*., 1976).

2.5.1 Structure of G6Pase enzyme

Human G6Pase (E.C.3.1.3.9) is a primal enzyme for endogeneous glucose production in specific organ, catalyzing the hydrolysis of G6P to glucose and inorganic phosphate (Chou *et al*., 2010). G6Pase is the key enzyme in the blood glucose homeostatic which catalyzes the terminal step in both gluconeogenesis and glycogenolysis (Shelly *et al*., 1993). As important enzyme in glucose homeostasis, problem regarding their activity will disrupt the production and release of glucose to the blood and causes the GSD type I, the most severe form of GSDs because of its exhibit latency (Shieh *et al*., 2005).

Human G6Pase is extremely hydrophobic andcontains nine putative membrane-spanning segments (Lei *et al.,* 1993) with a molecular mass of about 40 kDa (Smit *et al*., 2006). Topology analysis shows that G6Pase contains multiple transmembrane domains that hold it within the endoplasmic reticulum (ER) membrane (Pan *et al*., 1998). The N-terminus of G6Pase is located in the ER lumen and its C-terminus is located in the cytoplasm (Pan *et al*., 1998). A review by Ghosh and colleague (Ghosh *et al*., 2002) discovered that sequence alignment of mammalian G6Pase, lipid phosphatase, acid phosphatases and vanadium haloperoxidase (VCPO) shared a conserved phosphatase signature protein.

The active site of the protein comprised of Lysine-76 (Lys76), Arginine-83 (Arg83), Histidine-119 (His119), Arginine-170 (Arg170) and Histidine-176 (His176) (Ghosh *et al*., 2002). All the active sites are predicted to be situated in the luminal side of ER membrane, except for Lys76 which is predicted to be within helix 2. The helices 1, 3, 4, and 5 are proposed to be in close contact with each other for the binding of phosphate molecule and also hydrolysis process of G6P molecule, thus forming the core of the catalytic centre in the G6Pase enzyme (Hemrika & Weve, 1997; Pan *et al*., 1998).

The structure of the enzyme can be used to study the correlation between specific mutations and their effects. It is shown that the enzyme's stability and confirmation is determined by structural integrity of transmembrane helices in which protein with abnormal confirmation are rapidly eliminated through intracellular protein degradation. Furthermore, amino acid residues that are formed at the catalytic center and non-helical regions in G6Pase play no important role in the stability of the enzyme. Thus, mutation that resides in the amino acid residues of transmembrane helices will cause the degradation of protein products while mutation that resides in the coding sequence for the luminal loops or cytoplasmic loops will generally cause a reduction of protein activity (Shieh *et al*., 2002).

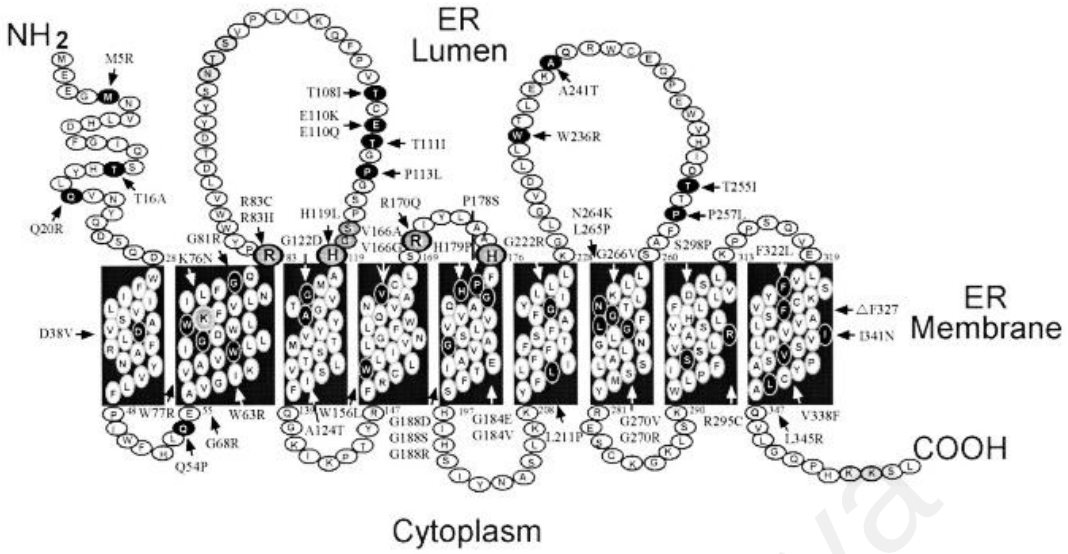


Figure 2.6: Molecular structure of G6Pase protein. Missense mutations that have been reported in the *G6PC* gene of GSD1a patients are marked in black (Figure adapted with permission from Shieh *et al*.,2002)

2.5.2 Activity of G6Pase in endoplasmic recticulum (ER) membrane

G6Pase is the hallmark step for steps glucogenesis and glycogenolysis. This enzyme is located inside the lumen of the endoplasmic reticulum (ER) membrane. Therefore, the substrate and the products of the enzyme have to cross the ER lumen first before published into the whole body. As referred to the figure 2.7, the G6Pase enzyme are associated with a calcium binding protein (SP) and three different transport systems or translocase protein for the substrates glucose-6-phosphate (G6P) via T1, the products phosphate (Pi) via T2 and glucose via T3 (Burchel, 1996).

These translocase proteins are required to respectively transport the substrate G6P, Pi and glucose across the liver ER membrane (Nordlie *et al*., 1993). Genetic evidence has proven that deficiency of endoplasmic facilitative transport protein of the G6Pase product will cause different symptoms of human genetic diseases and also have been reviewed elsewhere (Burchell, 1996). Hence, it is important to be able to cross the product over the ER to keep the G6Pase function normally (Burchell, 1996).

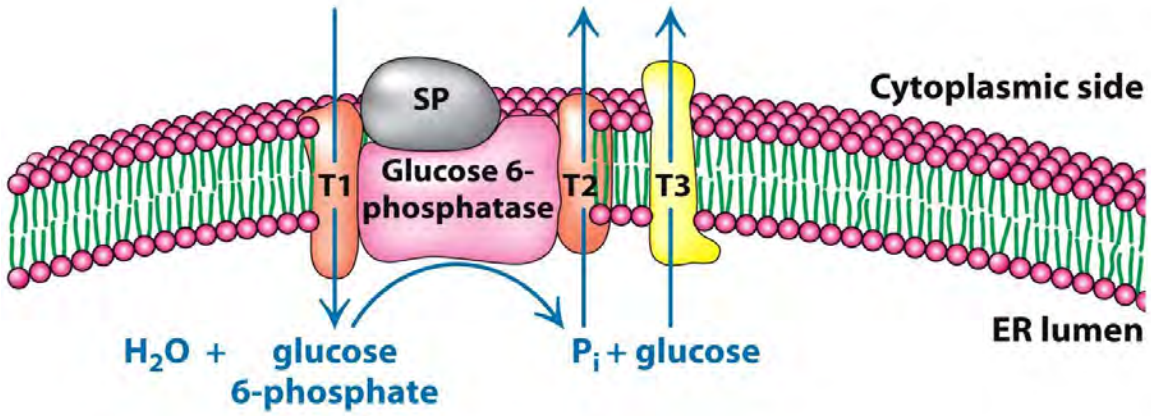


Figure 2.7: Scheme of the luminal of ER membrane and translocases to transport and released the glucose. SP: calcium binding protein; T1: translocase 1; T2: translocase 2; T3: translocase 3 (Figure adapted with permission from Berg *et al*., 2002)

2.6 Mutation screening in samples population

Mutation screening and detection of clinical samples are a crucial process in genetic testing of heritable disease. Traditionally, diagnosis of novel mutations of GSD1a has been confirmed by liver biopsy. However, this procedure is very invasive and is not well accepted by the patients and their parents. Nowadays, many quick and precise screening methods are available for the use in genetic testing such as DNA-based sequencing. For screening the population samples, other alternative methods are recommended to be applied in order to compare the novel mutations found with the huge number of panel of healthy control samples.

Thamhankar and associates (2011) used restriction endonuclease (RE) assay to screen the mutation in patients by applying specific restriction enzyme. This enzyme will cut into specific restrictions sites; either cut the normal strand or mutant strand. In this study, they used *Eae*l restriction enzyme to screen H119D mutation in Indian patients.

Other alternative to screen the mutation is by doing genotyping analysis. Gaedisk and colleague (2014) analysed their samples by TaqMan SNP genotyping assay. This assay

is widely used in the clinical and research settings for genotype analysis due to assay reliability, low cost and the availability of commercially available assays.

2.7 Functional analysis

To investigate the pathogenicity level of the novel mutations found, we need to do functional analysis as evidence of the infective of the mutation. Since GSD Ia is caused by the defective of the G6Pase enzyme in converting the glycogen into glucose, various functional test was done to check the absence of G6Pase activity in the liver, either by administration of glucagon or adrenaline or both, which stimulate the glycogenolysis and give results of the blood glucose level (Mahler, 1969).

Enzymological test is done in studies conducted by Reis and colleagues (2001) to confirm the mutation on the respective genes. Minigenes of the mutations construct is done and *in-vitro* transient expression is done to check the G6Pase activity and phosphohydrolase activity. In other studies, northern hybridization and western blotting analysis are done (Lei *et al*., 1993). Northern hybridization analysis is used to look into expression of the mRNA levels of G6Pase on *in-vitro* transient and western blotting is used to check the G6Pase protein level.

CHAPTER 3: METHODOLOGY

3.1 Study subject and samples

The subjects of this study comprises of Glycogen Storage Disease (GSD) type 1a patients either with their parents, sibling or alone, with no available information on family background (ethics approval and patient consent refer to Appendix C). Some of them are referral cases received from General Kuala Lumpur Hospital (HKL) and University Malaya Medical Centre (UMMC). A total of 42 DNA samples were obtained. Blood samples and extracted DNA samples of patients are kept in -20ºC for long term storage.

3.2 Genomic DNA extraction

DNA was extracted from the blood sample using QIAGEN DNA extraction kit (QIAGEN, Germany) following the instruction manual provided by the manufacturer. Prior to extraction, blood samples and RNase A solution stored in -20 ºC were first equilibrated to ambient temperature. To start, 20 µl of Proteinase K solution (provided with the kit) were transferred to the bottom of a sterile 1.5 ml microcentrifuge tube followed by 200 µl of blood. Two microliters of RNase A stock solution (100 mg/ml) was also added into the blood mixture to eliminate any trace of RNA. Equal volume of Buffer AL to the blood was then dispensed into the mixture and it was homogenised by pulse vortexing for approximately 15 seconds. The mixture was then incubated at 56 ºC for 10 minutes in a heating block to facilitate cell lysis.

After the incubation step was completed, the tube was removed from the heat block and the content of the tube was spun down briefly. Next, one volume of 100 % ethanol was added to the lysed sample. The mixture was pulse-vortexed for 15 seconds, centrifuged for another 15 seconds at 12,000 ×*g* and transferred into a fresh spin column (provided with the kits). The transfer has to be done carefully so as not to wet the rim of

the column. The column was then centrifuged at 6,000 ×*g* for one minute. Later, DNA washing step was carried out by adding in 500 µl of ethanol-diluted Buffer AW1 to the spin column followed by the centrifugation at 6000 ×*g* for one minute. After discarding the filtrate, the washing step was repeated by adding 500 µl of ethanol-diluted Buffer AW2 into the same spin column before it was centrifuged at 20,000 ×*g* for another time.

To avoid any risk of Buffer AW2 carryover from the previous centrifugation step, the spin column was transferred into new collection tube and centrifuged at 20,000 ×*g* for another minute. Then, the column was placed into a new labelled 1.5 ml microcentrifuge tube and 200 µl of Buffer AE was dispensed directly to the membrane filter of the spin column. The spin column was left to stand at room temperature for ten minutes before it was centrifuged at 6,000 ×*g* for one minute. Finally, the column was removed from the tube and discarded. The DNA elution was then assessed for its quality by gel electrophoresis and quantified using a nanophotometer. The extracted DNA product was stored at -20 ºC for future use.

3.3 DNA quantitation

Measurement of DNA concentration and purity was carried out using the Implen nanophotometer (IMPLEN, Germany). The appropriate measurement setting, and dilution factors were first keyed in before readings were taken. Then, diluted samples were transferred and placed into the photometer. The sample was measured, and the measurements of the DNA concentration, purity or OD_{600} readings were recorded for future reference.

3.4 Agarose gel electrophoresis

Agarose gel electrophoresis was done using agarose gel as the separation medium, to determine the quality and specificity of the product. The following preparation was done using electrophoresis system from Major Science (USA).

3.4.1 Gel preparation

In the preparation of separation gel, agarose powder (Promega, USA) was first weighed to the appropriate amount corresponding to the desired working concentration and placed into conical flask. Then, 1X Tris Borate EDTA buffer (TBE buffer; 445mM Tris base, 445 Mm borate and 10 mM EDTA) was added into a clinical flask with equal volume of the gel. The complete protocol for TBE buffer preparation can be found on Appendix A.

The mixture was boiled using a microwave oven for approximately 60 to 90 seconds to dissolve the agarose powder. One microlitre of Ethidium Bromide (EtBr, Sigma, USA) was added into the molten gel after cooling it to about 50 ºC. Later, the molten gel was poured into a gel caster which had been assembled in advance. The gel caster was fixed with a comb that has the desired size and number of tooth. The pouring process must be done as quickly as possible to avoid the gel from solidifying in the flask. The gel was left to set for about 15 to 20 minutes at room temperature.

3.4.2 Electrophoresis

The comb in the hardened gel was removed from the cast and the gel was then placed in the electrophoresis tank filled with 1X TBE buffer. Five microliters of DNA sample were mixed with 1 µl of 6X loading dye (30 % glycerol, 20mM EDTA, 0.05 % bromophenol blue and 0.0 5% xylene cyanol) before it was loaded into a well in the gel. Then, 3 µl of 100bp DNA marker (Seegene, Korea) was placed in a well next to the samples to estimate the expected size of the product. Electrophoresis was carried out at 120V for 25 minutes and the gel was viewed in the Alpha Imager system (Alpha Innotech Corp, USA).

3.5 Polymerase Chain Reaction

3.5.1 Primer design

Every exon of the *G6PC* gene was amplified using exon-specific primer pairs. Ten pairs of specific forward and reverse primers were designed, and sequence references were made based on the published sequence of the *G6PC* gene (Angaroni *et al.,* 2004). Primers were designed to complement sequences with adjacent exons at both ends to facilitate fragment joining in overlap extension process. Some necessary modifications of the primer sequences were done to generate the derived gene fragments according to the aims of this project. The primer sequences were checked using Sequence Manipulation Suite: PCR Primer Stat program. As shown in Table 3.1, different exons were amplified with different sets of primers.

3.5.2 PCR Master mix preparation

PCR reagents were kept at -20 ºC for long-term storage. Before use, the PCR reagents except for *Taq* polymerase (EURx, Poland) were thawed to ambient temperature, mixed by vortexing and spun down briefly to collect the contents to the bottom of the tube. In the meantime, the volumes of the PCR reagents per reaction multiplied to the desired number of reactions were calculated.

The master mix of the PCR was prepared by adding the following reagents to the final volume of 25 µl: 1X PCR buffer, 1.5 Mm $MgCl_2$, 200µM of each dNTP, 0.4 µM of each forward and reverse primers and 1 U *Taq* polymerase. Sterile distilled water was added to make the final volume up to 25µl. Then, the mixture was mixed and aliquoted into 0.2 ml labelled PCR tube. Approximately one hundred nanograms of purified DNA was pipetted into each PCR tube. Additional reactions were included in the master mix calculation and prepared to serve as negative control. The complete protocol for PCR master mix preparation can be found on Appendix A.

Table 3.1: List of primer sequences (10 µM) used in GSD 1a PCR screening.

Exon	Primer	Sequences	Size	Ta	Mg2+
Exon1	G6PC_1F	5' CACCTGAACATGTTGCATCAACC 3'	611bp	65 ºC	2.5 mM
	G6PC_1R	5' CCTCTCTCTGACTTTGGATCATCTAC 3'			
Exon 2	G6PC_2F	5' GAGGTCACAATGAGCCGAGATTG 3'	609bp	65 ºC	1.5 mM
	G6PC_2R	5' CTCCATCCAGGTTCTGTAATAGGC 3'			
	G6PC_2Fseq	5' TATCTCCCTCTCACACTTC 3'	470bp	60 ºC	
Exon 3	G6PCF3_a	5' GATGGTAAGATGGGTGGATGGATG 3'	473bp	65 ºC	1.5 mM
	G6PC3R_a	5' TACTGGATCCCTTTCAGCCCAAAC 3'			
Exon 4	G6PC_4F	5' CCAACAGGCATCTTTGGACTTTTG 3'	419bp	65 ºC	1.5 mM
	G6PC_4R	5' CTCATTCTTGACTTTCAACCCACAG 3'			
Exon 5	G6PC_5F	5' ATAAGCCAGGCGACCCTCCCATCTG 3'	827bp	65 ºC	1.5 mM
	G6PC_5R	5' GGAATAAGCCAGGCGACCCTCCAATC 3'			

3.5.3 Amplification of PCR

The PCR reaction solution were mixed and briefly centrifuged after the DNA was added. The tubes then were placed in the thermocycler and PCR was carried out using a program consisting of one denaturation step at 95ºC for 5 minutes followed by 30 cycles of 30 seconds of denaturation at 95 ºC, 30 seconds of primer annealing at specific temperature depends on optimum temperature primer set used and 1 minute of extension at 72 ºC. This was followed by final 5 minutes final extension step at 72 ºC and an incubation step at 25 ºC for another 5 minutes. PCR product were then stored at 4ºC for later use and either analysed immediately by AGE.

3.5.4 PCR Optimization

PCR optimization was done for all exons using healthy control donor before the amplification was performed on patients' DNA samples. The annealing temperature and the Mg^{2+} concentration for each exon were optimized and the parameters that give the brightest and most specific products were chosen to be used for subsequent PCR reactions.

To optimize the annealing temperature, 8 different temperatures ranging from 45 ºC to 65 ºC were tested using gradient PCR to find the optimum temperature that will give the brightest and most specific amplicons. Meanwhile, to optimize the Mg^{2+} concentration, concentrations ranging from 0.5 mM to 2.5 mM were used and the best Mg^{2+} concentrations were selected, based on their brightness and specificity of the PCR product. The best annealing and Mg^{2+} concentration were determined and chosen for each exons, and the optimum value was used for all PCR reactions performed for patients and healthy controls.

3.5.5 Direct DNA purification using QIAquick PCR purification kit

PCR product of the same samples replicates were pooled into a single tube. Then, 5 volumes of Buffer PBI to the volume of pooled PCR samples were added, mixed and vortexed briefly. The mixtures were then transferred into a labelled spin column placed in a 2 ml collection tube. The column was centrifuged for 1 minute to bind the DNA to the membrane. The filtrate was discarded and 750 µl of ethanol-diluted Buffer PE were added followed by 1-minute centrifugation. This washing step rinses away all the non-DNA components and stabilizes smaller pieces of DNA, so that they can be eluted from the column. Any filtration formed was discarded and the column was centrifuged again for another minute to ensure a complete removal of washing buffer form the column.

Later, the column was transferred into a sterile 1.5 ml centrifuge tube. Thirty microliters of Buffer EB was carefully dispensed directly into the centre of the membrane and left for 10 minutes to elute the DNA. After that, the column was spun for 1 minute and the eluted product was ready to use or kept for long-term storage at -20 ºC. The eluted DNA was checked for its quality by AGE as described in section 3.4.

3.5.6 Direct DNA purification using QIAquick gel extraction kit

PCR product contaminated with unspecific amplicons can be purified by separating them using electrophoresis on 1 % agarose gel for 30 minutes at 120V as described in section 3.4. PCR products and replicate reaction mixes were pooled into a single microcentrifuge tube and mixed with 1X loading dye before they were loaded onto the agarose gel. After electrophoresis was completed, the gel was view under UV illumination using the Alpha Imager system (Alpha Innotech Corp, USA)

During this process, the band with the desired size were identified and immediately cut from the gel using sterile scalpel. The gel cut was trimmed from excess gel and transferred into new labelled and weighed a 1.5 ml microcentrifuge tube. During this

step, it is advised to cut short the time to minimize UV exposure on the handler and the PCR product. Again, the tube was weighed to estimate the weight of the gel.

Five volumes of Buffer QG per one volume of the weighed gel were dispensed into the tube containing the gel. Next, the mixture was incubated at 50 ºC for 10 minutes using heat block. The mixture was occasionally vortexed to facilitate proper dissolution of the gel as well as to prepare the DNA for binding. A volume of 100 % isopropanol equal to the gel weight was added into the tube after the incubation step. The mixture was mixed and transferred into the labelled spin column in a 2 ml collection tube, then directly centrifuged at a maximum speed for 1 minute to bind the DNA to the column membrane.

Next, the filtrate was discarded and again 500 µl of Buffer QG was added into the column. The content was centrifuged at maximum speed for another 1 minute to get rid of any residual gel content. Washing step was carried out by adding 750 µl of ethanol-diluted Buffer PE into the column followed by the centrifugation step as done previously. The filtrate was discarded again and spun for 1 minute to remove all the ethanol, so that ethanol does not get into the final eluted product.

The spin column was then placed into a new labelled 1. 5 ml microcentrifuge tube. Thirty microlitre of EB buffer was dispensed directly into the centre of the membrane before it was left to stand for 10 minutes at ambient temperature. Last step, the column was centrifuged at a maximum speed for one minute to elute the DNA from the membrane. After the last step, the spin column was discarded, and the eluted product was stored at -20 ºC for future use. The quantity of the purified DNA was later checked by AGE as described in section 3.4.

3.6 DNA sequencing

The purified samples were sequenced using the automated 3130 *xl* Genetic Analyzer from Applied Biosystem (Applied Biosystem, USA). Sequencing was done using BigDye Terminator Cycle sequencing kit (Applied Biosystem, USA). Sequencing involved two steps; first was the cycle sequencing step, which is likenedto the process of PCR to amplify the DNA template. Second is the ethanol/EDTA precipitation step, which was done before the samples were loaded into the sequencer.

3.6.1 Cycle sequencing

In cycle sequencing, all the reagents used were slightly different from the ordinary PCR. The BigDye Terminator cycle sequencing protocol consists of an initial 60 seconds incubation at 96 °C, followed by 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 minutes. The sequencing reagents can be kept at -20 ºC for long-term storage. At first, the sequencing reagents were thawed to ambient temperature mixed by vortexing and spun down briefly to collect the contents to the bottom of the tube.

The master mix of the sequencing was prepared by adding the following reagents to the final volume of 20µl per reaction: 1 µl of BigDye (which already contained the replication enzyme and deoxyribonucleoside triphosphates (dNTPs), 3 µl of 5X sequencing buffer and 1 µl of 3.2 µM primer. Sterile distilled water was added to make the final volume up to 20 µl. Table 3.2 shows the primer sequences for DNA sequencing. Then, the mixture was mixed and aliquoted into 96-well sequencing plate. Desired amount of purified DNA was pipetted into each well of the sequencing plate. Next, the plate was placed into thermocycler to run the amplification programme. After the cycle sequencing was completed, the samples were either directly used and proceeded to ethanol/EDTA precipitation steps or stored at 4 °C overnight and

continued with ethanol/EDTA precipitation step on the next day. The complete protocol for cycle sequencing master mix preparation can be found on Appendix A.

Table 3.2: List of primer (3.2 µM) used for DNA sequencing.

Exon	Primer (3.2µM)	Sequences
Exon 1	G6PC_1F	5' CACCTGAACATGTTGCATCAACC 3'
	G6PC_1R	5' CCTCTCTCTGACTTTGGATCATCTAC 3'
Exon 2	G6PC_2F	5' GAGGTCACAATGAGCCGAGATTG 3'
	G6PC_2R	5' CTCCATCCAGGTTCTGTAATAGGC 3'
	G6PC_2Fseq	5' TATCTCCCTCTCACACTTC 3'
Exon 3	G6PCF3_a	5' GATGGTAAGATGGGTGGATGGATG 3'
	G6PC3R_a	5' TACTGGATCCCTTTCAGCCCAAAC 3'
Exon 4	G6PC_4F	5' CCAACAGGCATCTTTGGACTTTTG 3'
	G6PC_4R	5' CTCATTCTTGACTTTCAACCCACAG 3'
Exon 5	G6PC_5F	5' TAGCAAAGGTCCCAAATCCTTCC 3'
	G6PC_5R	5' CAAATAGTCCTCCTCAATCCC 3'

3.6.2 Ethanol/EDTA precipitation

The 96-well sequencing plate containing cycle sequencing reaction was vortexed and centrifuged after it was taken out from the machine or refrigerator. First, 5 µl of 125 mM of EDTA and 60 µl of absolute ethanol were added to each 96-well plate. The 96-well plate was mix by vortexing for a few seconds to homogenise it and was left stand for 15 minutes. After that, the plate was centrifuged at 2250 ×*g* at room temperature for 45 minutes. Then, the supernatant was discarded by inverting the plate until there was no supernatant left in the tube.

Next, 60 µl of 70 % ice-cold ethanol was added into the plate. Another centrifugation was done at 1650 ×*g* at 4 °C for 15 minutes. Again, the supernatant was discarded by inverting the plate until there were no supernatant left. After that, the pellet was dried using a thermocycler machine by leaving it at 90 °C for 1 minute, then followed by 50°C for 5 minutes. Ten microliters of Hi-Di Formamide was added to each well plate to bind the DNA template, before denaturation at 95 °C for 5 minutes using the

thermocycler. Immediately after the denaturation step was completed, the plate was transferred and placed on ice and directly put into the sequencing machine for sequencing process.

3.6.3 Sequence analysis

After the sequencing process has completed, the data obtained were analysed using Sequence Analysis Software v5.3 (Applied Biosystems,USA) and Chromas version 2.4.3.0 (Technelysium Pty Ltd, Australia).

3.7 Restriction Endonuclease (RE) assay

The DNA of unrelated healthy samples were kindly provided by Dr Ng Ching Ching from University of Malaya and Dr Azlina Ahmad Annuar from the University of Malaya Medical Centers (ethics approval and patient consent refer to Appendix C). Following PCR amplification of the desired regions, patients' and unrelated healthy control DNA samples were digested by using selected restriction enzymes to distinguish the mutant allele and normal allele. This is done to screen the samples for the presence of known and selected mutations.

3.7.1 RE design

Different restriction enzymes were used to cut the desired fragments of patients and healthy control samples to screen for the presence of selected mutations. The first restriction enzyme used is the *Mse*I enzyme (New England Biolabs, UK), which is used to cut the mutant allele (5'-TTAA-3') instead of the normal allele (5'-TAAA-3') The second restriction enzyme used is enzyme *Mbo*I (New England Biolabs, UK) enzyme that functions to cut the normal allele (5'-GATC-3')instead of the mutant allele (5'-GATT-3'). Figure 3.1 shows the schematic diagram of cutting site for *Mse*I enzyme and *Mbo*I enzyme.

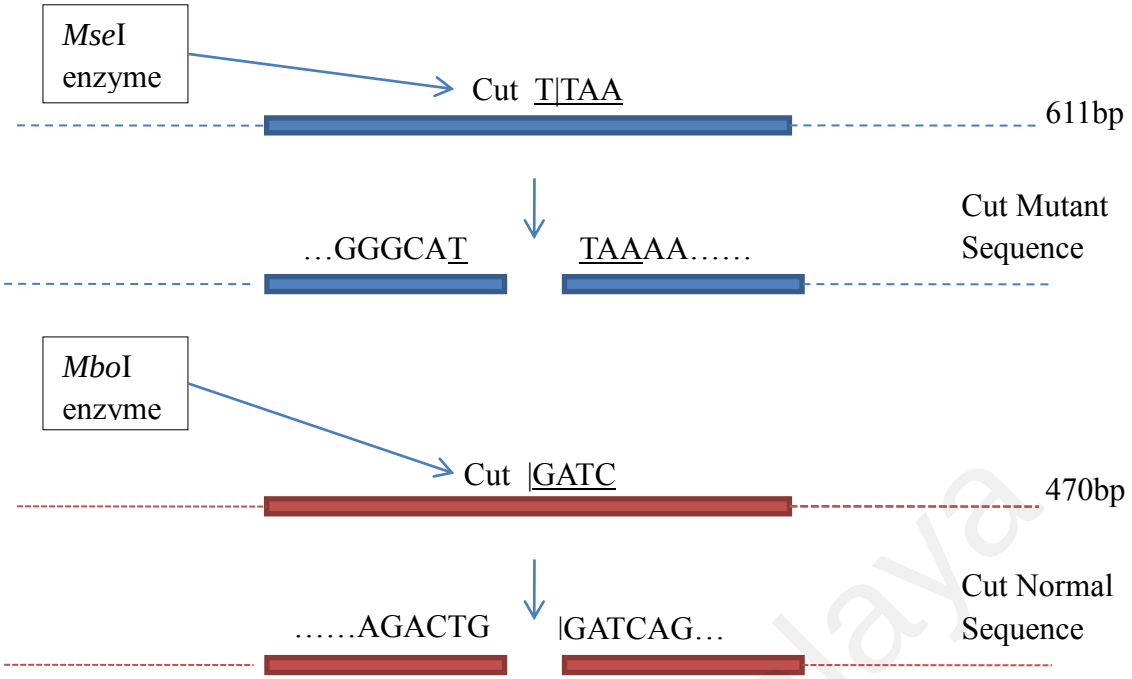


Figure 3.1: Schematic diagram of RE cut site for *Mse*I enzyme and *Mbo*I enzyme.

3.7.2 Samples preparation

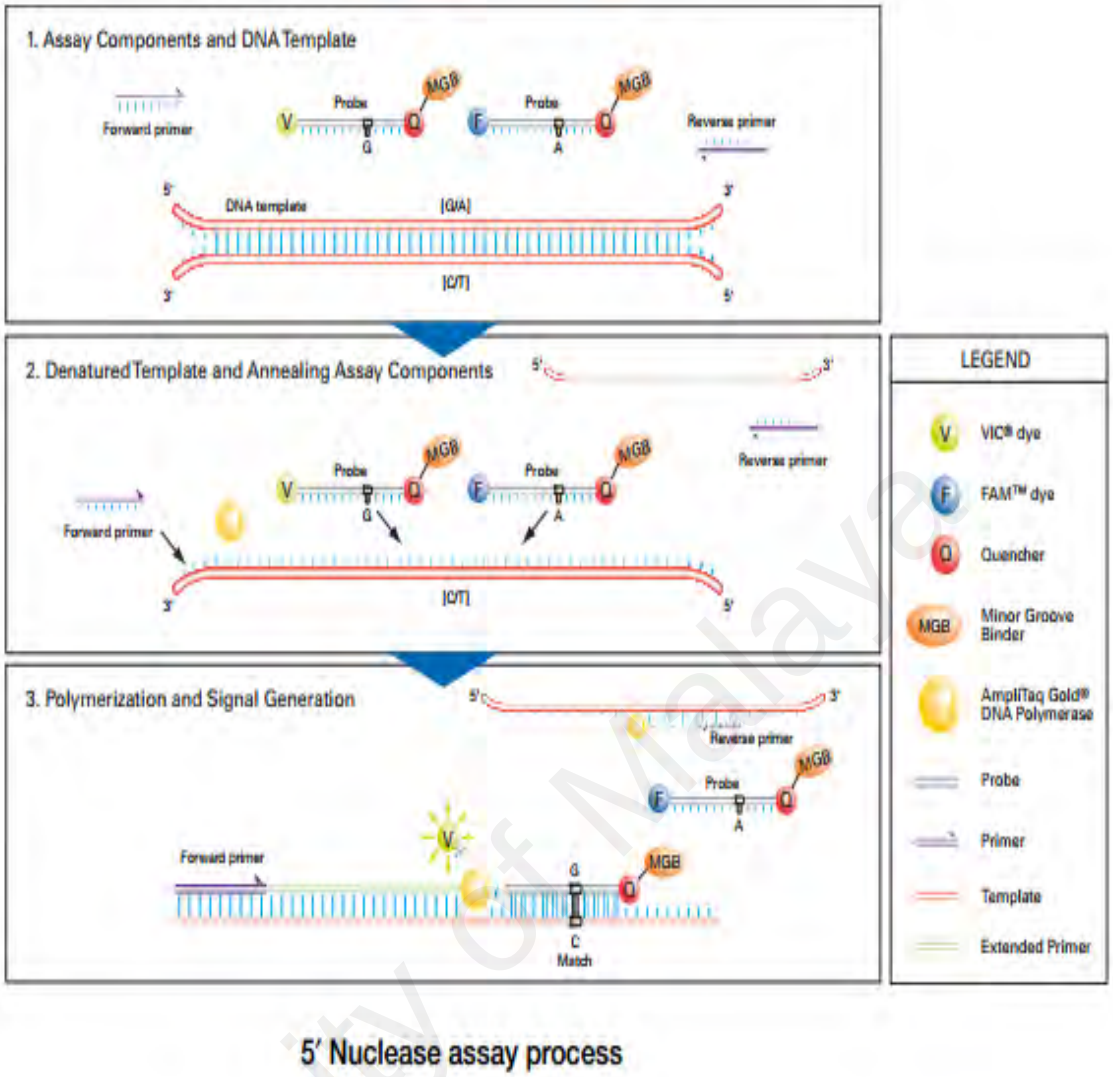
PCR of the patients' samples and 150 healthy control donor comprising multiracial Malaysians (n=50 Malays, n=50 Chinese, n=50 Indians) were carried out. The PCR products were then digested either by *Mse*I (New England Biolabs, UK) or*Mbo*I (New England Biolabs, UK) depending on the mutation to be screened for. For digestion, one unit of the *Mse*Ior*Mbo*I enzymes was added to a mixture containing 1X cutsmart NEbuffer and added with an appropriate amount of sterile distilled water to digest 10 µg of PCR product. The reaction mixture was prepared in 0.5 ml centrifuge tube with total volume of 20 µl. The reaction mixtures were incubated for 16 hours at 37 °C. Negative control reaction mixtures lacking the *Mse*I or *Mbo*I enzymes were also prepared. The complete protocol for the preparation of RE master mix is given in Appendix A.

After completing the 16-hour incubation, the samples were directly heated (to inactivate the RE) for 20 minutes at 60 °C. AGE was carried out with the 3 % of gel concentration (as described in section 3.4). Electrophoresis was carried out at 100V for 45 minutes and the gel was visualized under Alpha Imager system (Alpha Innotech

Corp, USA). 25bp, 50bp and 100bp markers were used to distinguish the fragments size of the product.

3.8 Custom TaqMan ® SNP Genotyping assay

TheTaqMan®SNP Genotyping assay is a single-tube PCR assay that exploits the 5' exonuclease activity of AmpliTaq Gold®DNA polymerase. It employs two locus-specific PCR primers that flank the SNP of interest, and two allele-specific oligonucleotide TaqMan®probes. These probes have a fluorescent reporter dye at the 5'end and non-fluorescent quencher (NFQ) with a minor groove binder (MGB) at 3'end. The process of TaqMan® SNP Genotyping assay is shown in Figure 3.2



5′ Nuclease assay process

Figure 3.2: Schematic representation of TaqMan® SNP Genotyping assay (Figure adapted with permission from *Custom TaqMan® SNP Genotyping assay Protocol, Applied Biosystem, 2010*)

3.8.1 Custom TaqMan ® SNP Genotyping design

The TaqMan assay was designed to target a highly conserved region of nucleotides to permit binding of both primers and the probes. Primers and probes were designed based on *G6PC* gene reference sequence (GenBank accession number: NG_011808.1) using Primer Express software package v.3.0 (Applied Biosystem, USA). Table 3.3 shows the list of primers and probes. Both used MGB-NFQ as a quencher at the 3'end.

Table 3.3: List of primers and probes used inCustom TaqMan ® SNP Genotyping.

TaqMan assay	Primers and probes' sequences	Amplicon size (bp)
c.155A>T	Primers Forward: 5′-AATGCCTTCTACGTCCTCTTCC-3′ Reverse: 5′-GAGTTTAATGCCCACAGCTTCC-3′ Probes Normal allele: 5′**VIC**-ATCTGGTTCCATCTTC-3′ Mutant allele: 5′**FAM**-ATCTGGTTCCTTCTTC-3′	63
c.1036G>C	Primers Forward: 5′-TGGCATCCGTCAGTGTCATC-3′ Reverse: 5′-CTCCACATCTCTTACAACGACTTCT-3′ Probes Normal allele: 5′**VIC**-ACTGCCTCGCCCAGGT-3′ Mutant allele: 5′**FAM**-CTACTGCCTCCCCCAGGT-3′	80

3.8.2 Custom TaqMan ® SNP Genotyping preparation

Five nanograms of genomic DNA of patients and 150 healthy control donors consisting of multiracial Malaysians (n=50 Malays, n=50 Chinese, n=50 Indians) were assayed by TaqMan GTXpress Master Mix (ABI, Applied Biosystems). The 10µl PCR mixtures contained 2X TaqMan® GTXpress™ Master Mix (ABI, Applied Biosystems), 20X TaqMan® Genotyping assay (ABI, Applied Biosystems) and appropriate amount of distilled water. The 20X TaqMan® Genotyping assay and the complete PCR mixtures were covered with aluminium foil to protect from direct exposure to light. Then, the mixture was mixed and aliquoted into MicroAmp Optical 8-tube strip (0.2 ml). A desired amount of purified DNA was pipetted into each well of the MicroAmp Optical 8-tube strip (0.2 ml) and closed with MicroAmp Optical 8-cap strip. Then, the plate was immediately transferred into QuantStudio™ 12K Flex Real-Time PCR System machine (Applied Biosystem, USA). The complete protocol for TaqMan ® SNP Genotypingmaster mix preparation can be found on Appendix A.

This amplification was carried out using a QuantStudio™ 12K Flex Real-Time PCR System machine (Applied Biosystem, USA) at 95 °C of AmpliTaq Gold enzyme activation holds for 10 minutes, followed by 40 cycles of 95 °C of denaturation for 15 seconds and 60 °C of annealing and extension for 60 seconds. Allele discrimination was performed by QuantStudio™ 12K Flex software.

3.9 DNA cloning in bacterial host *Escherichia coli* (*E.coli*)

3.9.1 Preparation of bacterial growth broth and plated media

Luria Bertani (LB) growth media was used to grow bacteria cells in the DNA cloning process. Preparation of LB broth was done by first dissolving 20 g of LB media powder (Pronadisa, Spain) in 1000 ml of distilled water in a clean beaker using a magnetic stirrer. Next, the mixture was added with an appropriate volume of distilled water to make the final volume of 1000 ml. An aliquot of 10 ml of media broth was transferred into clean universal bottles and autoclaved for 20 minutes (121 °C/15 1lb/sq). Autoclaved LB broth was kept at room temperature for future use.

LB agar plates were prepared by dissolving 6 g of LB agar powder mix (Pronadisa, Spain) in 300 ml of distilled water in a sterile Schott bottle using a magnetic stirrer. The media was then sterilised by autoclaving at the same setting as described above. After the sterilization process was completed, the LB media was pre-cooled under running tap water until the temperature reached approximately 50 °C. Next, 25 µg/ml of Kanamycin was added into the media before it was mixed by gently swirling. The 10 ml of LB media was then poured into labelled disposable plastic petri dishes and left to solidify at the ambient temperature for about 30 minutes. After the LB agar was completely hardened, the media was kept at 4 °C for future use. Both LB broth and agar were prepared fresh before being used in subsequent competent cell preparation, transformation and plasmid isolation steps.

3.9.2 Preparation component cells, TOP10 strain

The strain used in this cloning was *E.coli* strain TOP10 (Invitrogen, Life Technology, US). Single loop of TOP10 glycerol stock was taken and inoculated into 10ml LB broth and grown overnight at 37 °C, 5 ×*g* without added antibiotic. All the steps were done near to the flame to avoid contamination. Then, the bacterial culture was subcultured in another 10ml of LB broth at the same culture settings and grown to log phase for two to three hours. Next, the bacterial culture density was measured after 5 minutes until it reached OD_{600} value range of 0.4 to 0.6 which indicate log phase of growth.

After that, the subculture was transferred into a pre-chilled 15 ml tube and the bacterial culture was put into ice for 20 minutes and centrifuged at 4 °C, 1000 ×*g* for 5 minutes. The supernatant formed was carefully decanted. The bacterial pellet was re-suspended in 5 ml of ice-cold RF1 solution per 10 ml culture. The bacterial pellet was mixed by pipetting in and out and incubated on ice for 20 to 60 minutes. The mixture was then centrifuged at 4 °C, 1000 ×*g* for 10 minutes and the supernatant was discarded.

The bacterial pallet was re-suspended in 400 µl of RF2 solution and gently shaken until pallet dissolved. The cells were considered as competent cells. The competent cell suspension was then transferred into pre-chilled 1.5 ml microcentrifuge and a few seconds of cold shock treatment in liquid nitrogen and stored at -80 °C or either be used directly for transformation.

3.9.3 G6PC easy cloning pCMV6 Entry vector-based DNA cloning

The G6PC easy cloning (RC215623) human cDNA ORF with pCMV6-entry vector was bought from OriGene Technologies (OriGene, Rockville). To ensure that the cloning of the purchased was correct, the G6PC easy cloning pCMV6-entry vector was tested by transforming it in TOP10 competent cell. First, 100 µl of distilled water was

added into the clone stock so that the final concentration of the easy clone became 0.1 µg/ µl. Then, 250 ng concentration of easy clone was prepared by transferring 2.5 µl of easy clone into 1.5 ml microcentrifuge and 2.5 µl of distilled water was added. The mixture was vortexed and spun until it was homogenised.

3.9.4 Ligation

The vector used in this study was already in G6PC easy clone (OriGene, Rockville). One microlitre of T4 DNA ligase (New England Biolabs, UK) was added into a mixture containing 2 µl of 5 X rapid ligation buffer and 5 µl G6PC easy clone pCMV6-entry vector in a 0.5 ml microcentrifuge tube. The mixture was briefly centrifuged and incubated at room temperature for 5 minutes. The ligation reaction was incubated overnight at 4 °C.

3.9.5 Transformation

TOP10 competent cells were taken out from -80 °C and thawed in ice. Five microlitres of the ligation product were transferred into a pre-chilled 1.5 ml microcentrifuge tube containing 100 µl of TOP10 competent cells. The mixture was gently mixed and was then incubated on ice for 30 minutes. The mixture was heat shocked for 50 secs at 45 °C and immediately snap-chilled on ice for another two minutes to let the cells recover from heat stress.

A total of 900 µl of LB broth medium was added into the tube and incubated at 37 °C for three hours with shaking to allow cell growth. Next, the cells were spun down at 3000 ×*g* for 3 minutes at room temperature to concentrate the cell to the bottom of the tube. About 2/3 of the supernatant were thrown out using pipette and the remaining supernatants were re-suspended. About 100 µl of the cells suspension was transferred into LB agar plate containing 25 µg /ml of Kanamycin and spread evenly on the agar surface using a sterile glass rod spreader. Then, the plate was incubated overnight at 37

°C to allow the bacteria to grow. All the steps were carried out in an aseptic condition and near to the flame to avoid contamination by other microorganisms.

3.9.6 Colony selection

A single white colony growth on the agar medium surface was gently picked using a sterile wire loop. First, the selected colony was transferred into a 6 x 6 gridded labelled mini library agar plate. The remaining portion on the loop was then mixed with 30 µl of sterile distilled water in 0.5 ml microcentrifuge tube. The steps were iterated with other colonies until the mini library agar plate was full.

Selected colonies were boiled at 99 °C for 10 minutes and then centrifuged. Approximately 2 µl of the denatured cells were then used as DNA templates in PCR-based assay to screen for colonies that carry the clone insert. Insert with correct orientation was confirmed by using the combination of vector specific primers (Table 3.4). The PCR products were then analysed by AGE as described in section 3.4.

Table 3.4: List of vector primer for cloning.

Vector primer (10 µM)	Sequences
VP 1.5	5′ GGACTTTCCAAAACTGTCG 3′
XL 39	5′ AATAGGACAAGGCTGGTGGG 3′

3.9.7 Plasmid extraction

Bacterial colonies that had been positively identified as recombinants and carrying the inserts were cultured overnight at 3 7°C in 10ml LB broth containing 25 µg/ml of Kanamycin with agitation at 7 ×*g*. Once overnight culture was completed an 850 µl aliquot of the cell suspension was transferred into a labelled 1.5 ml microcentrifuge tube containing 150 µl of glycerol The mixture was mixed by pipetting and then stored at -80°C as glycerol stock. The remainder of the cell suspension was transferred into a labelled 1 5ml Falcon tube and spun at 11,200 ×*g* for 5 minutes or 4,000 ×*g* for 15 minutes at room temperature.

The supernatant was discarded, and the bacterial pellet was re-suspended in 200µl of solution I (25 mM Tris-Cl, 10 mM EDTA and 50 mM glucose) by vortexing and transferred into a sterile and labelled 1.5 ml microcentrifuge tube. After that, 200 µl of cold solution II (0.2 N NaOH and 1 % SDS) was added into the suspension and gently mixed for 4 minutes. Then, 200 µl of solution III (3M KOAc and 10 % acetic acid) was added, gently mixed at 0 °C for 15 minutes. Subsequently, the mixture was centrifuged at 19,000 ×*g* for 10 minutes and the supernatant was aspirated out into a new 1.5 ml microcentrifuge tube. Fifteen microlitres of 10 µg/µl of RNase A solution was pipetted into the supernatant and the mixture was incubated at 37 °C for 3 hours to eliminate any trace of RNA contamination.

The phenol/chloroform extraction was carried out to separate and remove plasmid proteins. Six hundred microlitres of phenol was added to the supernatant mixture and briefly vortexed. The mixture was centrifuged at 19,000 ×*g* for 3 minutes causing the mixture to separate into two distinct layers. The supernatant at the upper layer was carefully transferred into new sterile 1.5 ml centrifuged tube. Then, 600 µl of chloroform was added to the mixture and again mixed by vortexing and spun for 3 minutes at 19,000 ×*g*. The aqueous upper layer formed was collected as much as possible and transferred into a new 1.5 ml microcentrifuge tube.

One-tenth volume of 5 M NaCl and 2.5V of isopropanol were added to the suspension and left at 0°C for 20 minutes and followed by centrifugation at 19,000 ×*g* for 15 minutes. The supernatant was then removed gently to avoid accidental pellet removal. One millilitre of 70 % ethanol was then added to the DNA pellet and was spun again for 5 minutes at top speed 19,000 ×*g*. Then, the supernatant was discarded as much as possible by aspiration without disturbing the pellet.

The pellet was then dried for 10 minutes at 50 °C by using heat block. The tube was removed from the heat block with care to avoid the loss of pellet. Then, the pellet was reconstituted by adding 50 µl of TE buffer or sterile distilled water and the pellet was let to dissolve at 65 °C for one hour or kept overnight at 4 °C. The plasmid was stored at -20 °C for future use. The plasmid insert was sequenced as described at section 3.6 to verify the insert sequences.

3.10 Site-directed Mutagenesis (SDM)

All the mutations interest was introduced to the normal gene sequence by site-directed mutagenesis method using Phusion Site-Directed Mutagenesis Kit (Thermo Fisher Scientific, US) as shown in Figure 3.3. A new pair of primers that contains the desired mutation sequences and anneals the same sequence on opposite strands of plasmid was designed according to the manufacturer's guidelines.

3.10.1 Mutant strand synthesis reaction preparation

Twenty nanogram of DNA templates of each reaction were prepared by mixing the following reagents in a 1.5 ml microcentrifuge tube: 10 µl of 5X Phusion HF buffer, 20ng of DNA template, 1 µl of 10 mM dNTP mix, 2.5 µl of 10 µM of both forward and reverse primers, 0.5 µl of 2 U/µl of Phusion hot start II DNA polymerase and sufficient volume of sterile distilled water to make a final volume of 50 µl of each reaction. It is noteworthy to add all the reagents step by step and DNA polymerase was added last to the reaction as recommended by the protocol. Table 3.5 shows the list of all primers used in the site-directed mutagenesis method.

Another reaction was also prepared to perform as a control for site-directed mutagenesis step. The reaction was prepared by adding 10 µl of 5X Phusion HF buffer, 2 µl of 10 pg/µl of DNA template, 1 µl of 10 mM dNTP mix, 2 µl of 25 µM of both phosphorylated primer #1 and primer #2, 0.5 µl of 2 U/µl of Phusion hot start II DNA

polymerase and sufficient volume of sterile distilled water to make a final volume of 50µl.

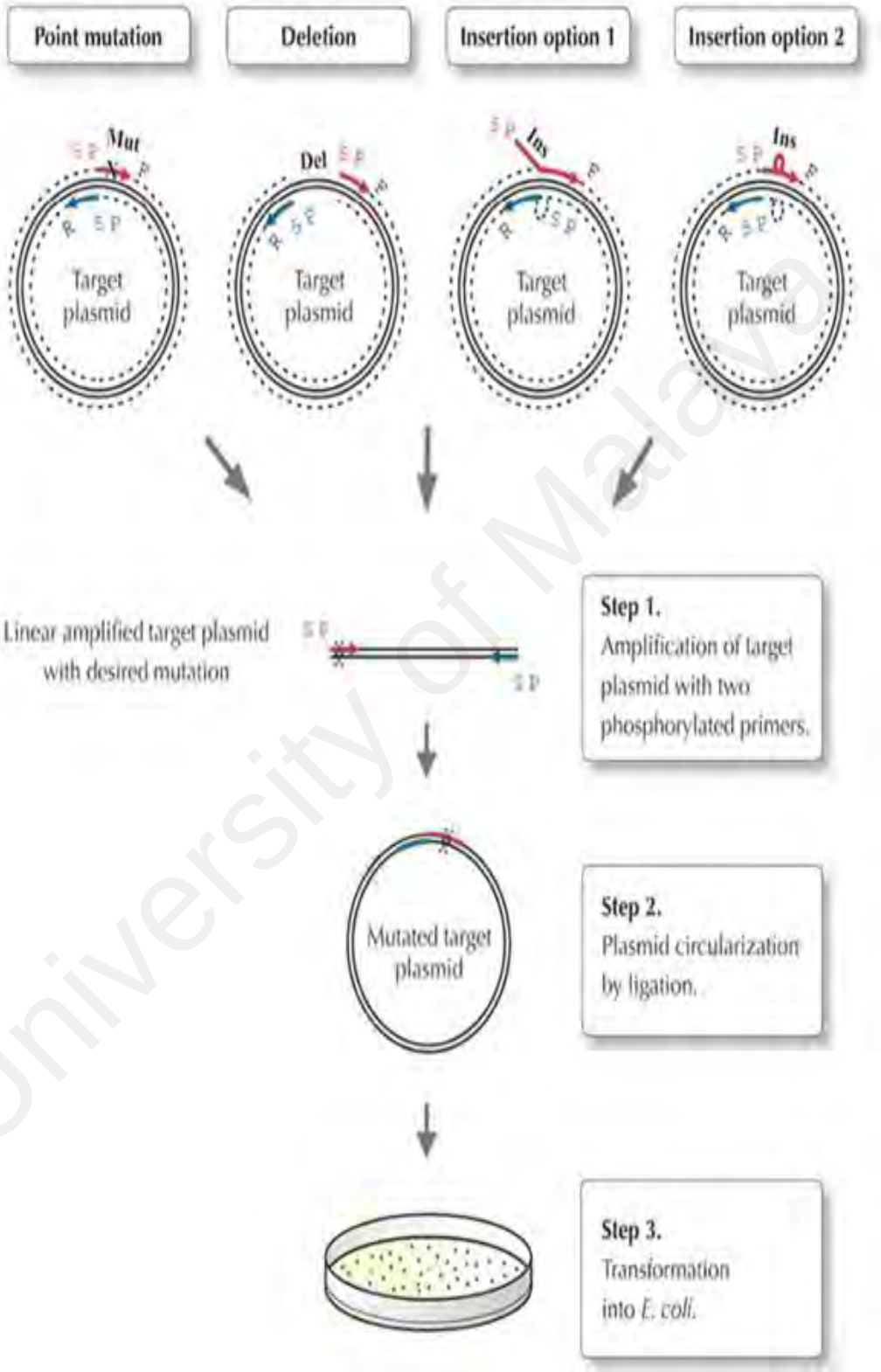


Figure 3.3: A schematic representation of site-directed mutagenesis.
(Figure adapted with permission from *Thermo Scientific Phusion Site-Directed Mutagenesis Kit Handbook, 2013)*

Table 3.5: List of phosphorylated primers used for site-directed mutagenesis method.

Primer name	Primer sequences	Ta
G6PC_52F G6PC_52R	5' CATCTGGTTCCTTCTTCAGGAAGC 3' 5' GGGAAGAGGACGTAGAAGGCATTC 3'	65°C
G6PC_76F G6PC_76R	5' CAACCTCGTCTTTTAGTGGATTCTC 3' 5' AGCCAGTCTCCAATCACAGCTACC 3'	67°C
G6PC_346F G6PC_346R	5' ATCCCCTACTGCCTCCCCCAGGTCCTG 3' 5' GACACTGACGGATGCCAGGGGCACTACC 3'	75°C
G6PC_STOP_F G6PC_STOP_R	5' AAGAAGTCGTTGTAAACGCGTA 3' 5' GTGCGGCTGGCCCAGGACT 3'	65°C

3.10.2 Thermal cycling parameters

Site-directed mutagenesis reactions prepared were placed in the programmable thermocycler machine. For mutagenesis reaction, the reaction was first heated to initial denaturation step at 98 °C for 30 sec, followed by 25 cycles of denaturation at 98 °C for 10 sec, annealing process at 65-75 °C for 30 sec and extension at 72 °C for 30 sec. After the cycles were completed, final extension step was then followed for 10 minutes at 72°C and hold at 4 °C. This product was stored in -20 °C for further used.

For control reaction, at first the reaction were heated for 98 °C for 30 sec then to 25 cycles of the following reaction cycling program: 98 °C for 10 sec and 72 °C for 45 sec. Later, final extension was done for 5 minutes at 72 °C and hold at 4 °C. The product was stored at -20 °C for further used.

3.10.3 Ligation, transformation and colony selection

Similar to the steps described in section 3.9.4, 3.9.5 and 3.9.5. Site-directed mutagenesis product were ligated and transformed into TOP10 bacterial competent cells. A few colonies from each mutagenesis product tested that grew on the selection plate were transferred to a library plate and at the same time inoculated in 10 ml of LB broth for plasmid extraction process.

3.10.4 Plasmid extraction and DNA sequencing

Plasmid extraction was completed by following the steps described in section 3.9.7 and the purified plasmid were directly involved with the DNA sequencing process to verify the presence of the insert of interest. DNA sequencing was successfully done as delineated at section 3.6. Table 3.6 shows the sequencing primers used to verify the plasmid product.

Table 3.6: List of sequencing primers for site-directed mutagenesis method.

Primer name	Primer sequences
VP 1.5-F	5' ACTTTCCAAAATGTCG 3'
XL 39-R	5' ATTAGGACAAGGCTGGTGGG 3'

3.11 Cell culture

Human liver hepatocellular carcinoma (HepG2) cell line was bought from American Type Culture Collection (ATCC, USA). HepG2 cell line was used as a transfection host for *G6PC* mutant construct preparation.

3.11.1 Preparation of culture medium

HepG2 cell line was cultured in one times Dulbecco's Modified Essential Medium (DMEM) (GIBCO, USA). The medium base was supplemented with 10 % of inactivated fetal bovine serum (FBS; GICBO, USA) and 1 % Penicilin Streptomycin (PenStrep; GIBCO, USA). 500 ml of the 1X DMEM growth media was prepared by taken out 20 ml of 1 X DMEM from the bottle and transferred into the new 50 ml falcon tube and labelled it as DMEM-0. Next, 50 ml of FBS and 5 ml of PenStrep were added into 1 X DMEM bottle. The mixture was then mixed by waggling the bottle and aliquoted into a new 50ml falcon tube.

Next, 10 ml of medium solution was aliquoted into 10 cm Petri dish and incubated at 37 °C in an incubator supplied with 5 % CO_2 to act as indicator that the media

preparation was free from any microbial contamination. All the steps of preparation medium were handled in sterile cell culture laminar flow cabinet, and thoroughly sprayed down with 70 % ethanol. The medium solution and supplemented reagents were stored at 4 °C.

3.11.2 Cell maintenance

After 3 to 5 days, cells that have reached 70-100 % confluence need to be apportioned to create room for new cells to grow. The old growth media was removed from the cultured cell by being aspirated out from the Petri dish. Later, the cells were rinsed with 3 ml of 1 X phosphate buffer saline (PBS; GIBCO, USA) to get rid of residual growth medium and aspirated out again. Trypsin-EDTA (GIBCO, USA) with the volume of 1.5 ml was carefully poured into the Petri dish and incubated for 15 minutes in the 37 °C incubator with 5 % CO_2 to allow trypsinization of cell detached.

Then, 5 ml of fresh growth medium were carefully dispensed into the Petri dish to deactivate the Trypsin activity. The cultures were mixed by pipetting up and down to detach and avoid the cell from being clumped. In a moment, the whole mixture of the culture was transferred into 15 ml falcon tube and centrifuged at 250 $\times g$ at 4 °C for 5 minutes to separate the cells from the protease. Once the cells were pelleted, the supernatant was aspirated out and the cells were re-suspended with 5 ml of fresh growth media into the tube. The cell suspension was split into two portions. One portion of the cell was prepared for cryopreservation and the other one was for cell maintenance.

Three millilitres of the culture were pipetted into the Petri dish and 7 ml of fresh medium were transferred and the Petri dish was gently shake before it was observed under an inverted microscope. Lastly, the culture was placed in the 37 °C incubator with 5 % CO_2 to allow the cells to grow.

3.11.3 Cell cryopreservation and revival

Prior to cryopreservation, cell culture was growing healthy and approximately 75 % confluence were checked microscopically. Before trypsinization, the old culture medium was removed, and the cell layer was washed with PBS and trypsin-EDTA solution was added. Cell detachment was monitored microscopically and 3ml of fresh medium supplemented with FBS was added to stop the trypsin-EDTA action. The trypsinized cells were transferred into sterile 1.5 ml microcentrifuge tube and the cells were harvested by centrifugation at 1500 ×g for 5 minutes at 4 °C.

The supernatant was aspirated carefully, and cell pellet was gently re-suspended in 1ml of 10 % cryoprotectants (90 % FBS, 10 % DMSO) transferred to 2 ml Cryovial tube (NUNC, Denmark). The cryoprotective agents were left cooled below 37 °C before suspended into the pellet to avoid cell damage. The cryosolution was handled with extra care to avoid contamination. Following this, the cryovial tube was transferred into -20°C first for 4 hours before being kept at -80 °C for overnight. Later, the cryosolution was transferred into a liquid nitrogen storage tank for long term storage.

Cryopreserved cells were revived by quickly thawing cryotubes in a 37 °C water bath for 5 minutes. Next, the cell suspension was transferred into 15ml centrifuge tube and centrifuged at 1500 ×g for 5 minutes. The resulting supernatant was decanted, and the pellet was re-suspended in 5 ml of 1 X DMEM-10. The mixture was mixed by pipetting and transferred into sterile 25 cm^2 culture flask and incubated at 37 °C incubator supplemented with CO_2 to allow the cells to grow. In the following day, the medium was changed and transferred to a bigger flask.

3.11.4 Cell enumeration

Cell counting was performed using a haemocytometer. The haemocytometer was cleaned first by wiping its surface with 70 % alcohol. Next, clean glass cover slip was

placed on the riled grids of the haemocytometer. Ten microliters of the cell suspension was mixed with 1µl of trypan blue solution. Then, the mixture was transferred onto the gridded plane of the haemocytometer by gently pipetting the pipette at notch at the edge of the glass cover slide.

The haemocytometer was observed under an inverted microscope once the mixture was completely spread over the surface. The viable cells were not stained by trypan blue while the dead cells appeared in blue colour. Therefore, the cells appeared colourless in two over four 1mm x 1mm large boxes in each corner of the gridded plane were counted as shown in Figure 3.4. The concentration of the cells (cells/ml) was dictated by averaging the total numbers of the cells from all two counting areas and multiplying the value to the dilution factor (x 10^4).

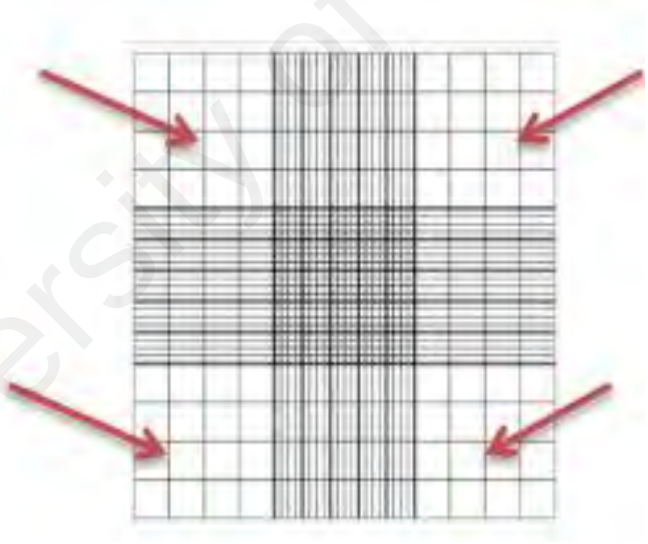


Figure 3.4: Haemocytometer gridded plane shows the 1mm x 1mm boxes. The red arrows show boxed area to be counted.

3.11.5 Cell seeding and transfection

In vitro expression of the mutant construct requires the expression vector to be transported into the host cells. In this study, the mutant construct was transfected into the hepatocarcinoma cell line HepG2 using calcium phosphate transfection protocol. Firstly, the cells were counted to 50 X 10^4 per each 6-well culture plate. Then, the cells

were mixed with 2ml of DMEM supplemented with 10 % FBS and seeded to 6-well culture plate. The culture then was incubated at 37 °C incubator supplemented with CO_2 overnight to allow the cells to grow.

On the next day, 1µg of DNA was top up with sterile distilled water up to 93.7µl. Then, 31.3 µl of 1M of $CaCl_2$ was added into the solution and left for 5 minutes at room temperature. One hundred and twenty-five microlitres of 2 X HBS (Appendix A) was added into the mixture and again left for 5 minutes. Lastly, the mixtures were gently flicked and slowly pipetted the mixture into the culture dish. The growth medium containing the transfection reaction was incubated overnight in 37 °C incubator supplemented with CO_2.

3.11.6 Stable transfection

After transfection session was completed, the cells were checked under microscope (1X73 Olympus) to check the confluence of successful cells transfected. Approximately 75 % of confluence of successful cells transfected was directed to stable transfected with 800 µg/ml of Geneticin (G418, GIBCO, USA). The cells were incubated in 37 °C incubator supplemented with CO_2 to allow the cell to grow for a week.

After a week, the cells were transferred into a 10 cm culture plate and incubated again until the cell reached 100 % confluence and transferred into 15 cm culture plate. The cells were left and kept subculture until the cell colonies formed. Twelve cell colonies formed were selected and transferred into 12 well-culture plate and cultured with DMEM supplemented with 800 µg/ml. Subculture and transferred into the bigger plates routine were continued.

3.11.7 Cell harvesting

The transfected cells were harvested after the cells were 100 % confluence. The cells were sub-cultured as mentioned in section 3.11.2. After the cells were trypsinized, 3 ml

of DMEM supplemented with 800 µg/ml of G418 were added into the culture plate and transferred into 15 ml falcon tube. Next, the cells were pelleted by centrifugation with speed of 1500 ×*g* for 5minutes at 4 °C and the supernatant formed was discarded.

Five hundred microlitres of HEPES lysis buffer (5 mM HEPES pH 7.4, 7 X inhibitor protease cocktail) was added into the tube and gently lysed and homogenized by passing them through a pipette several times. Then, the mixture cell were harvested into clean labelled 1.5 ml microcentrifuged tube and kept in -20 °C for direct used or stored at-80°C for future use.

3.11.8 DNA extraction from cell lysate

The DNA of the harvested transfected cell was isolated using QIAGEN DNA extraction kit (QIAGEN, Germany) in accordance to the instruction manual provided by the manufacturer. Anterior to extraction, the harvested cells were first equilibrated to ambient temperature. To start, 40 µl of Proteinase K solution (provided with the kit) were transferred to the bottom of the sterile 1.5 ml microcentrifuge tube followed by 400 µl of harvested cell and 400 µl of buffer AL (provided with the kit). The mixture was immediately homogenized by pulse-vortexing for approximately 15 seconds. Later, the mixture was then incubated at 56 °C for 10 minutes in a heating block to facilitate cell lysis.

After the incubation step was completed, the tube was taken out from the heat block and the content was briefly directly spun down. Next, 400 µl absolute ethanol was added to the lysed sample. The mixture was pulse-vortexed and centrifuged for another 15 seconds at top speed 19,000 ×*g*. Then, 750 µl of the mixture was transferred into a fresh spin column (provided with the kit) carefully to not wet the rim of the column. The column was centrifuged at 72 ×*g* for 1 minute. The column was discarded and replaced with a new column (provided with the kit) and 500 µl of buffer AW1 was

added and spun for another 1 minute at 72 ×g. The column was discarded and replaced with a new column (provided with the kit) and 500 µl of buffer AW2 was added and spun for 3 minutes at 22,000 ×g.

Again, the supernatant was discarded and spun for 1 minute at 72 ×g to eliminate buffer residues. The spin column was placed into a sterile 1.5 ml microcentrifuge tube and 200 µl of buffer AE was allotted directly to the membrane filter of the spin column. The spin column was left to stand at room temperature for 10 minutes before it was centrifuged at 72 ×g for one minute. Finally, the DNA elution was assessed for its quality by AGE and stored at -20 °C for future use.

3.11.9 DNA sequencing

DNA of the cell lysate was sequenced to verify the successful transfected construct as described in the section 3.6.3.

3.12 Protein analysis

3.12.1 Protein Quantification – Bradford assay (standard process for microlitre plates)

Amount of protein in mutant construct of G6PC was quantified used Bio-Rad Protein Assay (Bio-Rad, USA) based on Bradford method. According to the manufacturer's protocol, 1V of Dye Reagent Concentrate (provided in the kit) was diluted with 4V of ultrapure distilled water and afterward the solution was filtered through Whatman #1 filter (Whatman, UK) to remove particulates. Six dilution of BSA protein standard was prepared. The range of the BSA was 0.05 to 0.50 mg/ml. A total of 10 µl of transfected lysate and protein standard BSA were pipetted into separate microtiter plate wells (Greiner Bio-One, Germany).

Later, 200 µl of diluted dye reagent was added into the well and the mixture was gently mixed by pipetting up and down. Samples and protein standard were assayed in triplicate and incubated at room temperature for at least 5 minutes. The colour intensity or absorbance was measured at 595 nm using ELISA reader (TECAN, USA).

3.12.2 Sodium dedocylsulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The total protein from the harvested transfected lysate was separated using SDS-PAGE assay. The gel cassette was assembled according to manufacturer's manual, Mini-Protean®3 Cell system (Bio-Rad, USA). First, 12.5 % of resolving gel was prepared (Appendix A) and loaded into assembled glass slide in about 4ml. After that, 1ml of butanol was loaded into the gel to let the gel become linear and let it solidify for 20 minutes. As the resolving gel solidified, the butanol was sucked out and 4.5 % stacking gel (Appendix A) was prepared. About 1ml of stacking gel was loaded into the assembled glass and the comb was inserted between the spaces before the stacking gel solidified.

After the gel was solidified, the gel cassette was located into an electrode assemble and placed inside the mini tank. One time of electrophoresis buffer (25 Mm Tris, 200 mM glycine and 3.5 mM SDS) was filled into the tank. About 10 µl of protein lysate was prepared by mixing 1V of 2 X gel loading buffer (125 mM Tris-Cl [pH 6.8], 20 % glycerol, 4 % SDS and 0.25 % bromophenol blue) and β-mercaptoethanol in a 0.5 ml microcentrifuge tube. The mixture then was heated at 99 °C for 4 minutes and loaded into the well. About 3 µl of Precision Plus Protein^{TM} Standards (Bio-Rad, USA) was used as a reference of protein molecular weight. The electrophoresis was carried out at 180V for 60 minutes. The gel was stained by Coomassie Blue staining method.

3.12.3 Coomassie blue staining

After SDS-PAGE was completed, the gel was fixed with fixing solution (40 % methanol and 10 % acetic acid) for 30 minutes by gently shaking (Stovall, USA). Next, the previous solution was discarded, and the gel was stained with Coomassie blue solution (50 % methanol, 0.05 % Coomassie Brilliant Blue R250 and 10 acetic acids) for 60 minutes by gently shaking. After the previous solution was discarded, detaining solution (20 % ethanol and 7 % acetic acid) washed the gel for a few times until the bindings were clearly seen.

3.12.4 Western blot assay

The protein was undergoing SDS-PAGE as mentioned in section 3.12.2. In the meantime, nitrocellulose membrane Hybond^{TM}-C extra (Amersham Bioscience, USA) was treated with Transfer buffer (Appendix A) together with span and filter paper at 4°C for 1 hour. This procedure was carried out according to the manufacturer's manual. After electroporation was done, the gel was taken out from the gel cassette and was rinsed with distilled water. The gel was directly assembled into sandwich clamp together with membrane according to the manufacturer's manual and was transferred into container together with ice block and magnetic stirrer. Electroporation was done for 1 hour at 100V.

The membrane was transferred and incubated in blocking buffer (Tris buffered saline [TBS] containing 5 % of non-fat skim milk) at 4 °C overnight. Next, the membrane was soaked in probing solution (40 % blocking buffer) with polyclonal anti-rabbit G6PC antibody (OriGene, Rockville) and incubated at room temperature for 3 hours with low agitation. The membrane was then washed with TBST (washing buffer, 1 X TBS containing 0.2 % Tween-20) for 5 minutes for 5 times with medium agitation to remove the unspecific antibody binding. Again, the membrane was rinsed with 1XTBS before

the membrane was swirled in probing solution supplemented with rabbit anti-goat antibody as secondary antibody for 1 hour at room temperature with low agitation.

The membrane again was washed with TBST for 5 times with medium agitation. Lastly, the membrane was soaked in a Western blue ® stabilized substrate for alkaline phosphatase (Promega, USA) for colour development. The band was screened through Alpha Imager system (Alpha Innotech Corp, USA). The expected size of the band was estimated using Molecular Weight software with Precision Plus Protein ™ Standards (Bio-Rad, USA) as reference of protein molecular weight marker.

CHAPTER 4: RESULTS

4.1 Confirmation of mutations

4.1.1 PCR assay – PCR amplification of *G6PC* gene exons

All five exons of the *G6PC* gene were successfully PCR-amplified using primer pairs as described by Angaroni *et al*., 2004. Figures 4.1 (a-e) show the examples of successful PCR amplification of the 5 *G6PC* exons.

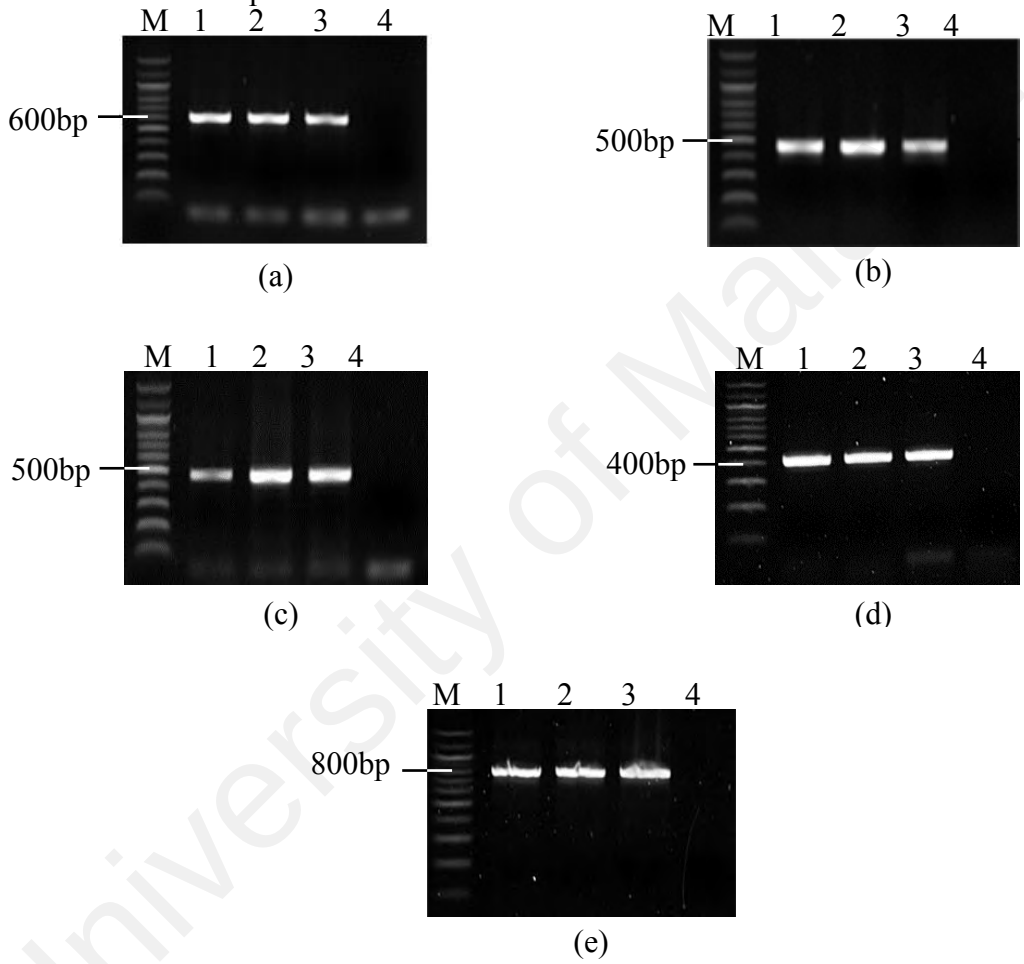


Figure 4.1: Examples of agarose gel electrophoresis of PCR products for (a) exon 1, 611bp, (b) exon 2, 470bp, (c) exon 3, 609bp, (d) exon 4, 419bp and (e) exon 5, 817bp.

Lane 1-3 : DNA patient's PCR products
Lane 4 : Negative control
Lane M : Marker (100bp ladder)

4.1.2 DNA sequencing assay

A total of 42 DNA samples (comprising GSD type 1a patients (n=30) and parental samples, when available) were sequenced in this study. As mentioned in section 3.6,

every exon of each sample was sequenced and compared with the gene reference sequence (GenBank Accession number: NG_011808.1). Any variation from the normal sequence were compared and cross checked against the *G6PC* gene mutation list in the Human Gene Mutation Database (HGMD).

Of the 30 patients, mutations were identified only in 21 individuals, while analysis on the remaining 9 patients revealed no variation from the normal sequence. A detailed description of the mutations found in this study is given in section 4.1.3 to section 4.1.12 (see below). Collectively, in the 21 individuals nine different mutations were identified, where five of them have already been reported in the literature and recognized as causative of GSD1a. The remaining four mutations have not yet been reported and do not match any recorded mutation in existing mutation databases. All four unreported mutations were found in Malay patients only. Table 4.1 shows the list of individuals screened and the mutations found.

In the present group of patients, the most common mutation found, which represent approximately 50 % of cases (n= 21/42 allele) was the c.648 G>T, p. L216L mutation which was first reported by Kajihara *et al*., in 1995. Although the is no change to the original amino acid Leucine (silent mutation), this mutation has been recognized to cause aberrant splicing of the *G6PC* gene. This mutation was found common in Malay patients (n: 17/28 alleles) and was found in lower frequency (n: 4/14 allele) in Chinese patients. The second most prevalent mutation was c.248 G>A, p. R83H mutation (Lei *et al.,* 1995). This mutation was found mainly in patients of Chinese ethnicity (n: 7/14 alleles). One Single Nucleotide Polymorphisms (SNPs) located in exon 5(c.547 A>G) was found in all of our patients.

Table 4.1: List of patients screened for GSD type 1a.

Sample	Ethnicity	Gender	*G6PC* Mutation	Effect	Remark
1a	M	M	c.337 C>T	P113S	**Unreported**
			c.648 G>T	L216L	Reported (Kajihara et al.,1995)
2a	M	F	c.337 C>T	P113S	**Unreported**
			c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
3a	M	F	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			-	-	-
4a	M	M	c.337 C>T	P113S	**Unreported**
			-	-	-
5b	M	M	c. 115 A>T	H52L	**Unreported**
			c. 115 A>T	H52L	**Unreported**
6b	M	M	c. 115 A>T	H52L	**Unreported**
			c. 115 A>T	H52L	**Unreported**
7	M	F	c.226 A>T	K76X	**Unreported**
			c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
8c	M	F	c.226 A>T	K76X	**Unreported**
			c. 1036 G>C	A346P	**Unreported**
9c	M	M	c.226 A>T	K76X	**Unreported**
			-	-	-
10c	M	F	c. 1036 G>C	A346P	**Unreported**
			-	-	-
11d	M	M	-	-	-
			-	-	-
12d	M	M	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)

Table 0.1 continued

			c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
13e	M	M	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
14e	M	M	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			-	-	-
15e	M	F	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			-	-	-
16f	M	M	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
17g	C	M	c. 248 G>A	R83H	Reported (Lei et al.,1995)
			c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
18g	C	M	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			-	-	-
19g	C	F	c. 248 G>A	R83H	Reported (Lei et al.,1995)
			-	-	-
20	M	F	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			c.226 A>T	K76X	**Unreported**
21	C	M	-	-	-
			-	-	-
22	M	M	-	-	-
			-	-	-
23	C	M	-	-	-
			-	-	-
24	-	-	-	-	-

Table 0.1 continued

25h	C	M	c. 248 G>A	R83H	Reported (Lei et al.,1995)
			c. 248 G>A	R83H	Reported (Lei et al.,1995)
26h	C	M	c. 248 G>A	R83H	Reported (Lei et al.,1995)
			-	-	-
27h	C	F	c. 248 G>A	R83H	Reported (Lei et al.,1995)
			-	-	-
28	C	M	c. 248 G>A	R83H	Reported (Lei et al.,1995)
			c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
29	M	-	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
30	C	F	c. 248 G>A	R83H	Reported (Lei et al.,1995)
			c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
31	M	F	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			c. 706 T>A	W236R	Reported (Lei et al.,1995)
32	M	M	-	-	-
			-	-	-
33	M	F	-	-	-
			-	-	-
34	C	F	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			c.664 G>A	G222R	Reported (Stroppiano et al.,1999)
35	C	-	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			c. 518 T>C	L173P	Reported (Li et al., 2007)

Table 0.1 continued

36	C	M	c. 248 G>A	R83H	Reported (Lei et al.,1995)
			c. 248 G>A	R83H	Reported (Lei et al.,1995)
37i	M	F	-	-	-
			-	-	-
38i	M	F	-	-	-
			-	-	-
29	M	F	-	-	-
			-	-	-
40	M	M	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
41f	M	F	c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
			c.648 G>T	L216L	Reported (Kajihara*et al.*,1995)
42	M	M	-	-	-
			-	-	-

[a] Sample1,2,3 and 4 are family
[b]Sample5 and 6 are siblings
[c]Sample 8, 9 and 10 are family
[d]Sample 11 and 12 are sibling
[e] Sample 13, 14 and 15 are family
[f] Sample 16 and 41 are sibling
[g]Sample 17,18 and 19 are family
[h]Sample 25,26 and 27 are family
[i]Sample 37 and 38 are siblings

4.1.3 c.155 A>T, (p. His52Leu) mutation

This mutation is located in exon 1, and the A to T substitution involved the second base of codon 52. This mutation has not been previously reported, and in this study this mutation was detected in sample 5 and 6 who are patients, and who are also siblings. Predictive analysis indicates that the sequence change will result in a missense mutation, where the original codon for Histidine will be changed to a codon for Leucine. Parental DNA sample was not available for these patients.

Example partial sequence chromatograms showing the mutations in the patients (and parents) are shown in figure 4.2.

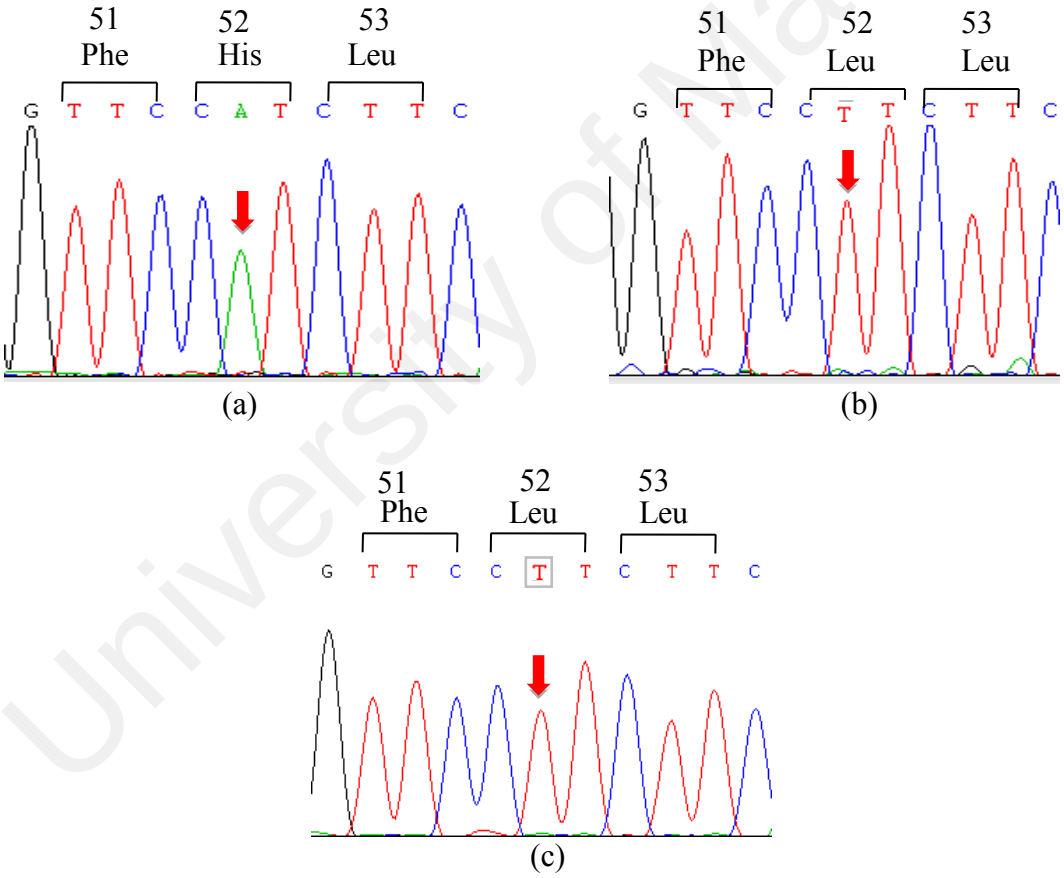


Figure 4.2: Partial chromatogram on cloned exon 1 sequence showing A>T nucleotide substitution (red arrows). **(a)** Partial exon 1 sequence from normal control showing adenine (A) residue at position 155. **(b)** and **(c)** The corresponding partial exon 1 from patients 5 and 6 showing thymine (T) residue at the same position.

4.1.4 c.226 A>T, (p.Lys76stop) mutation

This mutation is also located in exon 1, and the A to T substitution involved the first base of codon 76. This mutation has not been previously reported, and in this study this mutation was detected in samples 7 (mother), 8 (patient), 9(patient), who represent members of the same family, and also in sample 20 (patient, unrelated). Predictive analysis indicate that the sequence change will result in a nonsense mutation and generated a new stop codon (AAG>TAG) at codon position 76 that replaced the original codon for lysine (Lys) (p.K76X).

Parental DNA samples were available for patient samples 8 and 9, and subsequent analysis showed that the mutation is carried by the mother of the patient. Example partial sequence chromatograms showing the mutations in the patients (and parents) are shown in figure 4.3.

4.1.5 c.248 G>A, (p. Arg 83His) mutation

This mutation is located in exon 2, and the G to A substitution involved the second base of codon 83. This mutation had already been previously reported, and in this study this mutation was detected in sample 17 (patient) and his father (sample 19), patient samples 25, 27, and their father (sample 28) and in another unrelated patient sample 36. Predictive analysis indicates that the sequence change will result in a missense mutation, where the original codon for Arginine will be changed to a codon for Histidine. Parental DNA samples were available for patient's samples 17, 25 and 27, and subsequent analysis showed that the mutation is carried by the father of the patients.

Example partial sequence chromatogram showing the mutation in the patients (and parents) are shown in figure 4.4

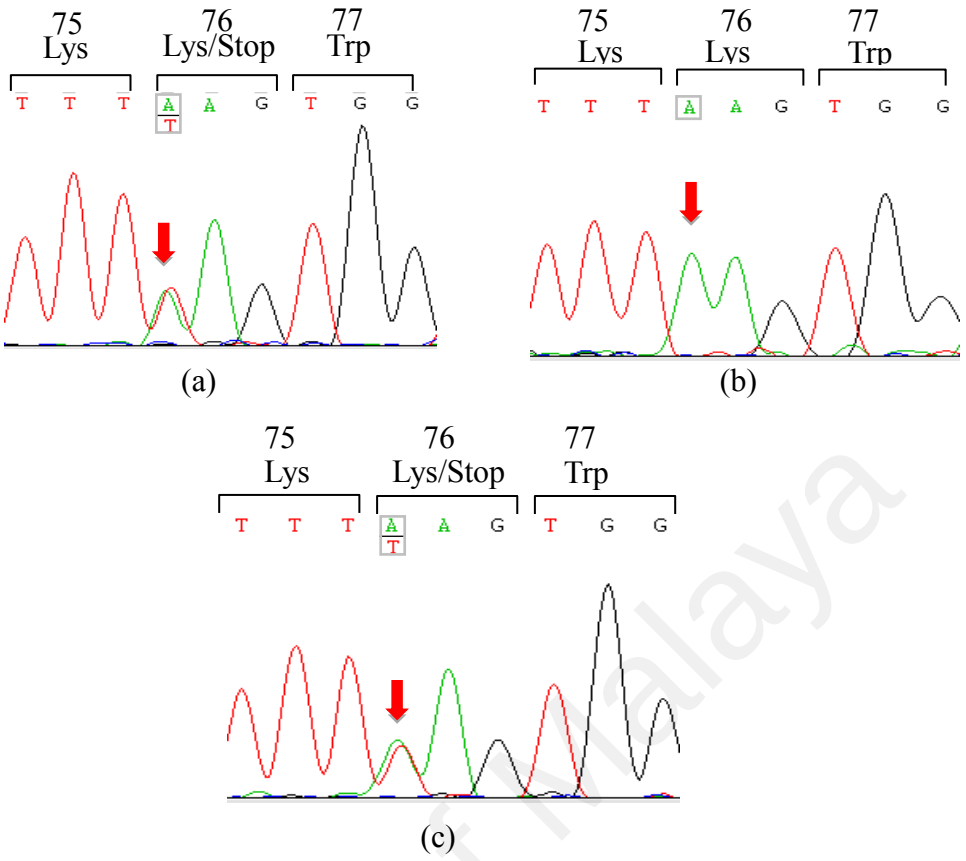


Figure 4.3: Partial chromatogram of exon 1 sequence showing A>T nucleotide substitution (red arrow). **(a)** Partial exon 1 sequence from patient 8 showing double peak, (A/T) at the first nucleotide position of codon 76. **(b)** The corresponding partial exon 1 sequence from the patient's mother showing the original alanine (A) residue at the same position. **(c)** The partial chromatogram exon 1 from the patient's father also shows double peak (A/T). Nucleotide substitution at this position creates a stop codon (TAG) at codon 76.

4.1.6 c.337 C>T, (p.Pro113Ser) mutation

This mutation is located in exon 2, and the C to T substitution involved the first base of codon 113. This mutation has not been previously reported, and in this study this mutation was detected in patient samples 1, 2 and their mother (sample 4). Predictive analysis indicates that the sequence change will result in a missense mutation, where the original codon for Proline will be changed to a codon for Serine. Parental DNA samples were available for these patients, and subsequent analysis showed that the mutation is carried by the mother of the patients. Example partial sequence chromatograms showing the mutations in the patients are shown in figure 4.5

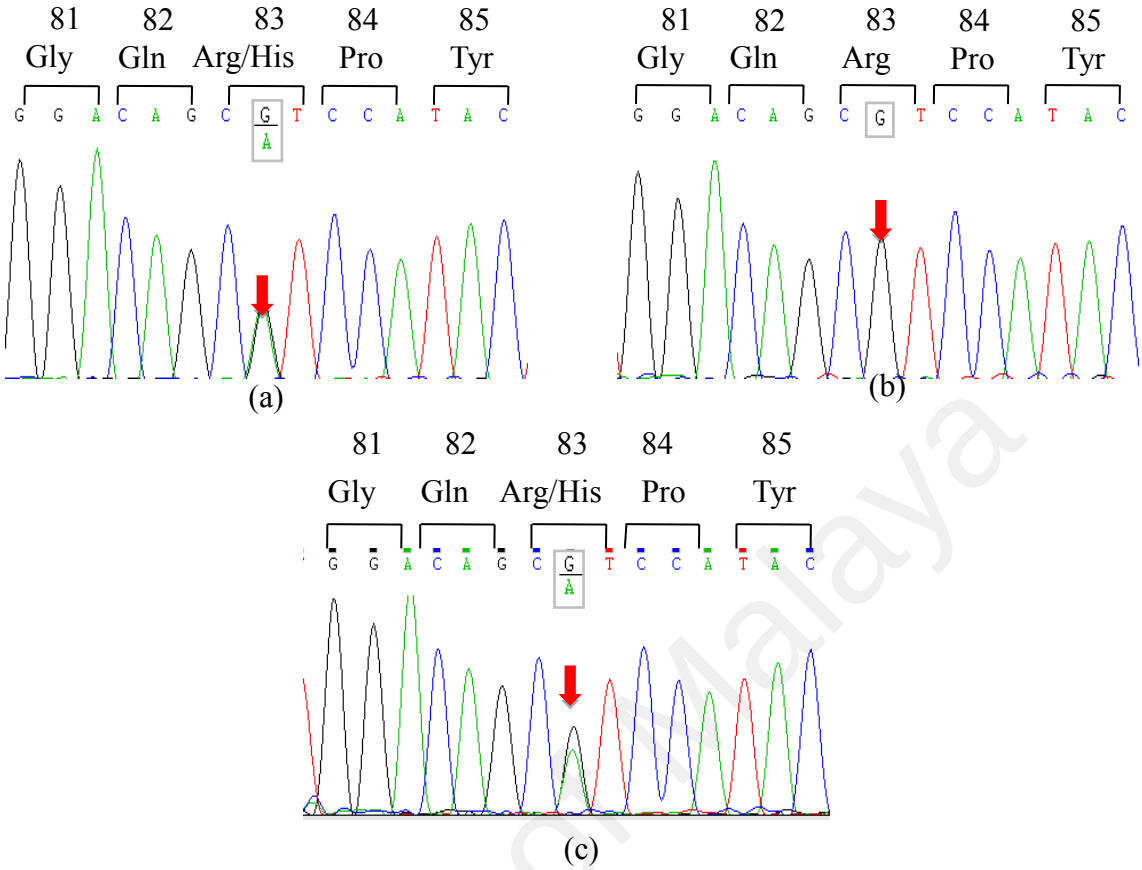


Figure 4.4: Partial chromatogram of exon 2 sequence showing G>A nucleotide substitution (red arrow). **(a)** Partial exon 5 sequence from patient 17 showing double peak (G/A) at position 83. **(b)** The corresponding partial exon 2 sequence from father showing the guanine (G) residue at the same position. **(c)** The partial chromatogram exon 2 from the father showing the double peak at the original position of guanine (G).

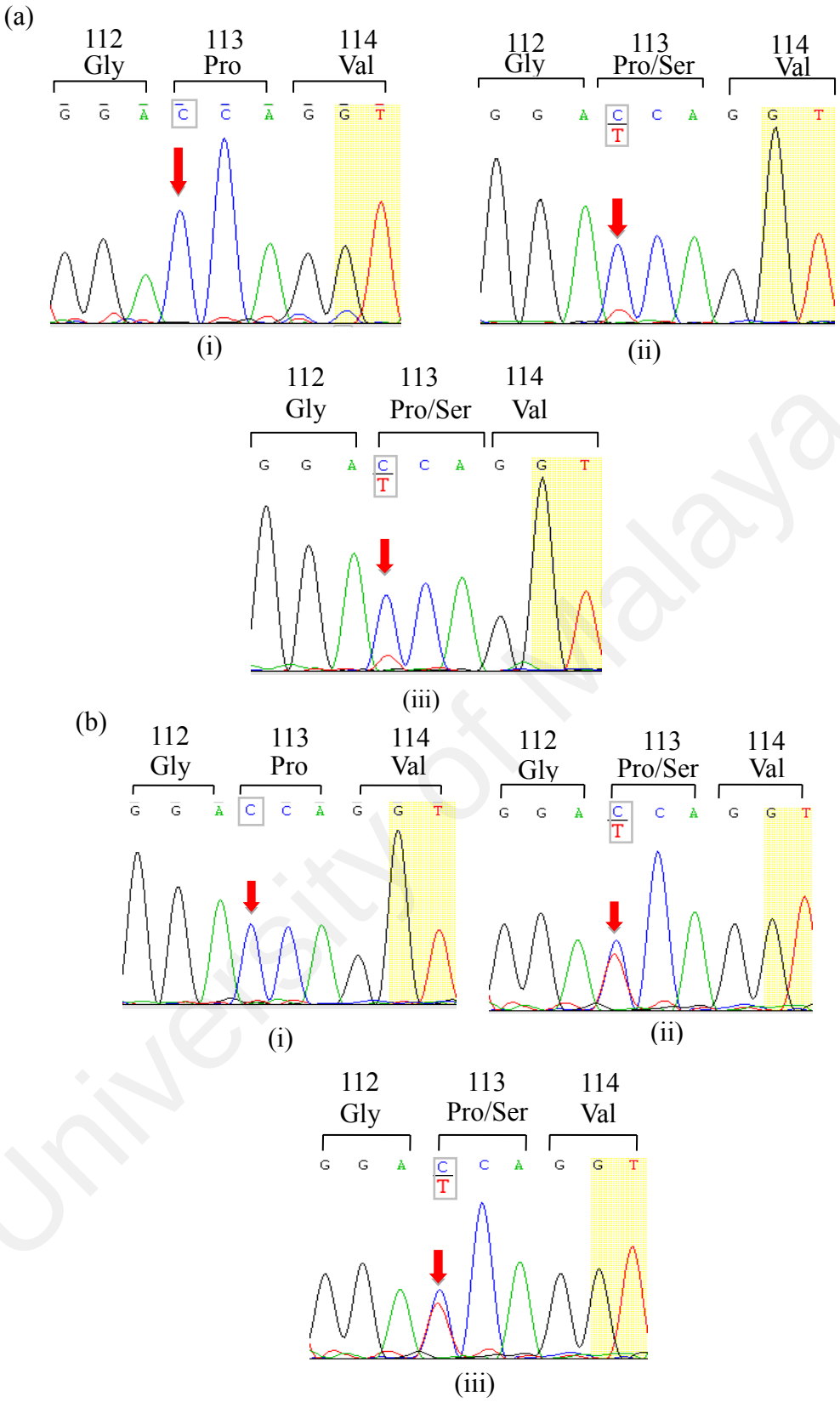
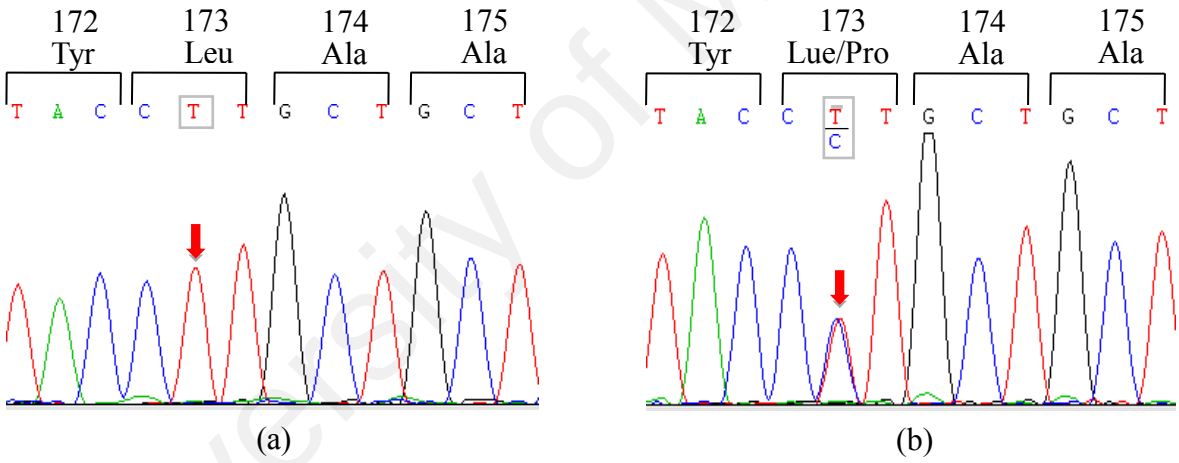


Figure 4.5 (a) and **(b):** Partial chromatogram of exon 2 sequence showing C>T nucleotide substitution (red arrow). Figure **(a)**shows the sequences using Primer 1 set and **(b)** Shows the sequences using new design forward primer (Primer 2). For both panels (a) and (b), **(i)** Partial exon 2 sequence from normal control showing cytosine (C) residue at position 337 (red arrow). **(ii)** and **(iii)** the corresponding partial exon 2 from patient 1 and 2 showing double peaks (C/T) at the same position at 337.

4.1.7 c.518 T>C, (p.Leu173Pro) mutation

This mutation is located in exon 4, and the T to C substitution involved the second base of codon 173. This mutation already been previously reported, and in this study this mutation was detected in patient sample 35. Predictive analysis indicates that the sequence change will result in a missense mutation, where the original codon for Leucine will be changed to a codon for Proline. Parental DNA sample was not available for these patients.

Example partial sequence chromatograms showing the mutations in the patients are shown in figure 4.6.



(a) (b)

Figure 4.6: Partial chromatogram of exon 4 sequence showing T>C nucleotide substitution (red arrow). **(a)** Partial exon 4 sequence from normal control showing thymine (T) residue at position 173. **(b)** The corresponding partial exon 4 from patient 35 showing cytosine (C) residue at the same position.

4.1.8 c.648 G>T, (p.Leu216=) mutation

This mutation is located in exon 5, and the G to T substitution involved the third base of codon 216. This mutation already been previously reported, and in this study this mutation was detected in most of the patients. Predictive analysis indicates that the sequence change will result in a silent mutation, where the original codon (Leucine) does not change.

Example partial sequence chromatograms showing the mutations in the patientsare shown in figure 4.7.

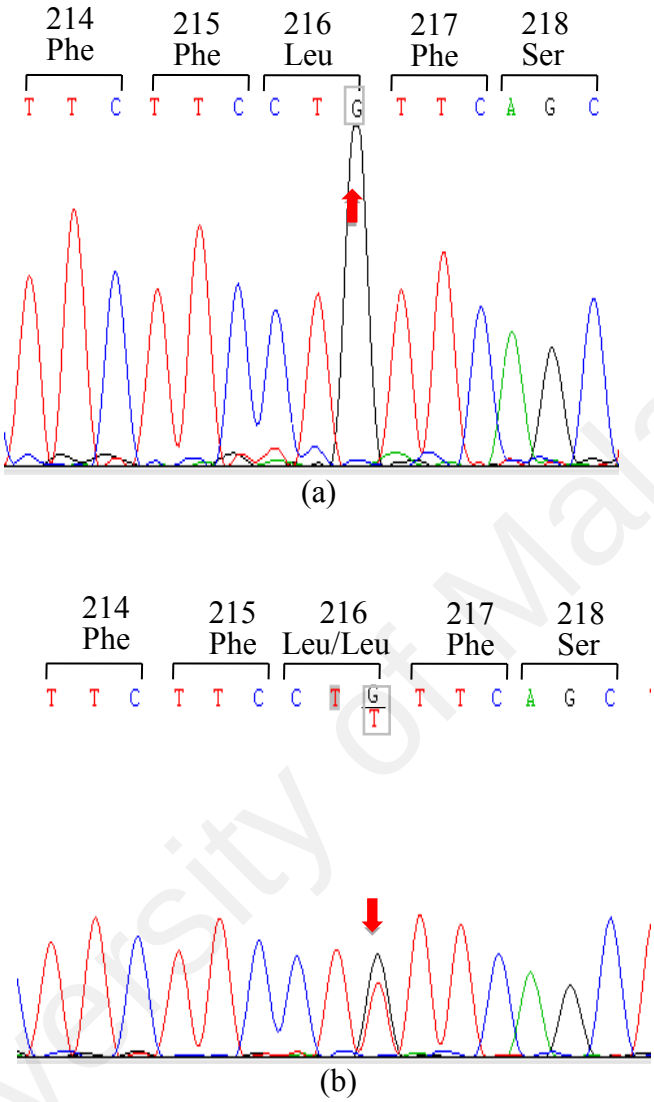


Figure 4.7: Partial chromatogram of exon 5. The first chromatogram **(a)** represents normal healthy control sequences. Chromatogram **(b)** represents the sequence from patients. The red arrow indicates the position of the mutation at position 648 which changes the guanine (G) to unidentified nucleotide (N) at codon 216.

4.1.9 c.664 G>A, (p.Gly222Arg) mutation

This mutation is situated in exon 5, and the G to A substitution involved the first base of codon 222. This mutation already been previously reported, and in this study this mutation was detected in patient 35. Predictive analysis indicates that the sequence change will result in a missense mutation, where the original codon for Glycerine will

be changed to a codon for Arginine. Parental DNA sample was not available for these patients.

Example partial sequence chromatograms showing the mutations in the patients are shown in figure 4.8.

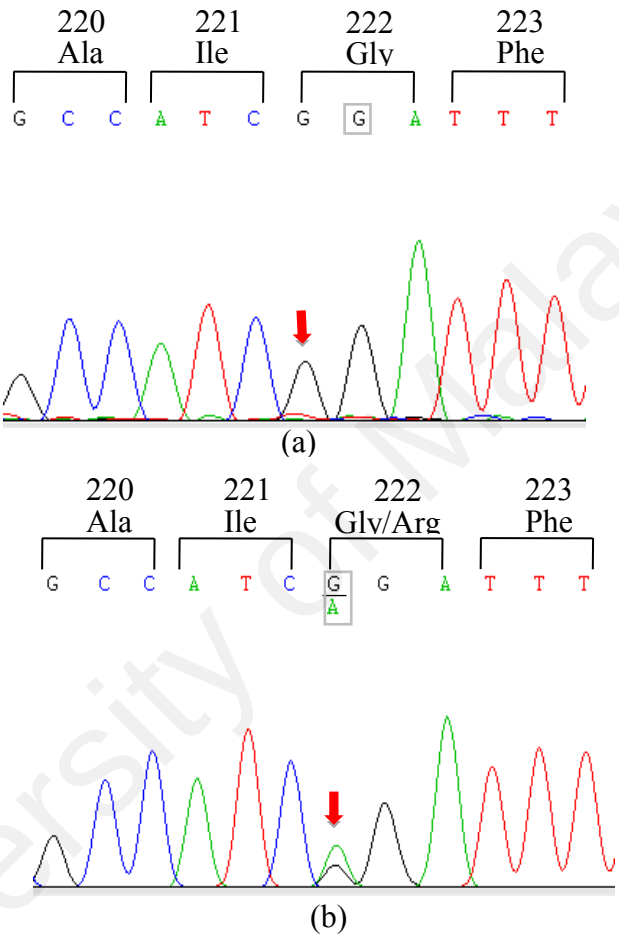


Figure 4.8: Partial chromatogram of exon 5 sequence showing G>A nucleotide substitution (red arrow). **(a)** Partial exon 5 sequence from normal control showing guanine (G) residue at position 222. **(b)** The corresponding partial exon 5 from patient 34 showing alanine (A) residue at the same position.

4.1.10 c.706 T>A, (p.Trp236Arg) mutation

This mutation is situated in exon 5, and the T to A substitution involved the first base of codon 236. This mutation already been previously reported, and in this study this mutation was detected in patient 31. Predictive analysis indicates that the sequence

change will result in a missense mutation, where the original codon for Tryptophan will be changed to a codon for Arginine. Parental DNA sample was not available for these patients.

Example partial sequence chromatograms showing the mutations in the patients are shown in figure 4.9.

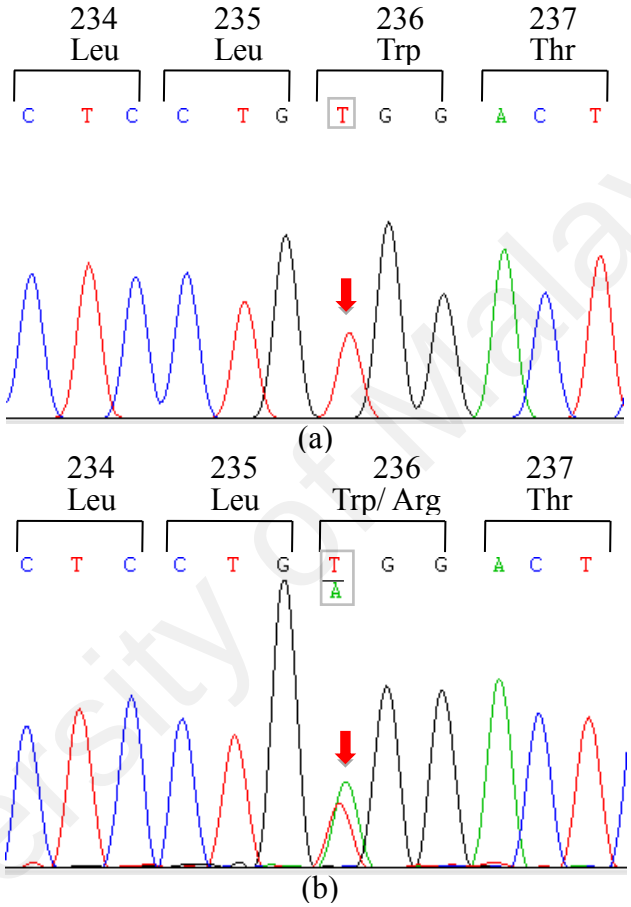


Figure 4.9: Partial chromatogram of exon 5 sequence showing T>A nucleotide substitution (red arrow). **(a)** Partial exon 4 sequence from normal control showing thymine (T) residue at position 236. **(b)** The corresponding partial exon 5 from patient 31 showing arginine (A) residue at the same position.

4.1.11 c.1036 G>C, (p.Ala346Pro) mutation

This mutation is in exon 5, and the G to C substitution involved the first base of codon 113. This mutation has not been previously reported, and in this study this mutation was detected in patients 8 and 10 (Father), who are family. Predictive analysis indicates that the sequence change will result in a missense mutation, where the original

codon for Alanine will be changed to a codon for Proline. Parental DNA samples were available for these patients 10, and subsequent analysis showed that the mutation is carried by the father of the patients.

Example partial sequence chromatograms showing the mutations in the patients (and parents) are shown in figure 4.10.

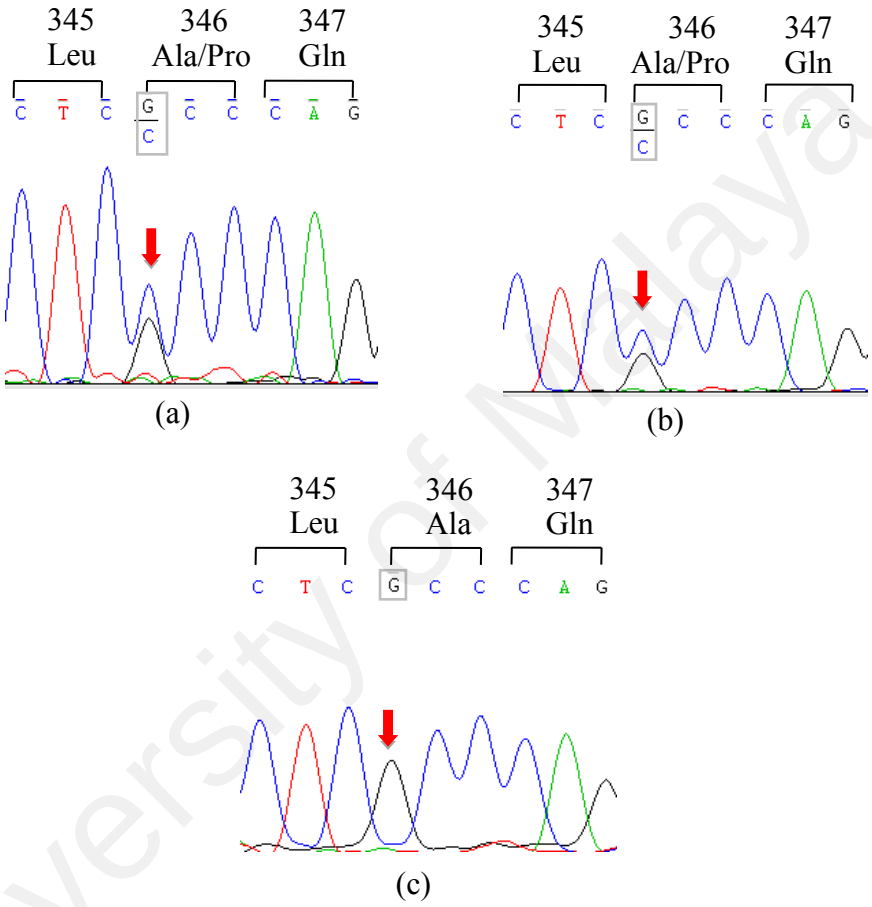


Figure 4.10: Partial chromatogram of exon 5 sequence showing G>C nucleotide substitution (red arrow). **(a)** Partial exon 5 sequence from patient 8 showing double peaks at position 346. **(b)** The corresponding partial exon 5 sequence from mother showing the cytosine (C) residue at the same position. **(c)** The partial chromatogram exon 5 from the father showing the original sequence at this position.

4.1.12 c.547 A>G, SNPs

This mutation is located in exon 5, and the A to G substitution involved the first base of codon 113. This mutation was been previously reported as single nucleotide polymorphisms (SNPs). Parental DNA samples were not available.

Example partial sequence chromatograms showing the mutations in the patients (and parents) are shown in figure 4.11.

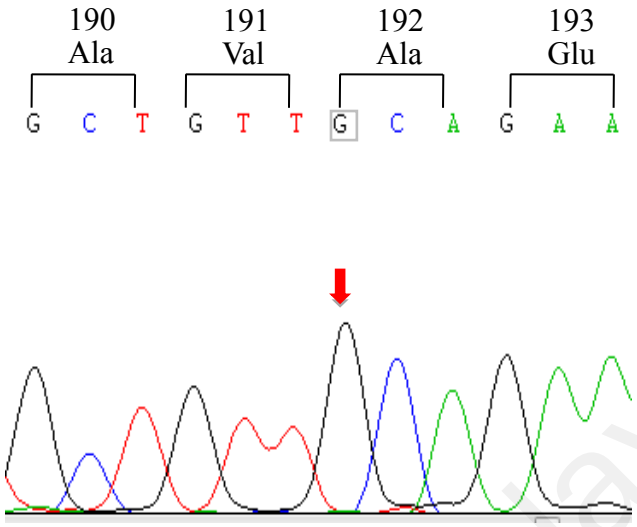


Figure 4.11: Partial chromatogram of exon 5 sequencing showing the position of SNPs in one of our patients. Red arrow represents position of guanine (G) at instead of original alanine (A) at position 547 (c.547 A>T).

4.2 Detection of mutant alleles in the healthy control sample population

To obtain support for the involvement of the unreported mutations as causative of the GSD1a in these patients, screening for the mutation in a panel of unrelated healthy control individuals were performed. The theoretical argument would be that if these mutations are neutral polymorphisms, there is a higher likelihood that they will be present in the normal population, and will be detectable at a frequency higher than 1% of the population sample tested. Assays to detect mutant alleles were designed either using restriction enzyme digestion or Taqman® SNP genotyping (when a suitable restriction enzyme assay is not available). A total of 150 control individuals were included as representative of the healthy population (Malay n=50, Chinese n=50, and Indian n=50).

4.2.1 Restriction Endonuclease (RE) assay

Two different RE enzymes were used to detect thepresence/absence of mutations in the healthy control population. *Mse*I and *Mbo*I restriction enzymes were used to digest the mutations c.226 A>T and c. 337 C>T respectively.

4.2.1.1 RE for mutation c.226 A>T, K76X

The *Mse*I enzyme was used to digest amplicons to detect the presence of mutation c.226 A>T located in exon 1. This enzyme will recognize and cut the amplicon twice at the sequence5'-T↓TAA-3', generating fragments with molecular weight of 48bp, 158bp and 405bp for the normal amplicon. The c.266A>T mutation abolishes one of the cutting sites, so that heterozygous individuals who carry the normal and the mutated amplicon will show four fragments of 48bp, 158bp, 206bp and 405bp in sizes. The 206bp is produced because the cutting site between the 48bp and 158bp fragment has been abolished as a result of the sequence change. The presence of the 206bp fragment can therefore be used as a marker to distinguish the mutant alleles from the normal allele. If the patient has homozygous mutation, it will generate fragments of 206bp and 405bp only (Table 4.2). Figure 4.13 shows the result of gel electrophoresis after treating with the *Mse*I enzyme. Screening results show that none of the 150 healthy control samples carried the mutated alleles.

Table 4.2: The number of DNA fragments produced after treatment with *Mse*I enzyme and expected size of each DNA fragments.

Sample	Expected size of fragment (bp)
Normal	48, 158, 405
Homozygous mutant	206, 405
Heterozygous mutant	48, 158, 206, 405

```
  1 CACCCTGAACATGTTTGCATCAACCTACTGGTGATGCACCTTTGATCAATACATTTTAGA
 61 CAAACGTGGTTTTTGAGTCCAAAGATCAGGGCTGGGTTGACCTGAATACTGGATACAGGG
121 CATATAAAACAGGGGCAAGGCACAGACTCATAGCAGAGCAATCACCACCAAGCCTGGAAT
181 AACTGCAAGGGCTCTGCTGACATCTTCCTGAGGTGCCAAGGAAATGAGGATGGAGGAAGG
241 AATGAATGTTCTCCATGACTTTGGGATCCAGTCAACACATTACCTCCAGGTGAATTACCA
301 AGACTCCCAGGACTGGTTCATCTTGGTGTCCGTGATCGCAGACCTCAGGAATGCCTTCTA
361                                        MseI  t|taa406
361 CGTCCTCTTCCCCATCTGGTTCCATCTTCAGGAAGCTGTGGGCAT TAAACTCCTTTGGGT
421                                MseI t|taa454
421 AGCTGTGATTGGAGACTGGCTCAACCTCGTCTT TAAGTGGTAAGAACCATATAGAGAGGA
481 GATCAGCAAGAAAAGAGGCTGGCATTCGCTCTCGCAATGTCTGTCCATCAGAAGTTGCTT
541 TCCCCAGGCTATTCAGGAAGCCACGGGCTACTCATGCTTCCAACCCCTCTCTCTGACTTT
601 GGATCATCTAC
```

Figure 4.12: Restriction enzyme map of *Mse*I enzyme for normal sequence of *G6PC* gene of exon 1. Red arrow indicates the cutting site of the enzyme. Red coloured letter indicate fragment of 405bp, blue colour letter for fragment 48bp and green colour letter for 158bp fragment.

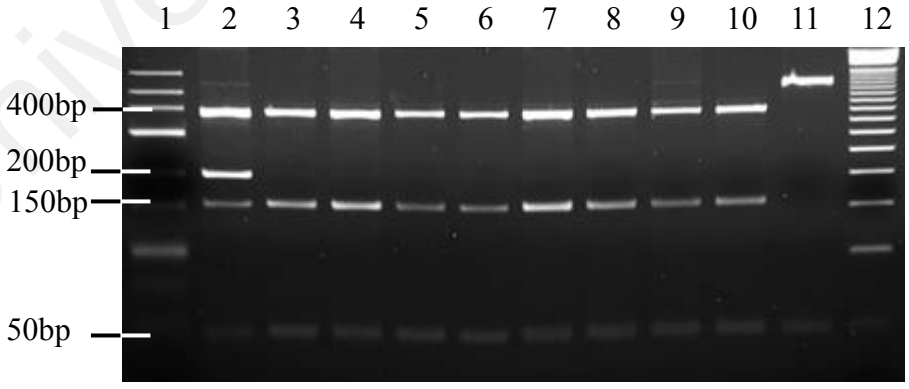


Figure 4.13: Gel electrophoresis on PCR product of exon 1 after being treated with *Mse*I restriction enzyme. Lane 2: Patient carrying c.226 A>T mutation at homozygous form (48bp, 158bp, 206bp and 405bp); Lane 3-10: Healthy control (48bp, 158bp and 405bp); Lane 11: undigested PCR product (611bp); DNA marker: 25bp and 50bp (Lane 1 and 12 respectively).

4.2.1.2 RE for mutation c. 337 C>T, P113S

The *Mbo*I enzyme was used to digest amplicons to detect the presence of mutation c.337 C>T located in exon 2. This enzyme will recognize and cut the amplicon at the sequence 5'-↓GATC-3'. The normal amplicon lacks this mutation site, and will produce a fragment with a molecular weight of 470bp. The c.337 C>T mutation create one cutting site, so that heterozygous individuals who carries the normal and the mutated amplicon will show three fragments of 213bp, 257bp and 470bp in sizes. The 213bp and 257bp are produced because a cutting site has been created in 470bp fragment as a result of the sequence change. The presence of the 213bp and 257bp fragments can therefore be used as a marker to distinguish the mutant alleles from the normal allele. If the patient has homozygous mutation, it will generate fragments of 213bp and 257bp only (Table 4.3). Figure 4.15 shows the result of gel electrophoresis after treating with the *Mbo*I enzyme. Screening results show that none of the 150 healthy control samples carried the mutated alleles.

Table 4.3: The number of DNA fragments produced after treatment with *Mbo*I enzyme and the expected size of each DNA fragments.

Sample	Expected size of fragment (bp)
Normal	470
Homozygous mutant	213, 257
Heterozygous mutant	213,257,470

```
  1 TATCTCCCTCTCACACTTCTCCCCAGCCACCCAGTTCTCCCTTCTAGAGGCAACATGTGA
 61 AATCCTTCTCAGGCTACACTCTTCTTGAAGGTGTAGGCTTTGGGCAAAAGCATTCATTCA
121 GTAACCCCAGAAACTTGTTCTGTTTTTCCATAGGATTCTCTTTGGACAGCGTCCATACTG
181 GTGGGTTTTGGATACTGACTACTACAGCAACACTTCCGTGCCCCTGATAAAGCAGTTCCC
241             MboI |gatc257
241 TGTAACCTGTGAGACTG▼GATCAGGTAAGCGTCCCAGCCCCTGCAGACAGAAGCTGAGTGG
301 ACCTCGTTTACCTGTTATGGATGAAACTGACCTTGAGGGGACATGAGGAGAGCCATTCCT
361 TTGTACTTTTGTCATGCTCTTCAATTGGCACAAATTAATTCACTTCTGCAATACTTTCCT
421 GAATAGCACAGTAGTATTGGAAATCTGCCTATTACAGAACCTGGATGGAG
```

Figure 4.14: Restriction enzyme map of *Mbo*I enzyme for mutant sequence of *G6PC* gene of exon 2. Red arrow indicates the cutting site of the enzyme. Red coloured letter indicate fragment of 213bp and blue coloured letter for fragment 257bp.

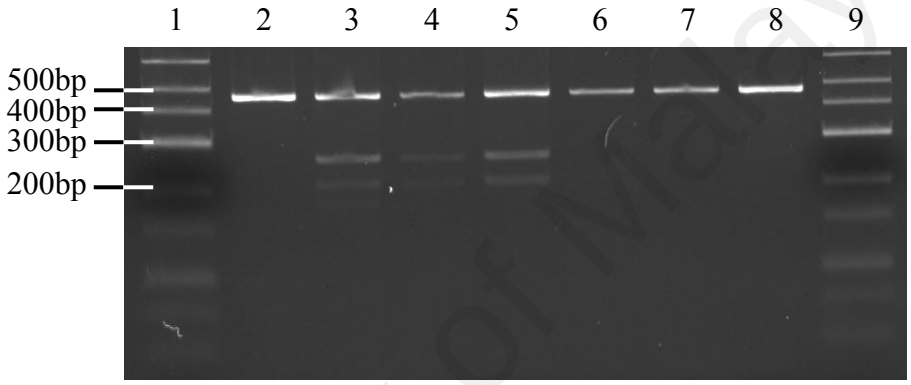


Figure 4.15: Gel electrophoresis on PCR product of exon 2 after treated with *Mbo*I restriction enzyme. Lane 2: mother; Lane 3: father; Lane 4-5: patients carrying P113S mutation at a heterozygous form (213, 257 and 470bp); Lanes 6-7: Healthy control (470bp); Lane 8: undigested PCR product (470bp); DNA marker: 25bp and 50bp (Lane 1 and 9).

4.2.2 TaqMan ® SNP genotyping assay

Another alternative method used in the screening of population samples is TaqMan® SNP genotyping assay. TaqMan® SNP genotyping assay was designed due to the absence of suitable restriction enzyme to cut either normal or mutant sequence. Probes and primers for mutations c.155 A>T and c.1036 G>C were designed according the requirements stated in the manufacturer's protocol. The universal cycling program for the genotyping assay was performed at the same temperature conditions for all probes designed.

4.2.2.1 TaqMan ® SNP genotyping for mutation c. 155 A>T, H52L

The results of TaqMan® SNP genotypingof the c.155 A>T mutation is presented as allele discrimination plots. Figure 4.16 shows the allele discrimination plot where there are four different clusters that can be categorized as homozygous mutant genotype (blue dots), homozygous normal genotype (red dots), heterozygous genotype (green dot) and no-template control (black filled squares). Healthy control samples (Homozygous 155A) were clustered at one end of the graph while homozygous mutated alleles (155T) were plotted at the upper end side of the graph. It is important to note that there were no heterozygous samples available for analysis. As such, a mock sample (heterozygous 155A/T) was prepared by mixing samples with homozygous 155A and homozygous 155T at a ratio of 1:1, and it was plotted to distinguish it from homozygous 155A and homozygous 155T, and to provide clarity to the allele discrimination plots. The overall results shows that none of the 300 alleles of 150 healthy samples carried the mutant allele c.155 A>T.

4.2.2.2 TaqMan ® SNP genotyping for mutation c. 1036 G>C, A346P

The results of TaqMan® SNP genotyping of the c.1036 G>C mutation is presented as allele discrimination plots. Figure 4.17shows the allele discrimination plot where there are three different clusters that can be categorized as homozygous normal genotype (red dots), heterozygous genotype (green dot) and no-template control (black filled squares). Healthy control samples (Homozygous 1036G) were clustered at one end of the graph while heterozygous mutated alleles (1036 G/C) were plotted at the upper side of the graph. It is important to note that there were no homozygous mutant sample(1036 C) available for analysis, so that no blue dot (blue circle area) are plotted in the graph.The overall results shows that none of the 300 alleles of 150 healthy samples carried the mutant allele c.1036 G>C.

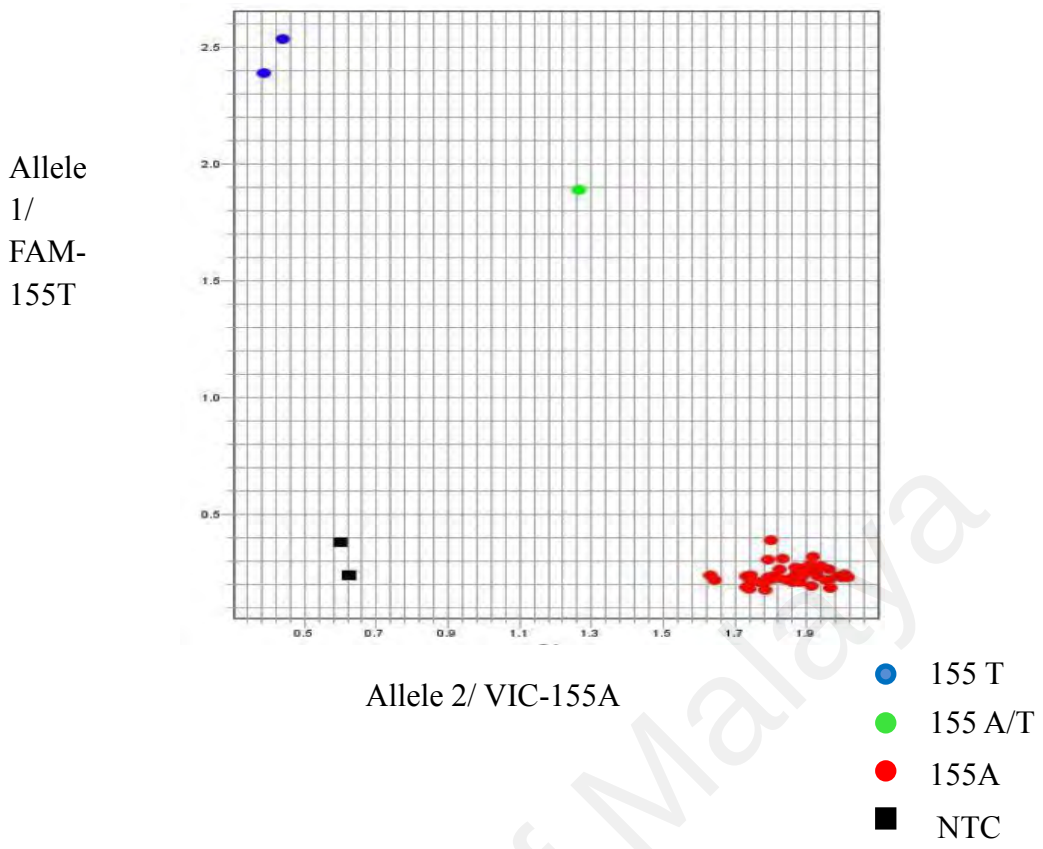


Figure 4.16: Allele discrimination plot shows clustering of genotypes for mutation c.155 A>T. The CT values for individual probes for all genotypes are presented. 155 T: Homozygous mutant; 155A: Homozygous normal; 155A/T: Heterozygous mock; NTC: no-template control.

Multicomponent plot

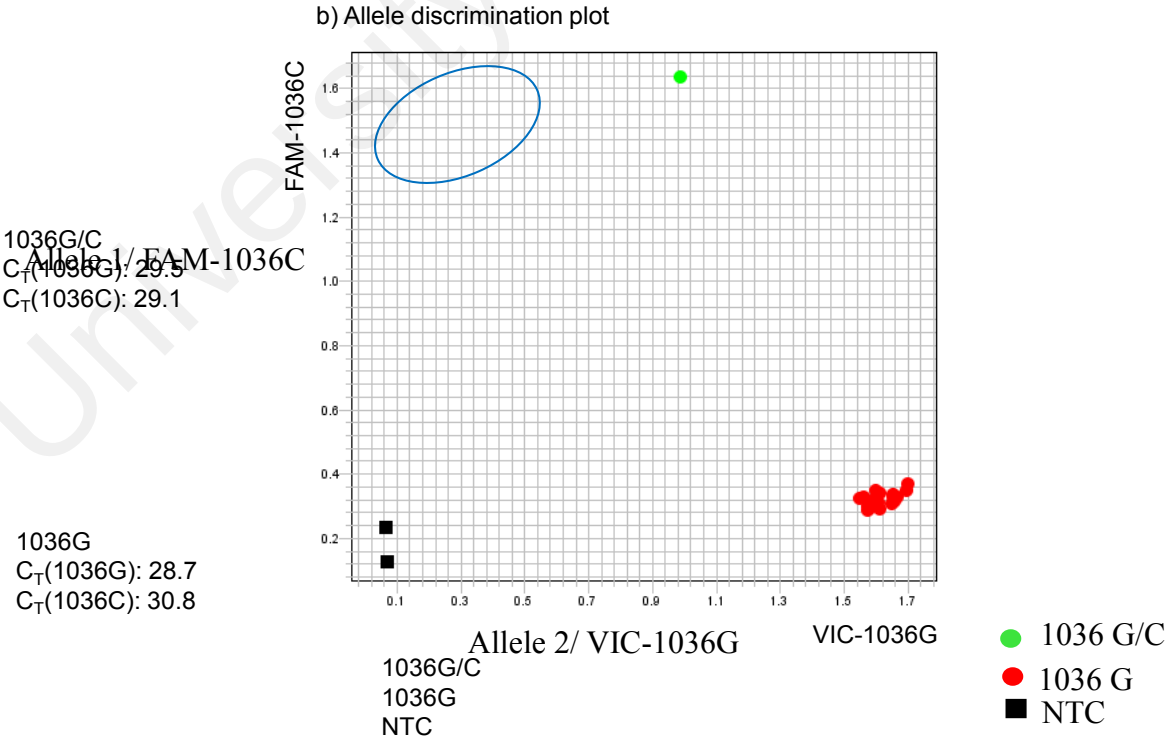


Figure 4.17: Allele discrimination plot shows clustering of genotypes for mutation c.1036 G>C. The CT values for individual probes for all genotypes are presented. 1036 G: Homozygous normal; 1036G/C: Heterozygous mutant; NTC: no-template control.

ROX
FAM
VIC

4.3 Functional analysis of the mutation

Ideally confirmation of the pathogenicity of newly described mutations requires protein functional assays that demonstrate the exact change or loss of normal function. These kinds of effort are usually laborious, and very much depends on the establishment of stable and consistent mutant protein expression systems, success of isolating and purifying the mutant protein and subsequently having the relevant tools and techniques to design protein functional assays that would allow one to observe and assess any shift from the normal protein function. As part of an on going project, attempts to obtain functional mutant proteins were made by first constructing mutant protein expression cassettes, followed by verification and expression of the desired protein.

4.3.1 Creation of mutant gene via SDM

Functional studies of mutant proteins first require the isolation of the mutant protein in a quantity that would be sufficient for functional assays to be carried out. To obtain this mutant protein, expression constructs were created and this is followed by gene expression in appropriate expression systems.

Mutant *G6PC* gene construct was created by site-directed mutagenesis (SDM). The size of pCMV6-empty vector used was 4929bp and the size of the G6PC gene was 1249bp. Amplification of the mutant insert by PCR after the mutagenesis step using construct specific primers produced a DNA band within the region of the expected length (6178bp) (Figure 4.18a) confirming that the plasmid was carrying the insert of interest. This was then cloned into the bacterial host *E.coli* strain TOP10 and later propagated into LB broth agar.

After the transformation into TOP10 cells, positive transformants were selected via PCR with pCMV6 vector primer set (VP1.5 and XL39 primer provided).Gel electrophoresis of mutant colonies selection was shown in Figure 4.18b. The mutant

construct transformant, a PCR-band of~1200bp was observed in agarose gel after amplification with vector primer (VP1.5: 5'-GGACTTTCCAAAATGTCG-3'; XL39: 5'-CCCACCAGCCTTGTCCTAAT-3').

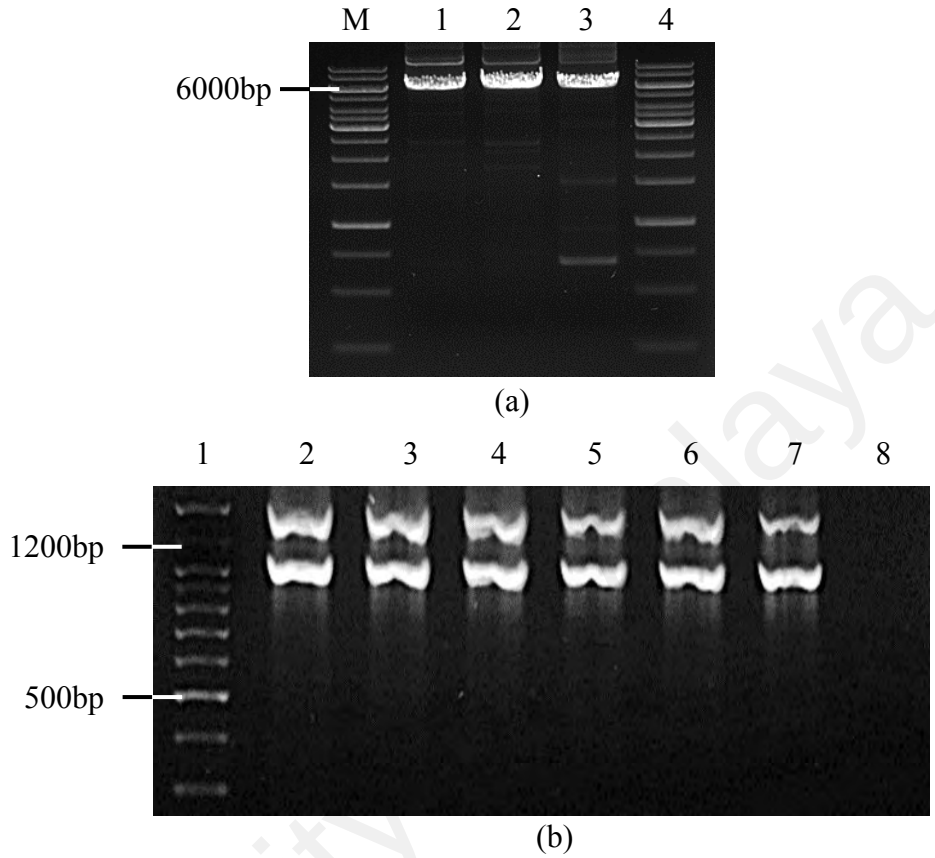


Figure 4.18:(a) Agarose gel electrophoresis of SDM product with size of 6178bp.
Lane 1 and 5: Marker (1kb ladder)
Lane 2: SDM_52 mutant
Lane 3: SDM_76 mutant
Lane 4: SDM_346 mutant

(b) Agarose gel electrophoresis of colony PCR after SDM. The size of the inserted gene is 1240bp.
Lane 1: Marker (100bp)
Lanes 2-7: Isolated colonies
Lane 8: Negative control

4.3.2 Sequence verification of mutant constructs

Three mutation constructs were subjected to sequence verification to confirm that mutagenesis has taken place at the intended nucleotide position. Figure 4.19 shows the result of sequencing of mutated sequence at the intended nucleotide position. The sequencing results showed that gene was successfully mutated at amino acid positions 52, 76 and 346.

4.3.3 Verification of transgenic cell line

Each of the successfully mutated cloned gene were then directly transfected into Human liver hepatocellular carcinoma (HepG2) cell line. Calcium phosphate transfection method was used to insert the mutant plasmid into the cell. Geneticin was used as a selective drug to stabilize transfection over the transgenic cell culture, and subsequently stable subcultured colonies were isolated and harvested. Total DNA was then extracted from positive transformants harvested cell lysate and was used for PCR and sequencing analysis to confirm that the clones were stable and maintained their mutated sequences. Sequencing results are shown in Figure 4.20.

4.3.4 Mutant Protein detection assay

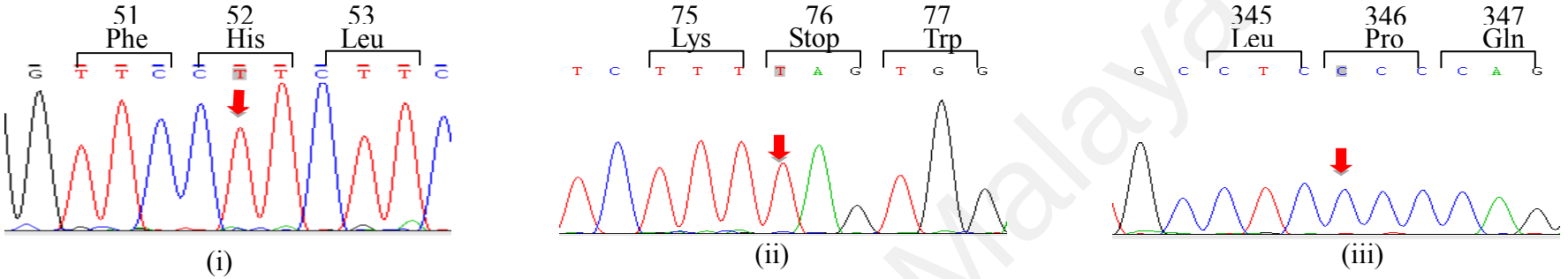
4.3.4.1 Detection of the mutant protein via SDS-PAGE and expression of the mutant protein on western blot assay

SDS-PAGE was carried out to check the expression of the G6PC mutant protein. Results shown in the Figure 4.21 showed a band of approximately 37kDa in size which could possibly be the G6PC mutant protein.

4.3.4.2 Expression of the mutant protein on western blot assay

Western blot analysis was used to detect and confirm *G6PC* mutant protein by polyclonal anti-rabbit G6PC antibody (OriGene, Rockville) as primary antibody and rabbit anti-goat antibody (kind gift from Dr. Hussain Al Rothan from The Medical Faculty, University Malaya) as secondary antibody. Unfortunately, despite repeated attempts, none of the mutant proteins were detectable through Western blot analysis. The expected size of the mutant protein is about 37kb. The expression of mutant construct of *G6PC* gene was very low and almost undetectable (Figure 4.22). It is noteworthy that the Western blotting experiment was repeated by using a different primary antibody, but the result remained the same.

(a)



(b)

```
ATGGAGGAAGGAATGAATGTTCTCCATGACTTTGGGATCCAGTCAACACATTACCTCCAGGTGAATTACC
AAGACTCCCAGGACTGGTTCATCTTGGTGTCCGTGATCGCAGACCTCAGGAATGCCTTCTACGTCCTCTT
CCCCATCTGGTTCCATCTTCAGGAAGCTGTGGGCATTAAACTCCTTTGGGTAGCTGTGATTGGAGACTGG
CTCAACCTCGTCTTTAAGTGGATTCTCTTTGGACAGCGTCCATACTGGTGGGTTTTGGATACTGACTACT
ACAGCAACACTTCCGTGCCCCTGATAAAGCAGTTCCCTGTAACCTGTGAGACTGGACCAGGGAGCCCCTC
TGGCCATGCCATGGGCACAGCAGGTGTATACTACGTGATGGTCACATCTACTCTTTCCATCTTTCAGGGA
AAGATAAAGCCGACCTACAGATTTCGGTGCTTGAATGTCATTTTGTGGTTGGGATTCTGGGCTGTGCAGC
TGAATGTCTGTCTGTCACGAATCTACCTTGCTGCTCATTTTCCTCATCAAGTTGTTGCTGGAGTCCTGTC
AGGCATTGCTGTTGCAGAAACTTTCAGCCACATCCACAGCATCTATAATGCCAGCCTCAAGAAATATTTT
CTCATTACCTTCTTCCTGTTCAGCTTCGCCATCGGATTTTATCTGCTGCTCAAGGGACTGGGTGTAGACC
TCCTGTGGACTCTGGAGAAAGCCCAGAGGTGGTGCGAGCAGCCAGAATGGGTCCACATTGACACCACACC
CTTTGCCAGCCTCCTCAAGAACCTGGGCACGCTCTTTGGCCTGGGGCTGGCTCTCAACTCCAGCATGTAC
AGGGAGAGCTGCAAGGGGAAACTCAGCAAGTGGCTCCCATTCCGCCTCAGCTCTATTGTAGCCTCCCTCG
TCCTCCTGCACGTCTTTGACTCCTTGAAACCCCCATCCCAAGTCGAGCTGGTCTTCTACGTCTTGTCCTT
CTGCAAGAGTGCGGTAGTGCCCCTGGCATCCGTCAGTGTCATCCCCTACTGCCTCGCCCAGGTCCTGGGC
CAGCCGCACAAGAAGTCGTTG
```

(i) (ii) (iii)

Figure 4.19:(a) Partial chromatogram sequence of mutant constructs **(i)** mutant construct 52;**(ii)** mutant construct 76;**(iii)** mutant construct 346 and **(b)** the nucleotide sequence of the G6PC vector gene. The nucleotide sequences in highlighted colour correspond to sequence of the mutant construct.

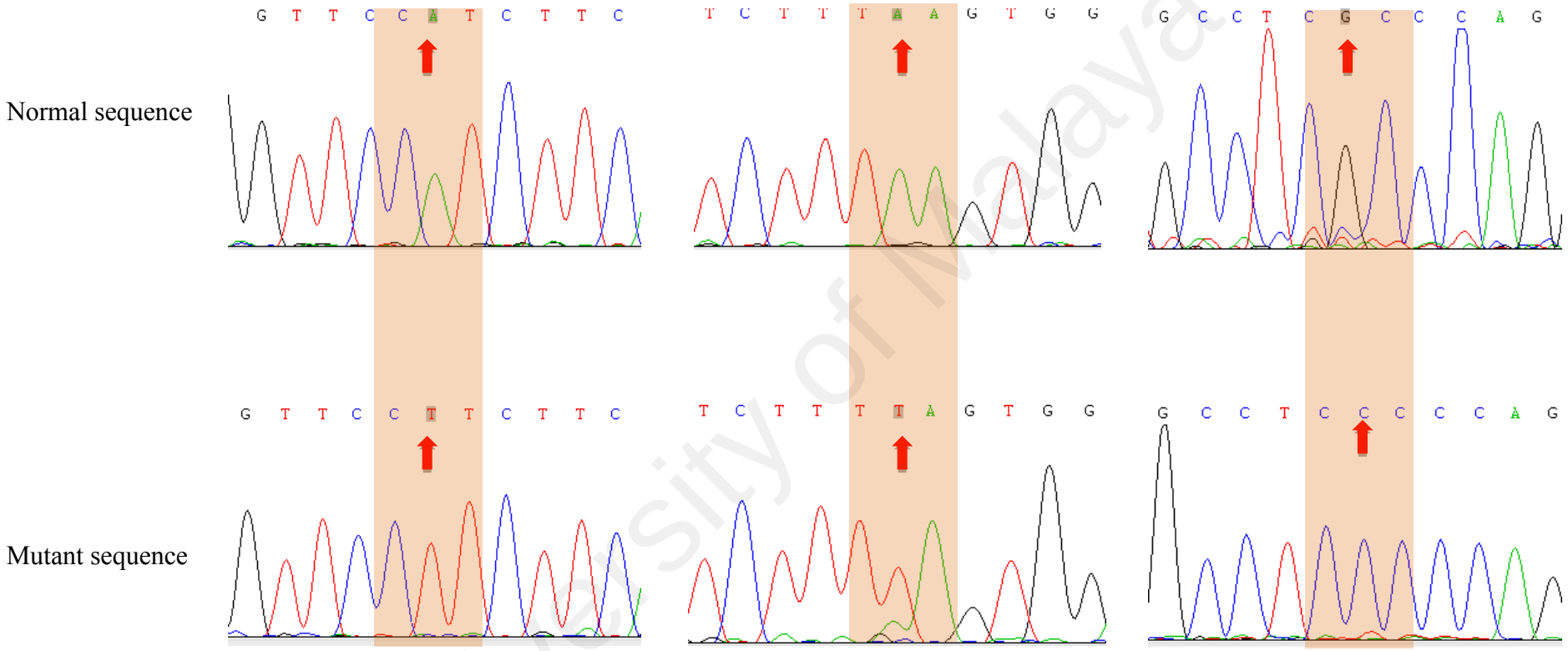


Figure 4.20: Shows the chromatogram sequences of normal and transgenic mutant. **(a)** Partial chromatogram sequence of mutant construct 52, **(b)** partial chromatogram sequence of mutant construct 76and **(c)** partial chromatogram sequence of mutant construct 346.

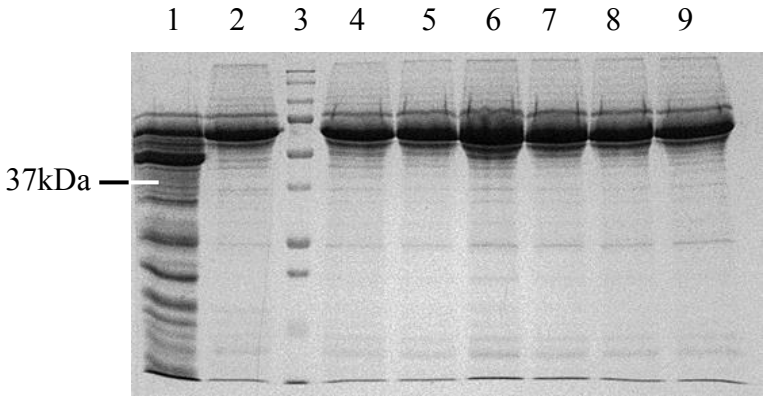


Figure 4.21: Example of SDS-PAGE and Coomassie blue staining mutant construct with size of 37kb.

Lane 1: HepG2 cell lysate
Lane 2: Wild type construct
Lane 3: Protein marker (Biorad)
Lane 4 and 5: Mutant constructs 52
Lane 6 and 7: Mutant constructs 76
Lane 8 and 9: Mutant constructs 346

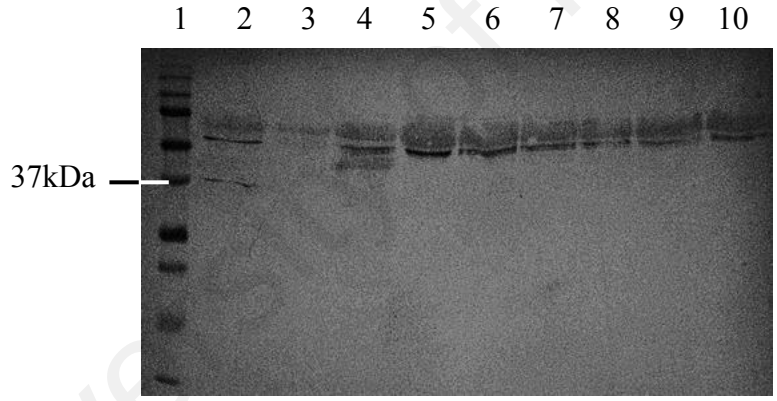


Figure 4.22: Example of western blot analysis of mutant construct with size of 37kb.

Lane 1: Protein marker (Biorad)
Lane 2: pCMV6 empty vector
Lane 3: G6PC lysate
Lane 4 HepG2 cell lysate
Lane 5: Wild type construct
Lane 6: Mutant constructs 52
Lane 7:Mutant constructs 54
Lane 8: Mutant constructs 76
Lane 9:Mutant constructs 72
Lane 10: Mutant constructs 346

CHAPTER 5: DISCUSSION

GSD Ia is one of the more common types of GSD representing approximately 80% of all GSD type 1 cases (Shieh *et al*., 2002). This disease is caused by the dysfunctional G6Pase enzyme -- a unique enzyme of carbohydrate metabolism that is active inside the lumen of ER membrane (Burchell, 1996). To date, more than 111 different germline mutations have been reported and recorded in Human Gene Mutation Database (HGMD). Over 550 unrelated patients affected with GSD Ia have been studied worldwide. In Malaysia however, the mutation spectrum and frequency of the *G6PC* gene has yet to be investigated.

There are three main parts to the current research project. The main part of this study is to screen the *G6PC* gene and identify the causative mutation(s) in the Malaysian GSD Ia patient cohort. The results obtained herein will to contribute towards the understanding and establishing the spectrum of pathogenic mutations found in GSD Ia patients in Malaysia. Screening in a panel of control samples representing the multi-ethnic Malaysian population was also performed to obtain support for pathogenicity of mutations that are yet unreported in the literature or in mutation databases. The absence of a particular mutant allele in the control population is taken as evidence to support the pathogenic status of the mutation and eliminate the notion that the mutation is a harmless polymorphism. Finally, the study of the functional properties of the affected protein that could be causative of GSD Ia is initiated in this study.

In this study, mutations within *G6PC* gene for most GSD Ia patients involved are identified. The types of the mutations with respect to each patient have been listed previously in Table 4.1 and are presented according to the format recommended by the HGMD. Among the 30 patients that were tested with molecular genetic analysis, only 21 of them are found to carry mutations. Altogether nine different mutations were

discovered, five of which are reported mutations and the other four are potentially novel mutations (Table 5.1).

The nine mutations identified include seven missense mutations, one nonsense mutation and one silent mutation that have been shown to result in abnormal splicing. All these point mutations (single nucleotide changes) are located at exon 1, 2, 4 and 5 (Figure 5.1) in either homozygous form or as compound heterozygotes.

Table 5.1: List of mutations found among 21 GSD Ia patients.

Reported mutation	Potentially novel mutation
c.248 G>A, R83H (Lei *et al*.,1995)	c. 155 A>T, H52L
c.518 T>C, L173P (Li *et al*.,2007)	c.226 A>T, K76X
c.648 G>T, L216L (Kajihara *et al*.,1995)	c.337 C>T, P113S
c.664 G>A, G222R (Stroppiano Lei *et al*.,1999)	c.1036 G>C, A346P
c.706 T>A, W236R (Lei *et al*., 1995)	

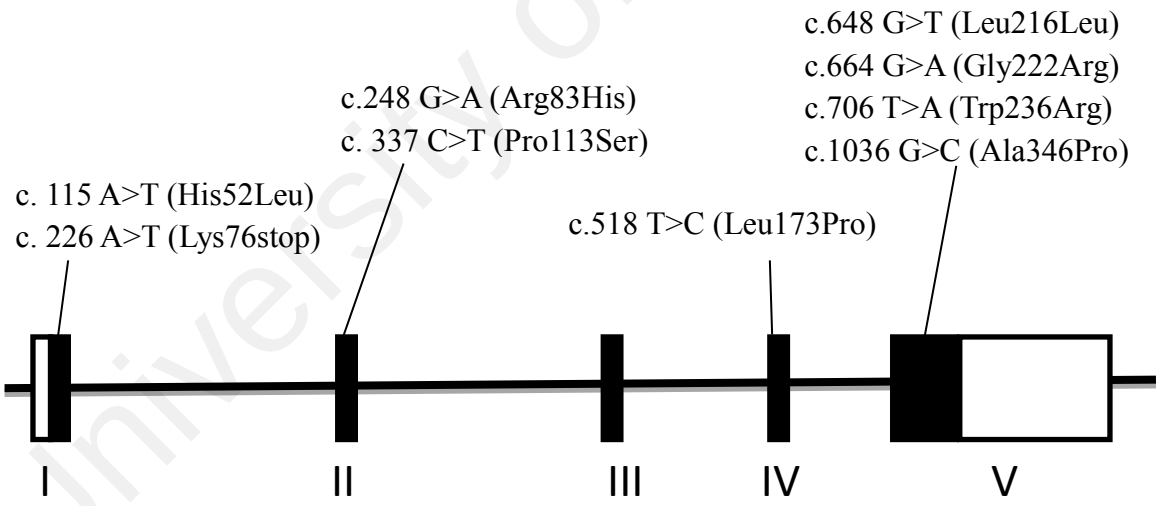


Figure 5.1: Position of identified mutation in *G6PC* gene in this study. The exonic regions are indicated by filled box (black box) and the untranslated regions by open boxes (white box). The Roman numerals represent the number of exons (*Source*: edited from Shieh *et al*., 2002)

5.1 Novel mutations

5.1.1 c.155 A>T, (p. His52Leu) mutation

One of the mutations that has not been reported the is c.155 A>T mutation. This mutation was located in exon 1 of the *G6PC* gene at nucleotide position 155, where the nucleotide A was changed to a T. This change is expected to results in an amino acid substitution, from Histidine coded by CAT to the amino acid Leucine, coded by CTT due to the change on the second nucleotide of codon 52.

This mutation has been found in patient 5 and 6 in homozygous form and this mutation is likely to be pathogenic and responsible for the development of GSD Ia disease. As studied by Chou and Mansfield (1999), His-52 residue is located at ER cytoplasmic loop, which is considered as less stringent for structural requirements, perhaps due to the low occurrence of mutation found in the ER cytoplasmic loop. However, this region has shown to be associated with the enzyme activity of G6Pase. In a published study on the effects of mutation on enzyme activity, it has indeed been shown that 12 out of 14 non-helical mutations have the tendency to significantly reduce to as low as 15 % of wild-type function, or even totally abolish the enzyme activity (Shieh *et al*., 2002).

To date, only one missense mutation and one nonsense mutation were found in these regions. The W50X mutation changes the Tryptophan to a stop codon and makes the expression of functional protein unlikely due to significant truncation of the mutant protein. Lei *et al.* (1995b) have also demonstrated that the stop codon caused loss of G6Pase catalytic activity. The Q54P mutation on the other hand was detected in the Caucasian population (Trioche *et al.*, 2000) and has also been observed to completely abolish the G6Pase enzyme (Shieh *et al.*, 2002).

There are 10 histidine residues located in the nine-transmembrane helical structure of the human G6Pase, which are denoted as H9, H17, H52, H119, H176, H179, H197, H252, H307 and H353. Each of the His residue are thought to carry different functions in G6Pase activity (Chou, 2001). For example, H176 acts as a nucleophile forming phosphohistidine enzyme-intermediate and H119 functions to provide the proton needed to liberate the glucose moiety (Chou, 2001). Both these His residues are located at the lumen side of ER which is the important region for enzyme activity. Almost all His residues (except H17) are conserved in human, mouse rat and dog (Chou & Mansfield, 1999). This provided additional support to suggest that mutations occurring in His residues will disrupt the function of G6Pase enzyme.

To obtain additional support that the mutation is indeed pathogenic to GSD Ia disease, we proceeded to show that this mutation is not present in a cohort of healthy unaffected individuals. The study of genetic variation within the population involves with frequency of the mutant alleles within the population was done to ensure that the mutations are not mere polymorphisms. As such, if this mutation occurs in normal population with the occurrence rate equal or more than 1%, it will be considered as a polymorphism. Otherwise, it may be considered as a mutation.

In screening for the H52L mutation in the healthy control population, the mutant c.155T allele can be efficiently assayed using TaqMan genotyping analysis. In this work, sensitive and reliable genotyping detection was used due to the lack of restriction enzyme assays that could be used to distinguish normal and mutant alleles. Using the Taqman approach, different probes were used for different alleles and each probe has a flurophore which transmits signal light in specific wavelength, making it possible to separate the different genotypes (Suzuki *et al*., 2005).

For the c.155C>T mutation, the patients involved are determined to be homozygous. As shown in Figure 4.16, homozygous mutant alleles and normal alleles were clustered differently. A mock heterozygous mutant was created to provide clarity during the interpretation of results, and to check the functionality of the assay and results showed that the alleles were clustered in different plot position. In a similar research, the minimum size of the population studied is about 200 healthy samples. In a study conducted by Okubo and associate (1997), 216 healthy samples were used. However, in the present study, due to limitation of time, sample availability as well as financial resources, only a total 150 (300 alleles) healthy samples were used. This number was deemed sufficient as published research screening lower numbers of sample in population studies have been encountered (Sellamutu *et al*., 2015; Federica *et al*., 2002)

5.1.2 c.226 A>T, (p.Lys76stop) mutation

Mutation K76X is a nonsense mutation located at codon 76. A new premature stop codon is created by this mutation and a truncated G6PC is predicted as a consequence. K76 is located within the transmembrane helices 2 (Pan *et al*., 1998) and it has been identified as one of the G6Pase active sites, together with Arg83, His119, Arg170and His 176 (Hemrika & Wever, 1997). K76 functions as the site for transition-state stabilization (Chou, 2001) and is predicted to be critical to G6P binding and hydrolysis.

In G6Pase, residues 76 and 108 shared a conserved phosphate signature motif K-X_6-RP-(X_{12-54})-PSGH-(X_{31-54})-SR-X_5-H-X_3-D. There is only one other mutation reported at codon 76, which is K76N. Mutation K76N showed a complete abolishment of the G6Pase enzyme activity (Lei *et al*., 1993) and confirmed that the K76 residue is important in G6Pase catalysis. This finding is also supported by a study that was conducted by Shieh and co-worker (2002), where K76N mutation altered the active site of G6Pase and directly abolished phosphate activity in transient expression assay. Thus,

the nonsense mutation identified here (K76X) is expected to result in total loss of function, and perhaps cause more severe effects on its enzyme activities. Its pathogenecity is also supported by the fact that the mutation is not present is all population control samples tested (300 alleles)

5.1.3 c.337 C>T, (p.Pro113Ser) mutation

Another unreported mutation found in the ER luminal loop is mutation P113S, which causes the substitution of Proline (CCA) to Serine (TCA) at codon 113. This missense mutation was found in exon 2 of *G6PC* gene at nucleotide position 337. This mutation is suggested to be a potential novel mutation since there is no confirmed similarity to any reported mutations in any previous studies on GSD Ia. This mutation has been found in patient 1 and 2 in compound heterozygote form, where the other mutation is L216L mutation. This c.337C>T mutation is carried in the mother while the father carried the L216L mutation.

Pro-113 residue is located at the helical portion within luminal loop 1 and 3 (Shieh *et al*., 2002). Shieh and co-worker depicted that any mutation located at luminal loop 1 may play a crucial role in the catalytic activity of the enzyme, because it will almost surely abolish the G6Pase activity (Zheng *et al*., 2014). There was another reported mutation, P113L which was previously found in this region resulting a substitution of Pro residue to a Leu residue at same position (Matern, 2002). This alteration also completely inactivated the G6Pase enzymatic activity (Shieh *et al.*, 2002). Luminal loop also plays a crucial part in catalytic activity of the enzyme because any mutation found in this loop will disrupt the activation of G6Pase (Shieh *et al.*, 2002). Because of the importance of this position P113 in enzymatic activity of G6Pase, the consequence of P113S mutation is expected to a give similar effect to G6Pase.

5.1.4 c.1036 G>C, (p.Ala346Pro) mutation

Finally, the last unreported mutation found in this study is A346P missense mutation. This mutation is located in the last transmembrane region of G6Pase and caused the change of amino acid Alanine (GCC) to Proline (CCC) at position 346. This mutation was found in exon 5 of the *G6CP* gene at nucleotide position 1036. In this study, this mutation was found in patient 8 in heterozygous form, where the father is the carrier for this mutation. This mutation is likely to be pathogenic and responsible for the development of GSD Ia disease as it is also not seen in the panel of healthy control samples.

There are several mutations found in the last transmembrane region of the G6PC protein. Mutations R295C, S298P, F322L, V338F, I341N and L345R were all found to abolish the G6Pase activity. From other neighbouring mutations found from previous studies, it can also perhaps be deduced that the mutation A346P might also have severe effects on enzymatic activity as reported (Lei *et al*., 1995; Lei *et al*., 1995a; Shieh *et al*., 2002).

The frequency of the mutant allele is only 0.33% (1/302) thus adding support that this unreported mutation found in this study is indeed pathogenic, causative GSD Ia disease.

5.1.5 Functional analysis of novel mutations

Functional analysis on potentially pathogenic mutations (H52L, K76X and A346P) found in the study was done to check the effects in G6Pase protein mutations. In the course of the study, the P113S mutation was not included to functional analysis because this mutation was only identified near the end of the research project, after the SDM work has been completed. All the clones that carry the mutant alleles were created via SDM and were directly transfected into mammalian cell culture (HepG2 cell line).

HepG2 cell line was selected as *in vitro* system in this study because it is derived from liver tissue, which is relevant to the fact that GSD Ia is dominantly found in the liver. The recombinant colonies was selected and harvested, and the DNA were extracted and their sequences were directly checked for verification.

The successfully transfected mutants were later screened for the expression of the mutant protein. Even though the mutants were successfully transfected and carried the mutant sequence, their proteins were unable to be detected in western blot assays. The predicted size of the mutant protein created is 40.47 kDa, 8.83 kDa and 40.52 kDa respectively (H52L, K76X and A346P). However, the predicted protein size was not present. It may due to the low expression of the protein itself (Figure 4.22). It this study, the polyclonal anti-rabbit G6PC antibody was used. Based on a study by Sheih *et al*., (2003), a specific antibody was used to check the structure function of the protein that would greatly facilitate the G6Pase protein expression. Since the polyclonal antibody is not specific, multiple bands appeared. However, because of the low concentration of the total protein of the mutant, the protein expression was undetectable in the blots.

5.2 Reported mutations

5.2.1 c.248 G>A, (p. Arg 83His) mutation

This mutation was first reported by Lei *et al.* (1995a) where it involved a transition from nucleotide G to A at nucleotide position of 248 in the exon 2 of *G6PC* gene. The original arginine residue is changed to a histidine at codon 83 (p.Arg83His). Research by Lei *et al.* (1995a) on liver biopsy of GSD Ia patient's shows that the level of phosphohydrolase activity is very low or merely undetectable. It indirectly inactivates the G6Pase enzyme due to the mutation. Sheih and colleagues (2002) also supported this notion, that changes of nucleotide (G>A) caused the alteration of codon 83 and totally abolished phosphate activity in transient expression assay.

Arg83 is predicted to be one of the active sites in the G6Pase enzyme which is an important catalytic activity of G6Pase (Shieh *et al.,* 2002). A study from Lei *et al* (1995b) shows that a conservative and also non-conservative change in the amino acid structure will lead to the abolition of protein activity. In addition, Arg83 occurred at the CpG nucleotide. Most mutation that occurred at this site will result in a 5-methylcytosine to T transition although transition of a G to A can occur as well. In addition, CpG nucleotide at codon 83 of the G6Pase gene seemed to be a hotspot for mutation in GSD Ia. As reported by Shieh *et al.* (2002), western blot analysis was done, and it was found that this mutation completely abolished the enzyme activity. Mutation altering the Arg codon is expected to distort the active center of G6Pase leading to adverse effect of this disease (Chou & Mansfield, 2008).

As reported by Matern and colleagues (2002), this mutation is among the most frequently detected mutations, accounting for 3.9 % of all Caucasian population. Arg83His is also most prevalently distributed in Chinese population almost 70 % frequencies (Lei *et al*., 1995b). However, another mutation is reported to be in the same amino acid position, c. 247 C>T (R83C) and it is widely distributed in many other populations including the Caucasian, Jewish, Hispanic, Turkish and Arabian (Chou & Mansfield, 2008). This strongly suggests that the mutation spectrums of *G6PC* among Asian and other countries are not similar.

5.2.2 c.518 T>C, (p.Leu173Pro) mutation

This mutation is located at exon 4 of the *G6PC* gene in the second position of codon 173. The transition from nucleotide T to C leads to the changes of codon CTT to CCT where the amino acid Leucine (Leu) is replaced by Proline (Pro). Leu173Pro mutation is first described by Li *et al.* (2007) where the mutation was found in heterozygous

carrier (compound heterozygote), of the parent for prenatal diagnosis using chorionic villus sampling in China.

Despite many mutations that have been studied worldwide, there is not much information available on L173P (Chou & Mansfield, 2008). Leu-173 resides on the second luminal loop (L2 domain) between transmembrane four and five. It is located at the non-helical outer region of ER membrane. Based on the previous studies by Shieh and colleagues (Shieh *et al*., 2002), 57 % of the mutation in the non-helical region of the lumen and cytoplasm domain will actually retain residual G6Pase activity. For example, other than the L173P mutation, its neighbouring R170R mutation will cause the G6Pase enzyme to be inactivated (Huner *et al*., 1998) and be unstable at the cellular level (Rake *et al*., 1998).

Therefore, hypothetically, the changes of the amino acid Leucine in 173 residues to other amino acid will give the same effects as other mutations on the non-helical region.

5.2.3 c.648 G>T, (p.Lue216=) mutation

Initially, this mutation was reported by Kajihara *et al*. (1995) as 727 G>T. This mutation is situated in exon 5, where G to T transversion has occurred. This mutation lies in the third nucleotide of codon position 216, where codon CTG will change to CTT. Both codons are coding for the same amino acid name (Leu, Leu216Leu). So, theoretically, this mutation is known as silent mutation that is not expected to be pathogenic.

However, cDNA analysis revealed that a 91-nucleotide deletion was detected at carboxyl-terminal as a result of this mutation (Kajihara *et al*., 1995). Kajihara is the first individual who is responsible for the detection and characterization of this mutation, and found that this mutation will actually cause an aberrant splicing at exon 5. The aberrant splicing that leads the deletion from the 5' end of the exon 5, which is from nucleotide

position 642 until 732, will alter the reading frame, and in turn will produced a termination signal in codon 202 of the aberrant mRNA (Ki *et al*., 2004). Therefore, it will produce a truncated protein product that only contains 201 residues compared to 357 in the normal G6Pase protein (Kajihara *et al*., 1995) which is 146 amino acids shorter (Karasawa *et al*., 1998).

This mutation was found to be the most prevalent mutations for GSD Ia patients in the Northeast Asia region (Lei *et al*., 2014). Based on the Eminoglu and colleague review (2013), 88% of Japanese alleles from the GSD Ia patients consist of this mutation. Besides, L216L mutation also accounts for 54 % of the affected alleles in Korean population. It is also common in the Chinese population as this mutation accounts for 54% (Lei *et al*., 2014) in the alleles of the GSD Ia patients. In total, this mutation is the second most prevalent mutation after p.Arg83Cys among Asian population with percentage occurrence of 16.4% (Chou & Mansfield, 2008). The fact that this mutation was frequently found in the Asia population, it suggested that the mutation might reflects the genetic relationship among different races in Asia and also due to the founder effect (Okubo *et al*., 1997).

5.2.4 c.664 G>A, (p.Gly222Arg) mutation

This mutation was first reported by Stroppiano and colleague (Stroppiano *et al*., 1999) when they found it in a compound heterozygote patient. This mutation resides on nucleotide position 664 in exon 5 of *G6PC* gene in which the change occurs in the first nucleotide of codon 222. As a result, it changes codon GGA to AGA and this will produce a missense mutation where the original amino acid Glycine (Gly, G) changes to Arginine (Arg, R), c.664 G>A. Lei and colleagues (1994) also reported the same amino acid changed (p.Gly222Arg), but that is due to another nucleotide change (c. 664G>C, GGA>CGA).

According to Matern and friend (Matern *et al*., 2002), this mutation is the lowest frequency mutation of which it accounts to only 0.2% of total allele in GSD Ia patients in Caucasian population. G-222 amino acid resides in the sixth transmembrane helix of G6Pase protein, which is anchored in the ER membrane. Several studies by other researchers have shown that mutation in this site will greatly reduce the enzymatic activity.

One of the earliest researches performed by Lei *et al.* (1995a, 1995b) found that the changes from amino acid of Glycine, which is non-polar amino acid, to Arginine which has the basic property, has resulted in a great decrease in the enzymatic activity to only 4%. The results of the research raised a speculation that codon 222, which resides in ER membrane that requires a hydrophobic environment, cannot tolerate large polar or charged amino acid, such as Arginine (Lei *et al*., 1995a).

Shieh and team (2002) proved that this mutation will stabilize the G6Pase protein and reduce the enzyme synthesis, whilst at the same time retaining the residual activity. The study also proposed that p.Gly222Arg mutation affects the amino acid residing in the transmembrane helices, which thus affects the structural integrity of the helices by altering the helical structure, hence causing misfolding and abnormal conformations of the protein which eventually leads to its degradation.

5.2.5 c.706 T>A, (p.Trp236Arg) mutation

Mutation p.Trp236Arg was first found by Lei *et al*. (1995a). This mutation has changed T to A at nucleotide position 706 in exon 5 of the *G6PC* gene (c. 706 T>A) and occurred in the first position of codon 236, where TGG is replaced by AGG. As a result, these mutations change the amino acid from Tryptophan to Arginine (p.Trp236Arg), a missense mutation. The frequency occurrence of the mutant allele in GSD Ia patients is low and only accounts about 0.1% of total population (Matern *et al*., 2002).

Trp-236 residue is located inside the ER lumen, on the third luminal loop of the G6Pase protein. Like most of the other mutations occurred in the non-helical region, the changes of the amino acid at this side has resulted in a great decrease in the G6Pase activity (Lei *et al*., 1995a). Furthermore, Shieh and colleagues (2002) depicted that the mutant allele bearing this mutation has proven to synthesize similar amount of G6Pase protein like the normal allele, and reduced the protein activity.

5.2.6 c.547 A>G, SNPs

Genetic variation, such as Single Nucleotide Polymorphism (SNPs), is a natural occurring characteristic of genome that differentiates individuals of a species. This variation may have arisen due to mutation, duplication, inversion, unequal recombination, insertion or deletion of the DNA sequence (Futuyma, D. J, 2005). In this study, a substitution of adenine (A) nucleotide into guanine (G) nucleotide at position 574 in exon 5 (c. 574 A>G) occured. This mutation causes the change of amino acid Threonine (Thr, ACA) to Alanine (Ala, GCA). Initially, this mutation is expected to be a missense mutation of GSD Ia. However, Okubo and team (Okubo *et al*., 1997) have cited this mutation as non-pathogenic mutation and reported as SNPs.

This SNPs mutation was found in all of our patients and in some of our healthy control. A research by Okubo *et al*. (1997) claims that this mutation was also found in all Japanese patients, control samples and in nine of Caucasian patients. It is thus proven that this mutation is one of variations in *G6PC* gene. This mutation could also be accessed in National Center for Biotech Information database (NCBI accession number- NG_011808.1).

5.3 Unsuccessful mutation detection

In this study, 10 out of 42 individuals screened for GSD 1a were found with no mutation. There are several reasons in the failure of mutation detection in the patient

with no mutation. One of the reasons is region of mutation screening. The process of mutation screening in the present study only involved in coding region of *G6PC* gene and partly intronic sequences within 10bp from the exon-intron boundary. Accordingly, if there are any changes located in 5' and 3' of untranslated region (UTR) of *G6PC* gene, it would not be detected in the mutation screening. The mutation screening in the UTR has shown to be important in some cases as it had been known to affect mRNA stability (Chatterjee & Pal, 2009).

The failure in screening of mutation may also due to misdiagnosis of the disease of the patients. These patients have not undergone additional testing using any enzyme assay, which is normally carried out as confirmatory assays for the purpose of diagnosis. The current diagnosis in hospital is based only on clinical observation and the presence/appearance of the symptoms. According to other published literatures, several types of GSDs share the almost similar clinical manifestation with only slight variations. For example, all GSDs patients are affected with hypoglycemia which is low blood sugar. But GSD1b for example has association with neutropenia (abnormal neutrophils). So, similar clinical features can often lead to misdiagnosis.

CHAPTER 6: CONCLUSION

This mutation screening of the *G6PC* gene has successfully led to the identification of nine different mutations; five were reported mutations (c. 248 G>A, c.518 T>C, c. 648 G>T, c. 664 G>A and c.706 T>A) and four (c.115 A>T, c.226 A>T, c.337 C>T and c.1036 G>C) potentially pathogenic mutation in GSD Ia patients. The reported five mutations' protein effects had been extensively studied by other researchers. Meanwhile, the other four potentially pathogenic mutations have not been reported by any publication and thus their pathogenicity needs to be confirmed.

The study on mutation in the Malaysian population on all four potentially novel mutations was done by screening the mutant allele in patients and in a panel of healthy volunteer control (300 alleles). Restriction endonuclease (RE) assay and TaqMan® SNP Genotyping assay were designed to use in identifying the mutant alleles in the patients and healthy individual samples. After screening 150 healthy individuals' samples consisting 50 Malays, 50 Chinese and 50 Indians in Malaysian population, all the mutant alleles were found to be absent. It concludes that none of the 150 healthy controls carry mutant alleles, thus suggesting that these potentially novel mutations are potentially pathogenic mutation. To support the evidence of the pathogenicity of the mutations, future studies should focus on functional characterization of the potentially novel mutation in the G6Pase gene in order to increase our understanding of the GSD1a.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Paper presented:

1. **Abd Rahman, A. A**., Abdullah, I. S., Teh, Ser-Huy., Khaidizar, F. D., Yap, Sufin., Keng, Wee-Teik., …Mohamed, Z. (2014). Determining Pathogenicity Of Potential Novel Mutations in GSD1a Patients using Restriction endonuclease and TaqMan probe-based assays. *Oral presentation at the 19th Biology Science Graduates Congress,12th-14th December 2014, National University of Singapore, Singapore.*

2. **Abd Rahman, A. A**., Abdullah, I. S., Teh, Ser-Huy., Khaidizar, F. D., Yap, Sufin., Keng, Wee-Teik., …Mohamed, Z. (2016). Novel mutations of the *G6PC* gene in Malaysian von Gierke disease patients. *Oral presentation at the International Postgraduate Research Awards Seminar 2016, 7th-8th March 2016, University of Malaya, Malaysia.*