1.0 INTRODUCTION

1.1 Overview

Primary immunodeficiency disease (PID) is a disorder caused by an inherited flaw in the immune system. PIDs are mainly seen in children and are characterized by recurrent and severe infections with susceptibility to unusual organisms. The infections tend to be overwhelming and fail to resolve with conventional treatment (Marodi & Notarangelo, 2007). The simplest classification of PID is based on the dysfunctional immune system component that is involved i.e. T lymphocytes, B lymphocytes, phagocytes and complement. Phagocytes are cells of the immune system and the first line of defense in an infection. Neutrophils are professional phagocytes and neutrophil dysfunction constitutes a subset of primary immunodeficiency disease (Burns & Davies, 2010). Whilst the collective incidence of primary immunodeficiency is estimated to be 2.82 per 100,000 live births in Australia (Baumgart et al., 1997) and 8 per 100,000 live births in Sweden (Fasth, 1982), the nature and incidence of PIDs is not known in Malaysia.

This research is conducted to study the neutrophil dysfunction disorders in a Malaysian primary immunodeficiency patient. This study is part of a larger project to determine the PID frequency and distribution in Malaysia. The study would lead to the establishment of diagnostic tests for neutrophil dysfunction disorders and the detection of genetic abnormalities causing these disorders. The study design is shown in Figure 1.1. The index case in this study was indicative of having impairment in his neutrophil function based on a less sensitive chemiluminescence based phagocytic function test. Hence, specific and definitive testing methods by flow cytometry were developed. The genetic studies were performed to establish the disease status in this patient.

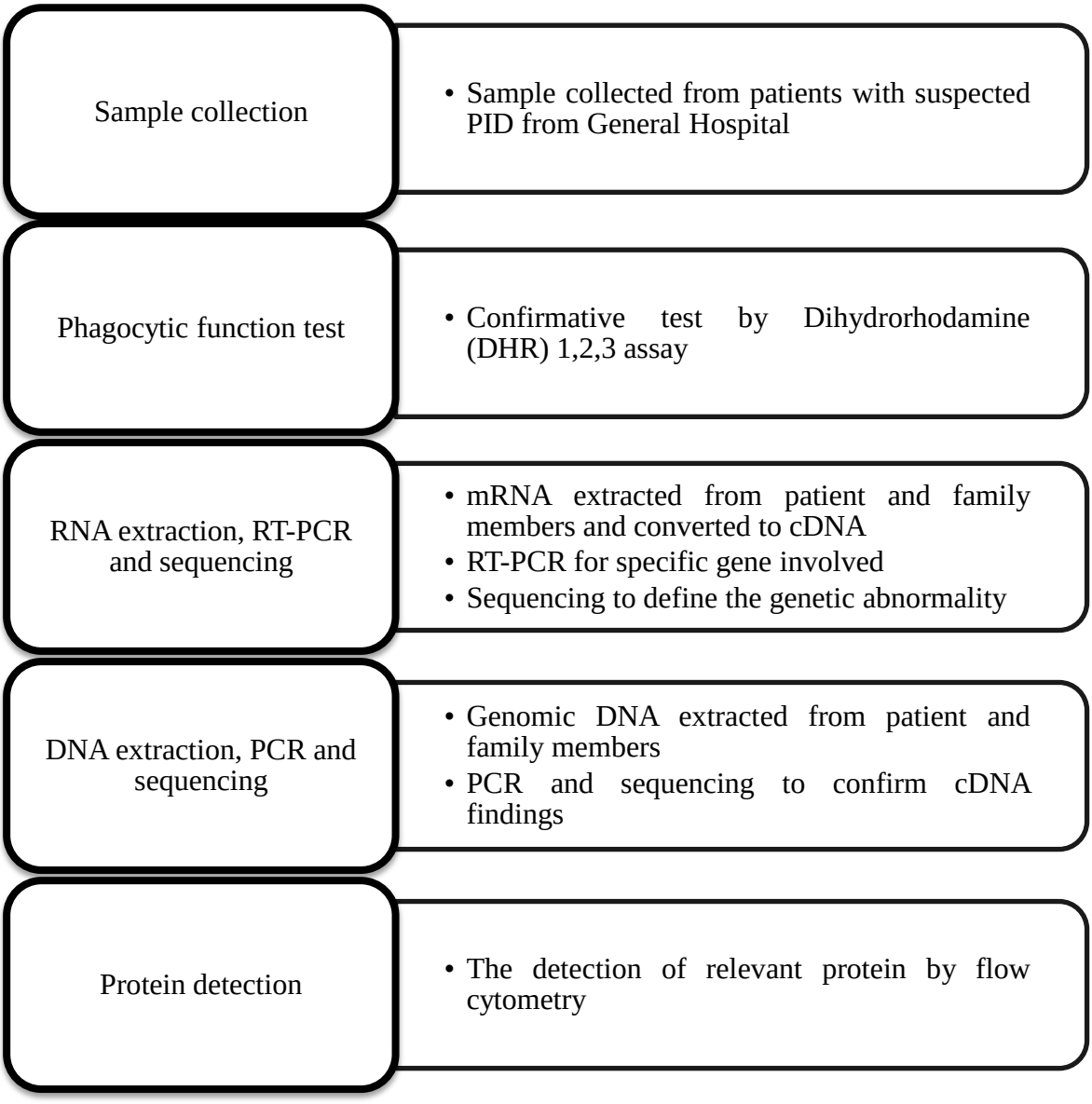

Figure 1.1: Study design for the neutrophil dysfunctional project.

1.2 Rationales and Objectives

The previous inceptive studies of PIDs with neutrophil dysfunction have highlighted the need to establish tests and techniques to investigate these PIDs and emphasized the importance of including the family members in the study. As PIDs are generally due to a single gene defect, the criteria for diagnosis tend to include genetic information. Treatment options, such as bone marrow transplant and gene therapy require the genetic abnormality of the patient to be defined. Most PID patients and their families tend to carry genetic abnormalities that are unique to the patient and his family. In the case of X-linked disease, the establishment of carrier status and confirmation of the abnormality would be important to female siblings so that accurate genetic counseling can be provided. The molecular genetic studies on the family studies would further elucidate whether the mutation occurred in the patient is *de novo* or whether it was inherited.

Hence, the objectives of this study are:

1) To define the genetic abnormality in a patient suspected with hereditary neutrophil dysfunction and the carrier status of his family members,
2) To determine the relationship between the genetic abnormality and the clinical presentation of neutrophil dysfunction disorders,
3) To establish diagnostic tests for neutrophil dysfunction related disorders for the clinical usage in the Institute for Medical Research, Malaysia.

1.3 Thesis Organization

This thesis consists of six chapters. The first chapter is an overview of Primary Immunodeficiency Disease (PID), neutrophil dysfunction and the status of PID cases in Malaysia. Chapter two is the literature review whereby it describes the immune system, primary immunodeficiency disease, neutrophil dysfunction disorder and Chronic Granulomatous Disease in depth. The research methods employed in this study are illustrated in chapter three. Chapter four depicts the results obtained from the flow cytometry assay and the genetic studies. Chapter five discusses the findings on Chronic Granulomatous Disease suffered by the index case recruited in this study. The summary of this research is written in chapter six and followed by the bibliography and appendix.

2.0 LITERATURE REVIEW

2.1 Immune System

The human immune system is composed of constellation of cells and molecules that confers protection and host defense against invading foreign particles (Akira, Uematsu & Takeuchi, 2006). Cells of the immune system distinguish self from non-self before mounting an immune response in order not to destroy their own cells (Janeway, 2001). The identification of own cell is aided by the presence of markers provided by the major histocompatibility complex (MHC) that are usually present on the cell membrane (Lakshmikanth, Karre & Sreerama, 2011). Antigens are termed as non-self-molecules or foreign particles that induce an immune response. Cells of the immune system are known as leukocytes or white blood cells (WBCs) that are derived from pluripotent hematopoietic stem cells in the bone marrow. Upon maturation, cells migrate to tissues for safeguarding, circulate in the bloodstream or reside in the lymphatic system (Murphy, 2012). The host immune system responds to an infection by different mechanism provided by different cells of the immune system namely the innate and adaptive system (Oberbarnscheidt, Zecher & Lakkis, 2011).

The innate immunity is the first line of defense presented since birth that provides protection in a non-specific manner (Kurtz, 2004). Though the innate immunity provides the front line defense, it is not long lasting as the response mounted is not specifically targeted to the causal infectious agent (Janeway et al., 2001). Innate leukocytes include natural killer cells, mast cells, eosinophils, basophils, complements and phagocytes (Turvey & Broide, 2010). The main task of the innate immune system is to recruit the innate leukocytes to the infection site, to identify and remove foreign substances and to activate the adaptive system by means of antigen presentation

(Beutler, 2004; Janeway, 2001). The innate immune response is initiated by the pathogen recognition (Janeway, 2001). Serum proteins of the complement system add a molecular tag to the pathogens to label them as infectious or dangerous for elimination (Janeway et al., 2001). The effector cells such as the phagocytes are recruited to kill and eliminate the foreign entity in body (Silva, 2010). Inflammation is the immune system's response towards infection. The innate immunity is restricted to the recognition of limited number of receptors and secreted protein coded in the germ line (Oberbarnscheidt et al., 2011).

On the other hand, adaptive immunity is acquired through the infection encountered throughout life (Janeway, 2001). It is an antibody (humoral) and cell-mediated response carried out by B lymphocytes and T lymphocytes respectively (Kurtz, 2004). The lymphocytes have a vast range of antigen receptors that allows the immune system to recognize and extinguish any antigens presented to them (Alberts et al., 2002). Somatic cell gene rearrangement produce colossal range of antigen receptors that is able to finely discern even the slightest disparity in closely related molecules (Medzhitov, 2007; Oberbarnscheidt et al., 2011). In other words, different lymphocytes bear different variants of the same basic receptor that would take part selectively in immune response towards the encountered pathogen. This type of immunity is fleshed out during the lifetime of an individual as a result of acclimatization to the specific pathogen causing the infection and hence has immunological memory (Medzhitov, 2007). Therefore, it confers lifelong immunological protection against the same pathogen (Bonilla & Oettgen, 2010). Re-infection with the similar pathogen would be managed with a more robust and rapid adaptive immune response (Hofmeyr, 2000). The humoral responses conferred by the B lymphocytes are mediated by antibodies or known as immunoglobulins secreted by B cells (Bonilla & Oettgen, 2010). There are five types of antibodies namely IgA, IgG, IgE, IgM and IgD that has its own biological

properties with different ability to react with different antigens (Vale & Schroeder, 2010). The T lymphocytes are associated with cell-mediated immunity in which they move in the blood vessel until encountering specific antigens to provide protection against those particular pathogens (Broere et al., 2011). T cells that have not encountered pathogen are known as naive T cells. Upon stimulation with pathogen, the naive T cells are activated to differentiate into effector T cells. The T cells are divided into two different classes of cells, cytotoxic and helper cells that identify peptide antigens from different type of pathogen (Broere et al., 2011). The cytotoxic T cells or also known as CD8 T cells recognize antigens presented by intracellular pathogens that divide in cytoplasm and eliminate them (Alberts et al., 2002). Antigens from pathogens multiplying in intracellular vesicles and those resulting from ingested extracellular bacteria and toxin are presented to another class of T cells known as helper CD4 T cells (Janeway et al., 2001). The effector T cells which are short lived generate memory T cells that are long lived to respond rapidly with re-infection with the same pathogen (Buchholz et al., 2013).

Both the innate immunity and the adaptive immunity are essential arms of the immune system that works together to provide the utmost protection to the host. A defect in either the innate immunity or the adaptive immunity system predisposes individual to enhanced susceptibility to infection that results in primary immunodeficiency disease.

2.2 Primary Immunodeficiency Disease

Primary immunodeficiency disease (PID) is a rare inherited disorder caused by an intrinsic flaw in the immune system (Boyle & Buckley, 2007). PIDs are mainly seen in children and are exemplified by recurrent and severe infections with susceptibility to

unusual organism (Lekstrom-Himes & Gallin, 2000). In other words, microorganisms that are not harmful to a normal person can cause lethal infections in PID patients (Stasia & Xing, 2008). The infections tend to be overwhelming and fail to resolve with conventional treatment. Other clinical symptoms include autoimmunity, inflammation, allergy and malignancy (Parvaneh et al., 2013). Besides the hallmark feature of recurrent infection, PID patients are also usually presented with bacterial septicaemia, bronchiectasis, pneumonia and sinusitis (Lim et al., 2003). PIDs are also associated with poor physical and/or mental development and have a family history of similar clinical disorders (Lim & Elenitoba-Johnson, 2004; van de Berg et al., 2009). Though PID is thought to be a pediatric disease, it is often detected in adulthood due to delayed diagnosis of childhood illness and late disease manifestations (Marodi & Casanova, 2009). PID is multidisciplinary in that it requires intervention of medical experts from various fields as it affects not only the immune system, but also many organs such as the liver and lung (Parvaneh et al., 2013; Marodi & Casanova, 2009).

The combined population prevalence of PID is 1 in 1,200 live births in the United States (Boyle & Buckley, 2007) and 2.82 per 100,000 live births in Australia (Baumgart et al., 1997). It is highly heterogeneous as phenotypic variability of PID is due to the result of genetic and environmental factor interaction, disease penetrance and expression variability (Notarangelo, 2010). However, it may be more common in countries with high rate of consanguineous marriage (Yu et al., 2008; Notarangelo et al., 2009). Frequency of certain PID mutations is also higher in certain population owing to founder effect and genetic drift (Notarangelo et al., 2009). However, the nature and frequency of the PID problem in Malaysia is not known.

More than 200 different disorders have been reported to date and recognized by the World Health Organization (Marodi & Notarangelo, 2007). Of that, more than 170 disease related genes have been identified at molecular level (Abraham, 2011). Defects

in the development and maturation of cells of the immune system lead to increased vulnerability to infections (Notarangelo, 2010; Kuijpers, Weening & Roos, 1999). Protein molecules that are involved in important biological process such as signal transduction are diminished in expression and function in PID patients (Marodi & Notarangelo, 2007). Mutations that lead to PID can arise from germ line, *de novo* or could be somatic (Casanova & Abel, 2007). Most mutations are inherited as single gene defect by autosomal recessive, autosomal dominant or X-linked inheritance pattern from parental genome (Lim et al., 2003; Casanova & Abel, 2007).

Early and precise diagnosis by means of immunological and molecular genetics analysis is vital for the administration of prompt and appropriate therapy for PID patients to prevent morbidity and mortality (Lim et al., 2003). The establishment of the laboratory screening and confirmatory testing for PID is essential in establishing the disease status in the patient (Lim et al., 2003). Besides, it provides an opportunity and platform for screening of family members and prenatal diagnosis to be carried out (Lim et al., 2003; Jones & Gaspar, 2000) as most PID patients and their families tend to carry similar genetic abnormalities that cause the disease or as carrier that would need genetic counseling for future family planning (van de Berg et al., 2009). The treatment rendered for PID patients includes prophylactic antibiotics, intravenous immunoglobulin treatment, bone marrow transplant and gene therapy (Casanova & Abel, 2007).

New genetic basis of known PIDs are being established with the use of latest high throughput technology such as next generation sequencing (Parvaneh et al., 2013). The underlying genetic defect predisposes PID patients to varying clinical presentation or severity (Cunningham-Rundles & Ponda, 2005). Different type of mutations in a gene can cause unique phenotypic difference in each individual. For instance, a nonsense mutation would cause the reading frame of an mRNA of a protein to stumble with a sudden stop codon. This would lead to the translation of an incomplete protein

that is dysfunctional and inactive. Patient with nonsense mutation would suffer from severe clinical features as the protein that is needed for the function of the immune system is defective. However, a missense mutation in the same gene whereby a single nucleotide is substituted may result in a protein that is partially altered. This patient would suffer from a milder form of the disease.

The defect affecting any of the humoral, cellular or innate immunity will cause PIDs and they are classified by the type of immunological mechanisms that are disrupted by the particular gene defect (Song et al., 2011). The classification of PID is based on the dysfunction immune system arm that is involved i.e. adaptive immunity and innate immunity (McCusker & Warrington, 2011; Samarghitean, Ortutay & Vihinen, 2009). The broad classification of PID is shown in Table 2.1. Antibody deficiency is the most commonly implicated PID, followed by the phagocytic disorders that could lead to profound immunodeficiency (Lim et al., 2003; Noh et al, 2013). Phagocytes are cells of the innate immune system that provide immediate defense against infection (Rosenzweig & Malech, 2010). Professional phagocytes in the blood stream include monocytes, macrophages, neutrophils and dendritic cells (Marodi & Notarangelo, 2007). Professional phagocytes are defined by the presence of receptors on their surface and based on their efficiency in phagocytosis. The first line of defense against infection of bacterial and fungal origin is rendered by the neutrophils (Andrews & Sullivan, 2003). Neutrophil defects may be quantitative, in which the circulating neutrophils in the bloodstream are reduced in number or qualitative whereby the function of neutrophils are greatly compromised (Bogomolski-Yahalom & Matzner, 1995). Both the quantitative (extrinsic) defect and the qualitative (intrinsic) defect result in increased susceptibility to severe and lethal infections (Kuijpers et al., 1999; Roberts & Bonilla, 2013). The defect involving neutrophil function has been well documented.

Table 2.1: Broad classification of primary immunodeficiency.

Innate immunity disorders	Phagocyte defects
	Complement defects
Adaptive immunity disorders	T cell immunodeficiency
	B cell immunodeficiency
	Combined immunodeficiency

(Source: McCusker & Warrington, 2011; Samarghitean, Ortutay & Vihinen, 2009)

2.3 Neutrophil Dysfunction Disorder

Neutrophils are professional phagocytes that constitute about 60-70% of total white blood cells in the blood circulation (Burns & Davies, 2010). The cardinal role of neutrophil is to execute a rapid response by ingesting and killing any pathogen that has entered the body, thereby eliminating infection before the adaptive immune response takes over (Dinaeur, 2007). Bone marrow is the storage or reserve pool of neutrophils. Peripheral neutrophils are marginated neutrophil pool that are attached to the endothelium and are readily available to be released into the blood stream during stress, i.e. infections (Kyono, 2003). During infection, chemotactic substances and bacterial peptides (antigen) that are released stimulate the movement of neutrophils from the marginated pool to the infection site (Kyono, 2003). Neutrophils possess a battery of receptors that enables them to recognize and eradicate the foreign particles by phagocytosis (Lakshman & Finn, 2001).

The phagocytosis process facilitated by neutrophils is illustrated in Figure 2.1. Neutrophils and other professional phagocytes respond to pathogen in a sequence of events that includes: 1) diapedisis of neutrophils out of the vasculature to the infection site, 2) adherence to target, 3) ingestion of target, 4) phagolysosomal fusion and 5) intracellular killing by respiratory burst (Lakshman & Finn, 2001). A defect or failure in any of these steps will lead to neutrophil function disorders and impairs the immune system's ability to eliminate pathogens (Burns & Davies, 2010). For example, Leukocyte Adhesion Deficiency (LAD) is a result of the inability of neutrophils to move to the site of infection (Lakshman & Finn, 2001) and Chronic Granulomatous Disease (CGD) is due to a defect in the intracellular killing step (Dinauer, 2005). Hyper-IgE syndrome is an example of intrinsic disorder of chemotaxis in which neutrophils chemotaxis and respond to inflammation is diminished (Bogomolski-Yahalom &

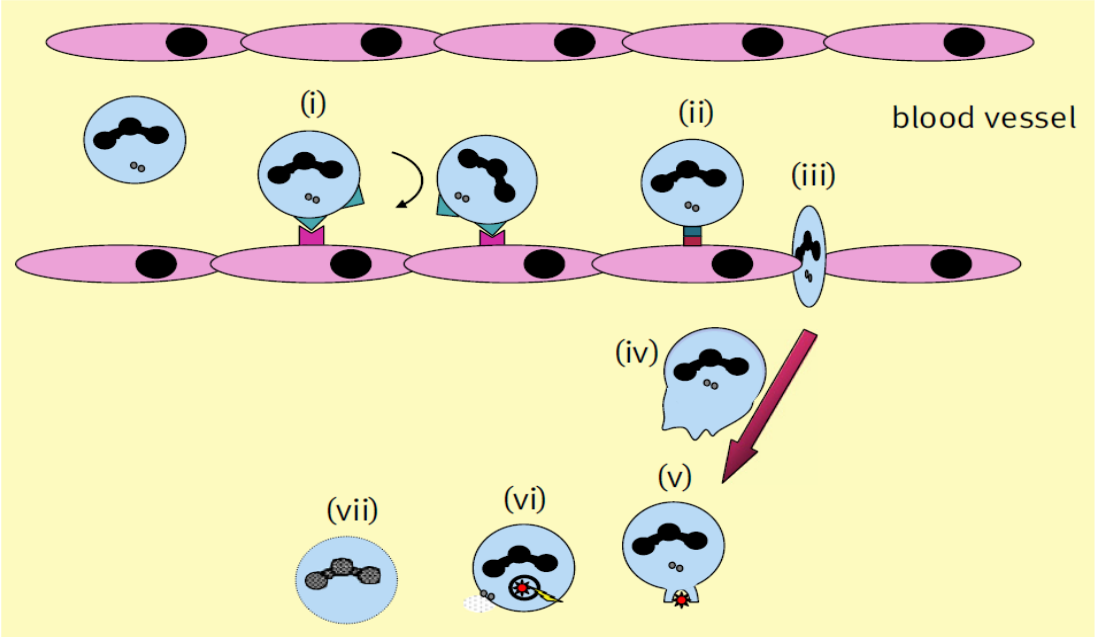

Figure 2.1: Phagocytosis process by neutrophils.

i. Neutrophil adhesion
ii. Before diapedesis through the vessel wall
iii. Migration to infection site
iv. Ingestion of microbe
v. Respiratory burst and enzymatic killing in phagolysosome
vi. Antimicrobial compound is secreted extracellularly
vii. Neutrophil dies by apoptosis

(Source: Burns & Davies, 2010)

Matzner, 1995). These disorders share characteristic clinical and microbiological trait that define the neutrophil intrinsic defect. Table 2.2 summarizes diseases associated with phagocytosis pathway defect.

Neutrophil dysfunctional disorders are normally diagnosed early in life due to the severity of the infection and the type of microorganisms contracted (Kuijpers et al., 1999). However, some patients escape diagnosis until adulthood due to delayed revelation of the disease phenotypes (Roberts & Bonilla, 2013). Patients are often predisposed to skin and mucosal infections, abscess and lymphadenitis (Dinauer, 2007). Recurrent otitis media and upper respiratory infection also provide a clue as to indicate a phagocytic defect (Kuijpers et al., 1999). Infections with *Staphylococci* and *Streptococci*, enteric Gram-negative bacteria such as *E. coli* and *Pseudomonas* sp. and fungi such as *Aspergillus fumigatus* should constitute a cue to suspect the underlying defect of neutrophil dysfunction (Rosenzweig & Malech, 2010). The essential factors and indications to be regarded while considering a phagocytic defect includes: 1) recurrent infection, 2) severity of infection, 3) infection site and 4) infection causal agent i.e. bacteria and fungi (Dinauer, 2007).

The most common and best characterized dysfunctional neutrophil disease is Chronic Granulomatous Disease (CGD) in which the defect lies at the intracellular killing step as the neutrophil is unable to generate a respiratory burst (Yu et al., 2008; (Stasia & Xing, 2008).

2.4 Chronic Granulomatous Disease

Chronic Granulomatous Disease (CGD) is the most commonly encountered neutrophil dysfunction defect with a frequency of 1 in 250,000 live births whereby the neutrophils are unable to produce reactive oxygen intermediates (ROIs) leading to

Table 2.2: Types of neutrophil functional disorders.

Phagocytosis pathway defect	**Disease**
Neutrophil adhesion defect	Leukocyte Adhesion Deficiency Type-1 (LAD-1)
	Leukocyte Adhesion Deficiency Type-2 (LAD-2)
	Leukocyte Adhesion Deficiency Type-3 (LAD-3)
Intracellular killing defect	Chronic Granulomatous Disease
	Myeloperoxidase deficiency
	G6P dehydrogenase deficiency
Chemotactic defect	Hyper IgE syndrome
	Neutrophil actin dysfunction
Granule formation and functional defect	Chediak-Higashi syndrome
	Griscelli syndrome
	Neutrophil specific granule deficiency

(Source: Burns & Davies, 2010; Lakshman & Finn, 2001)

defective killing of bacteria and fungi (Dinauer, 2005; Song et al., 2011). CGD is a rare inherited disorder of the phagocytic function characterized by a profound defect in the intracellular killing of pathogen (Roos et al., 2006). CGD occurs when the enzyme Nicotinamide Adenine Dinucleotide Phosphate (NADPH)-oxidase, that catalyzes the conversion of molecular oxygen to the negatively charged superoxide ion, is defective (Abraham, 2011; Teimourian, de Boer & Roos, 2010). The enzyme is a multi-component protein of gp91-phox, p22-phox, p47-phox, p67-phox, p40-phox and Rac2. Defect in any of its component can result in the disease (Heyworth, Cross & Curnutte, 2003). Hence CGD is a heterogeneous disease which may be X-linked (X-CGD) when gp91-phox is defective, and autosomal recessive (AR-CGD) when either p22-phox, p47-phox, p67-phox, p40-phox or Rac2 defective (Assari, 2006; Lekstrom-Himes & Gallin, 2000).

2.4.1 Clinical Features and Presentation

As in all primary immunodeficiencies, CGD presents in childhood as recurrent, chronic and often life threatening infections (Oliveira & Fleisher, 2010). It is also frequently associated with deep-seated abscesses and formation of inflammatory granulomas, which may be obstructive (Kyono, 2003). Lymphadenitis, hepatosplenomegaly and bronchopneumonia are other common presenting features of CGD (Bogomolski-Yahalom & Matzner, 1995). Patients with X-CGD are often diagnosed early in childhood due to severity of disease manifestation and high mortality rate. Autosomal recessive CGD patients sometimes escape diagnosis in early life as the clinical symptoms surface late and are less severe (Stasia & Xing, 2008).

Bacteria and fungi are the predominant pathogens seen in CGD patients. They are particularly prone to catalase-positive bacteria such as *Staphylococcus aureus* and the Gram-negative enterobacteriacea including *Salmonella, Klebsiella, Aerobacter* and

Serratia (Rosenzweig, 2009). Catalase-negative bacteria usually do not cause infections in CGD patient as they fail to neutralize the residual reactive oxygen intermediates produced by other cells and hence are harmless to CGD patients (Lekstrom-Himes & Gallin, 2000). *Aspergillus* species, *Candida albicans* and *Nocardia* are the most commonly implicated fungus in CGD patients (Agudelo-Florez et al., 2004). However, in Malaysia, five CGD cases have been reported to have contracted *Chromobacterium violaceum* (Mohd et al., 1994; Sureisen, Choon & Tai, 2008; Gill et al., 2012). *C. violaceum* is an opportunistic pathogen (Brito et al., 2004). Infection with *C. violaceum* would lead to severe symptoms that may be lethal as they are highly pathogenic and rarely cause infection in human (Kaufman, Ceraso & Schugurensk, 1986). This finding may suggest that Malaysian CGD patients are particularly prone to *C. violaceum*.

2.4.2 NADPH-Oxidase and CGD Molecular Basis

The generation of reactive oxygen intermediates is catalyzed by NADPH-oxidase (DeLeo & Quinn, 1996). It consists of a membrane bound flavocytochrome b_{558} and several cytosolic proteins; p47-phox, p67-phox, p40-phox and Rac2 (Gill et al., 2012). The flavocytochrome b_{558} comprises an α-subunit p22-phox and a β-subunit gp91-phox and occurs as heterodimer in the membranes of specific granules and secretory vesicles (Rosenzweig, 2009). Heterodimerization is required by p22-phox and gp91-phox for stable expression (Yu et al., 1998). The structure and activation of the NADPH-oxidase enzyme is depicted in Figure 2.2. In a resting cell, the NADPH-oxidase components exist independently and are inactive (DeLeo & Quinn, 1996).

Upon activation by infection, the cytosolic components of NADPH-oxidase form a complex and translocates to the membrane to associate with flavocytochrome b_{558} (Goldblatt & Trasher, 2000). The presence of src-homology 3 (SH3) domains in p47-phox, p67-phox and p40-phox facilitates the translocation of the cytosolic

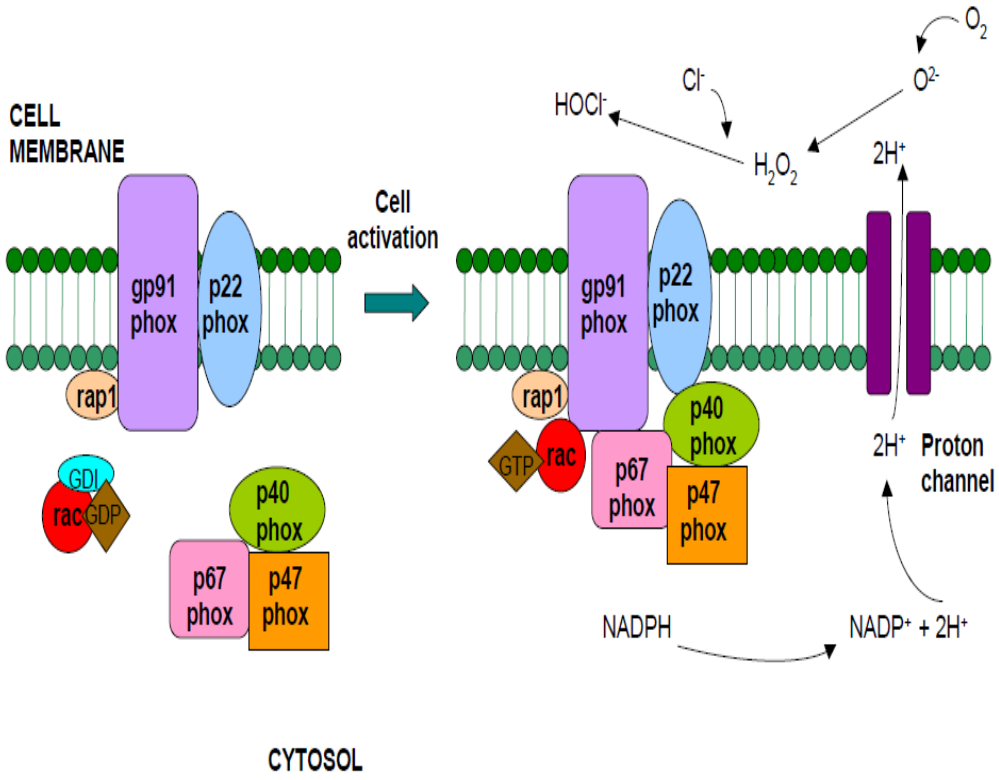

Figure 2.2: NADPH-oxidase components and activation in neutrophils.

(Source: Assari, 2006)

components to the flavocytochrome (Rae et al., 2000). Study had shown that the binding of Rac2 with p67-phox is important in the assembly of active NADPH-oxidase (Clark, 1999). The assembled NADPH-oxidase complex catalyzes the generation of superoxide anion, O_2^-, by transferring electrons across the membrane to molecular oxygen in the phagolysosome (DeLeo & Quinn, 1996). The superoxide anion which is extremely unstable and weakly bactericidal, is converted to more potent oxidants such as hydroxyl radical; OH^-, hydrogen peroxide; H_2O_2, and hypochlorous acid; HOCl to destroy the pathogens (El-Benna et al., 2009; Assari 2006). The most common molecular defect of CGD is caused by mutations in the *CYBB* gene that codes for the β-subunit gp91-phox and leads to X-linked CGD (Green et al., 2001). The autosomal recessive CGD are caused by mutations arising in other NADPH-oxidase components such as p22-phox, p47-phox, p67-phox, p40-phox or Rac2 (Teimourian, et al., 2010). The molecular defect of CGD is shown in Table 2.3.

2.4.3 X-linked CGD

A defect in the gp91-phox protein results in X-linked Chronic Granulomatous Disease (X-CGD) that accounts for about 65% of all CGD cases (Abraham, 2011). The *CYBB* gene that codes of gp91-phox is located on the short arm of the X chromosome at region p21.1 (Kuijpers et al., 1999) and is expressed on the leading DNA strand. It is composed of 13 exons that code for 570 amino acids, which would form a functionally active gp91-phox protein (Sun et al., 2012). Figure 2.3 depicts the *CYBB* gene and gp91-phox protein structure.

The structure of gp91-phox subunit is divided into two separate domains, which is the N-terminal domain and C-terminal domain. The N-terminal domain is the heme-binding domain with six putative transmembrane segments. The C-terminal domain has the NADPH binding site (Pessach et al., 2006). Missense, nonsense, splice site

Table 2.3: CGD molecular defect.

Disease	Molecular defect	Gene	Chromosome location	Frequency
Chronic Granulomatous Disease	gp91-phox	*CYBB*	Xp21.1	65%
	p47-phox	*NCF1*	7q11.23	25%
	p67-phox	*NCF2*	1q25	5%
	p22-phox	*CYBA*	16q24	5%
	p40-phox	*NCF4*	22q13.1	~1%

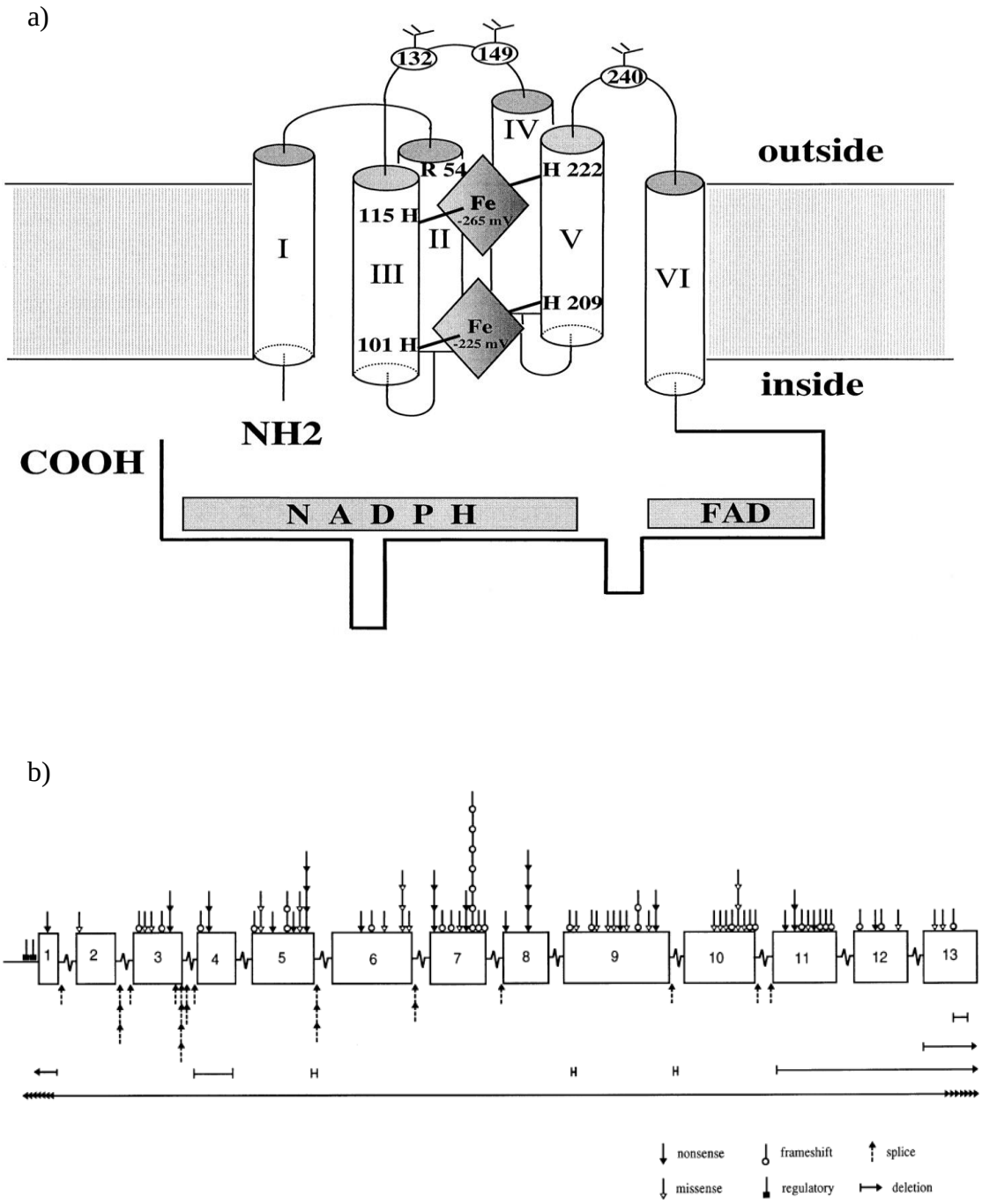

Figure 2.3: (a) gp91-phox protein structure. (Source: Yu et al., 1998)
(b) *CYBB* gene. (Source: Rae et al., 1998)

mutations and frame shift mutations due to deletion and insertion have been defined in this gene (Rae et al., 1998; Song et al., 2011). Single nucleotide polymorphisms (SNPs) that include missense, nonsense and splice site mutations account for about 70% of gene defects. Meanwhile, deletion, insertion and mutations in the promoter region accounts for about 30% of defects (Abraham, 2011). Severe mutations like nonsense, deletion and insertion in the *CYBB* gene could demolish protein expression due to the mRNA and protein instability (Sun et al., 2012).

Males are more likely to be affected since it is an X-linked disease (Lewis et al., 2008). Female carriers usually do not display clinical symptoms due to the presence of two X chromosomes that protects them with at least one functional gene (Wolach et al., 2005). The occurrence of X-CGD is very rare in female, but there are reports showing female to have manifested the disease (Anderson-Cohen et al., 2003; Mills, Rholl & Quie, 1980; Wolach et al., 2005). In rare situations like skewed X-inactivation, females tend to suffer from the disease as the defective X allele is selectively activated to be expressed in their neutrophils (Anderson-Cohen et al., 2003). Hence, without a functional gp91-phox protein, the NADPH-oxidase enzyme loses its function to generate reactive oxygen intermediates. Subsequently, female carriers become susceptible to infection and hence manifest the CGD phenotypes (Lewis et al., 2008).

2.4.4 Autosomal Recessive CGD

Autosomal recessive CGD (AR-CGD) occurs when there is a defect in any of genes that code for p22-phox, p47-phox, p67-phox and Rac2 (Abraham, 2011). Each of these protein components are coded by a different gene located on different chromosomes (Martire, et al., 2008; van de Berg et al., 2009) (Table 2.2). The AR-CGD affects male and female patients at equal proportion and accounts for about 35% of all CGD cases (van de Berg et al., 2009; Fattahi et al., 2011).

Defect in *NCF1* gene that codes for p47-phox reports the highest frequency of AR-CGD cases (Heyworth, Noack & Cross, 2002). The most common mutation encountered in the *NCF1* gene is a characteristic dinucleotide deletion in the beginning of exon 2 that causes a premature stop codon and subsequently leads to absent p47-phox protein in neutrophils (Roos et al., 2006). Mutations in the *NCF2* gene and *CYBA* gene that codes for p67-phox and p22-phox are rare that they contribute about 5% each of the CGD frequency (Agudelo-Florez et al., 2004). The occurrence of CGD due to mutation in p40-phox is extremely rare that there has been only a single case reported to date (Matute et al., 2009). Mutations in p22-phox, p47-phox, p67-phox, p40-phox and Rac2 are highly heterogeneous that includes insertion, deletion and single nucleotide substitution that may result as nonsense or missense mutations (Wolach et al., 2008).

Several studies had shown that the p47-phox deficient CGD patients tend to have residual ROI production that somehow protects them from infections and they are better survivors compared to other AR-CGD patients (van de Berg et al., 2009; Kuhns et al., 2010; Fattahi et al., 2011). In general, AR-CGD patients have a better prognosis of survival rate compared to X-CGD patients due to the milder clinical course (Song et al., 2011).

2.4.5 Diagnosis

Nitrobluetetrazolium (NBT) reduction was established as the diagnostic test for CGD patients as early as 1967 (Bogomolski-Yahalom & Matzner, 1995). This method is still used widely to determine the respiratory burst as it is cost efficient. A more sensitive and robust test using Dihydrorhodamine 1,2,3 (DHR 1,2,3) assay by flow cytometry is now used to determine the hydrogen peroxide production in the neutrophils (Abraham, 2011). Figure 2.4 depicts the classical response pattern of neutrophils in the DHR assay upon stimulation with phorbol myristate acetate (PMA). The production of

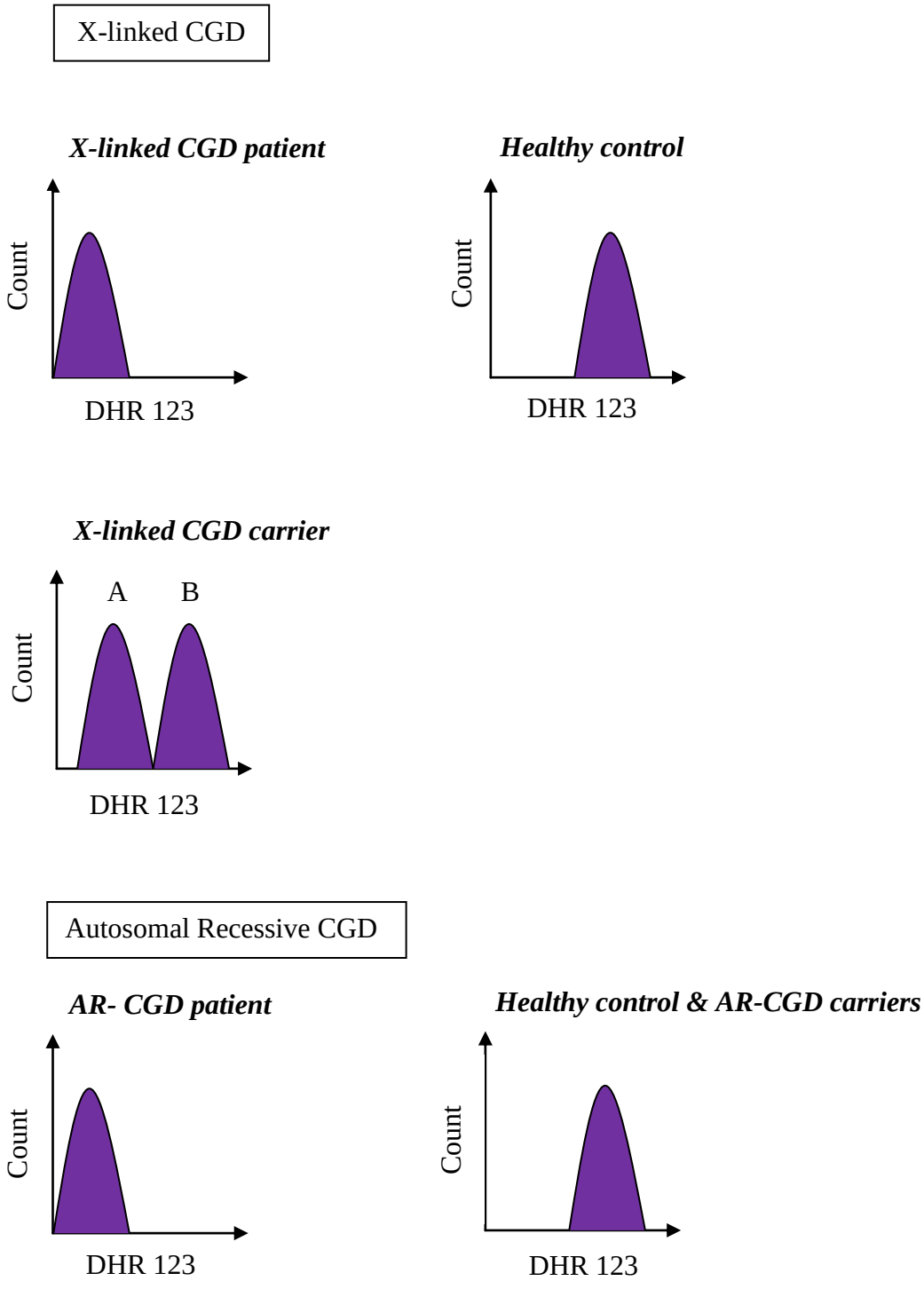

Figure 2.4: The classic DHR result pattern showing of X-linked and autosomal recessive CGD patients, healthy control, carriers of X-linked CGD and AR-CGD. Area under the curve denotes H_2O_2 production.

respiratory burst in the neutrophils is indicated by the peak shift to the right. A normal healthy person would generate a respiratory burst when the neutrophils are activated with foreign particle and hence would show a peak shift to the right in the DHR assay. Typical X-linked and autosomal recessive CGD patients do not show a peak shift upon PMA stimulation. The X-CGD carriers would exhibit two distinct neutrophil populations whereby one of the neutrophil populations is defective in producing a respiratory burst (A) and the other population will generate a respiratory burst (B) (Figure 2.4). The autosomal recessive CGD carriers however demonstrate the same response pattern as the healthy control. The AR-CGD carriers could not be detected by flow cytometry and the only means of determination is by molecular studies (Stasia & Xing, 2008). Hence, if the flow cytometry assay does not reveal a bimodal response pattern in the family members especially in the mother and sister, it can be postulated that the inheritance pattern of CGD in the patient is autosomal recessive. The NBT and DHR assay serve as a screening test for CGD patients to say whether they have functional and non-functional neutrophils.

Once the status of patient is established, it is important to determine the missing NADPH-oxidase component and the mutation(s) that had arised (Abraham, 2011). Western blot is the traditional method to determine the presence of missing protein (Stasia & Xing, 2008). The transmembrane protein, gp91-phox is difficult to be isolated as it is anchored to the membrane and difficult to maintain the native conformation for functional study (Marques et al., 2007). Hence, direct surface staining method has been developed to study the expression of g91-phox protein by flow cytometry (Yamauchi et al., 2001). The presence of other NADPH-oxidase components such as p22-phox, p47-phox and p67-phox can be determined by Western blot (Lakshman & Finn, 2001). Molecular mutational analysis is carried out to define the type of mutation encountered

and to classify patients into specific subtypes by amplifying the gene of the respective missing protein (Abraham, 2011).

Identification of the underlying molecular defect of CGD has led to several improvements such as accurate molecular diagnosis of the disease, institution of proper and lifesaving treatment, carrier detection and prenatal diagnosis (Holland, 2010; Teimourian et al., 2010; Abraham, 2011).

2.4.6 Treatment

The management of CGD patients includes prophylactic antibiotic to prevent serious infections. Antimicrobials such as trimethoprim sulfamethoxazole and itraconazole are administered to wade off bacterial and fungal infections respectively (Notarangelo, 2010). It was proven that CGD patient's prognosis improves with the use of antimicrobial prophylaxis.

The use of interferon gamma as inflammatory cytokine has shown to reduce the rate of serious infections in CGD patients (Roos, Kuijpers & Curnutte, 2007). It is recommended as lifelong therapy to contain infections in patients with CGD.

Hematopoietic stem cell transplantation is one of the definitive curative treatments for CGD (Holland, 2010). A complete HLA-matched donor is needed for the transplant to take place successfully (Seger, 2007). The major risk associated with bone marrow transplant is the graft-versus-host-disease (GVHD) and inflammatory reactions at infection sites (Dinauer, 2005; Stasia & Xing, 2008).

Gene therapy is now considered as a possible treatment for CGD (Newburger, 2006). As in X-CGD, female carriers with skewed X inactivation with at least 10-20% functional neutrophils do not show a defect in respiratory burst (Dinauer, 2005; Seger, 2007). Hence, it is postulated that partially corrected granulocytes could provide protection to the patients especially those without a HLA-matched donors (Assari,

2006). However, clinical trials of gene therapy are still under development. Several rectifying factors need to be improved such as vector design, in vivo selection of transduced hematopoietic stem cells and bone marrow conditioning (Assari, 2006; Ott et al., 2007).

Even though the definitive curative treatments, namely stem cell transplant and gene therapy are now available, they are associated with great risk of host rejection and complications in the recipient. Hence, a proper and accurate diagnosis is vital to ensure the quality of life of the patient.

3.0 MATERIALS AND METHODOLOGY

Blood was obtained from the CGD patient and the family members with informed consent. Blood samples were collected in lithium heparin tubes for the neutrophil function assay. As for the gp91-phox protein expression, RNA and DNA extraction, blood samples were withdrawn in EDTA tubes. The experimental work flow is shown in Figure 3.1.

3.1 Sample size

This is a single case study of X-linked CGD. The small sample size (n=1) is the limitation in this study. This is because CGD is a rare disease with a prevalence of 1 in 250,000 live births. Hence, neutrophil dysfunction disorder cases especially CGD are not seen very often in Malaysian population. Therefore, during the study period there was only a case of X-linked CGD being referred from the government hospital for molecular analysis.

3.2 Patient and Family Members

The index patient, EKL is a male child of non-consanguineous Chinese parents, born in 2010. This patient came to our attention without a definitive diagnosis and had severe clinical conditions. He suffered an episode of meningitis at day 27 of life. This was followed by BCG lymphadenitis at 4 months and at 6 months he was admitted to hospital for prolonged fever. He was noted to have recurrent left axillary lymphadenitis.

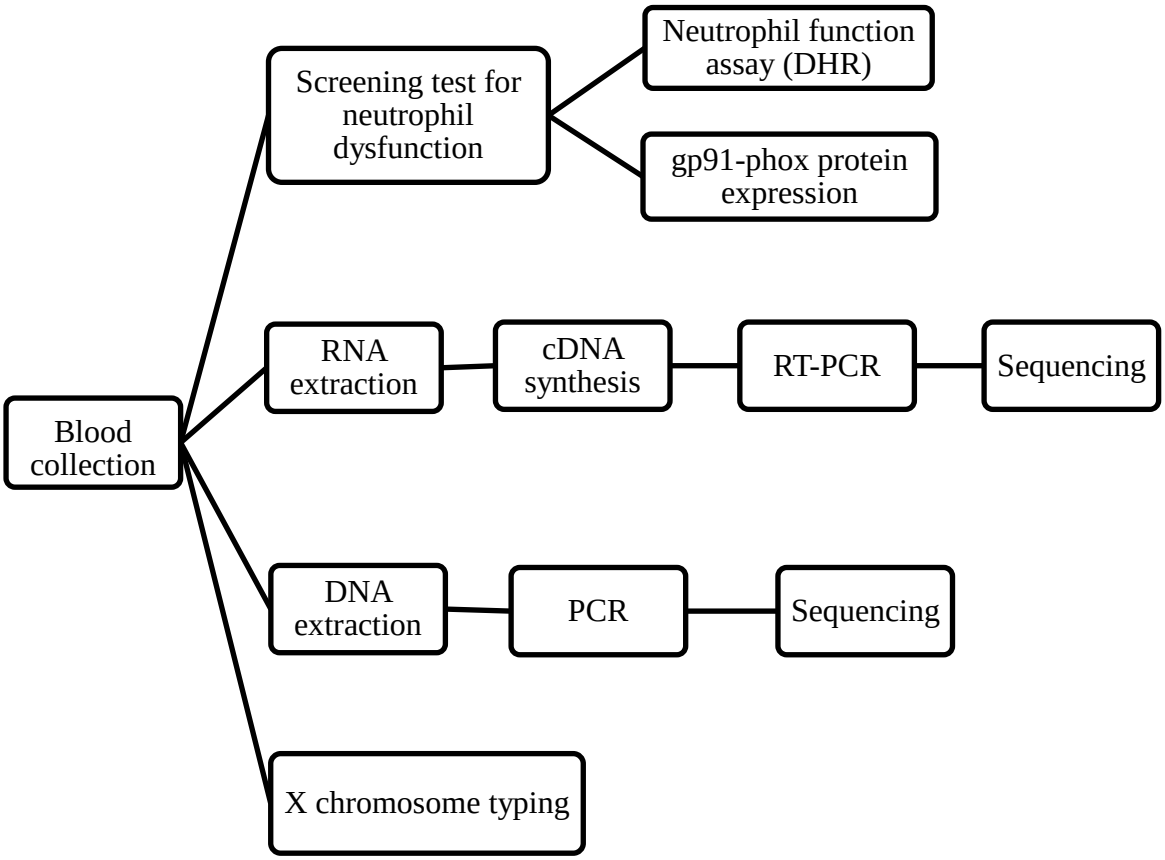

Figure 3.1: Experimental work flow in screening and confirmation of neutrophil dysfunction in patient.

3.3 Neutrophil Function Assay

The hydrogen peroxide productions by neutrophils were measured by the Dihydrorhodamine (DHR) assay using the BURSTTEST kit (Orpegen Pharma, Germany). Samples were processed according to the manufacturer's instructions. BURSTTEST allows the indirect measurement of the neutrophil oxidative burst and it is an effective screening test for diagnosing CGD.

Heparinized whole blood (100 µL) was dispensed at the bottom of Falcon tubes and incubated on ice bath for 10 minutes to cool down the cells. PMA solution was added to the final concentration of 8 µM, mixed well and incubated at 37.0°C for 10 minutes in a shaking water bath. After the incubation, the tubes were removed from the water bath and 20 µL substrate (DHR 1,2,3) was added. The tubes were then vortexed and incubated again in the shaking water bath with similar condition. After 10 minutes, the tubes were removed simultaneously from the water bath. The red blood cells were lysed by the addition of 2 mL lysing solution. The tubes were vortexed thoroughly and incubated for 20 minutes at room temperature. The tubes were centrifuged at 250 x g for 5 minutes at 4°C and the supernatant were discarded. The samples were then washed with 3 mL washing solution under same condition. Finally, 200 µL DNA staining solution was added to each tube and incubated in the dark for 10 minutes on ice. The cell suspension was analyzed with FACSCalibur flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA). Neutrophils were gated and evaluated for their respiratory burst activity.

3.4 gp91-phox Protein Expression

The expression of gp91-phox protein was determined with a modified two tube flow cytometry protocol recommended by the manufacturer using PE labeled anti-flavocytochrome monoclonal antibody clone 7D5 (MBL, Nagoya, Japan). The first tube held 20 µL of CD45 PerCP, meanwhile the second tube held 20 µL CD19 FITC / 7D5 PE / CD45 PerCP. CD45 is a cell surface marker for leukocytes (white blood cells) and CD19 is a cell surface marker for B lymphocytes and they are also known to express the gp91-phox protein. Whole blood sample (50 µL) were added to each tube and incubated for 30 minutes in the dark at room temperature. After incubation, samples were washed with 1 mL of stain buffer (BD Biosciences, San Jose, CA). The tubes were centrifuged at 500 x g for 3 minutes. FACS lysing solution of 1 mL (BD Biosciences, San Jose, CA) was added to each tube and incubated for 10 minutes in the dark at room temperature to lyse the erythrocytes, followed by centrifugation at 500 x g for 3 minutes. The samples were then washed with stain buffer. Finally 500µL stain buffer was added to each tube to resuspend the cells for further analysis by FACSCalibur flow cytometer. Samples were acquired and analyzed using CellQuest software. Neutrophils were gated and the expression of gp91-phox protein was reported as percentage.

3.5 RNA Extraction

Total RNA was extracted from whole blood using the RNeasy Mini Blood Kit (Qiagen, Germany) as recommended by the manufacturer. Blood samples were kept on ice bath immediately after collection as RNA molecules are labile and extremely susceptible to degradation.

One volume (1 mL) of whole blood was mixed with five volumes (5 mL) of EL buffer (erythrocyte lysing buffer) and incubated on ice for 15 minutes. The tubes were mixed twice during incubation at 5 minutes interval. Tubes were centrifuged at 400 x g for 10 minutes at 4°C. The supernatant was removed completely and 2 mL EL buffer was added and the cells were resuspended by brief vortexing. Tubes were then centrifuged again at 450 x g for 10 minutes at 4°C and the supernatant was removed completely without leaving any red tint on the pellet. RLT- β-ME buffer was added to the pelleted leukocytes and mixed by pipetting. The lysate was pipetted directly into QIAshredder spin column with collection tube and centrifuged for 2 minutes at maximum speed. One volume (700 µL) of 70% ethanol was added to the lysate and mixed by pipetting. The sample were then transferred to QIAspin column and spun at 8,000 x g for 15 seconds and the flow through were discarded. Cells were washed once with RW1 buffer and twice with RPE buffer. The QIAamp spin column was transferred to a clean microcentrifuge tube and 35 µL RNase-free water was pipetted directly onto the QIAamp membrane and centrifuged at 8,000 x g for 1 minute to elute the RNA. The concentration and purity of RNA was determined by using the NanoDrop 1000 instrument (Thermo Scientific, USA). Nucleic acids including RNA and DNA have maximum absorbance at 260 nm and 280 nm respectively. Hence the ratio of absorbance at 260 nm and 280 nm is used as a measure of purity in both nucleic acids. A ratio of ~ 2.0 is generally considered pure for RNA sample. A lower ratio may indicate the presence of protein, phenol or other contaminants that absorb at the 280 nm wavelength.

3.6 Reverse Transcription Polymerase Chain Reaction

First strand complementary DNA (cDNA) was synthesized from 1 µg RNA in a 15 µL total reaction mix containing 1 µL random primers, 500 µg/mL (Promega, Madison, USA) and volume of nuclease free water adjusted accordingly. The mixture was heated at 70°C for 5 minutes and chilled on ice. First strand buffer of the volume 8 µL, 4 µL 0.1 M DTT, 4 µL 10 mM dNTP, 1 µL recombinant RNasin® Ribonuclease inhibitor, 40 u/µL (Promega, Madison, USA), 7 µL nuclease free water and finally 1 µL SuperScript™ II Reverse Transcriptase (Invitrogen, USA) was added to the mixture and incubated at 42°C for 1 hour in a thermocycler. The reaction was inactivated by heating at 90°C for 5 minutes. The cDNA was used as for template Polymerase Chain Reaction (PCR).

PCR was carried out with the converted cDNA to amplify the NADPH-oxidase components i.e. p22-phox, p47-phox, p67-phox and gp91-phox to identify which of the protein component is defective in function. A housekeeping gene, actin was run alongside as a positive control. The primers used for the PCR reactions of p22-phox, p47-phox and p67-phox are shown in Table 3.1. cDNA was amplified in a 50 µL PCR mix of 800 µM/L dNTPs, 250 nM/L forward and reverse primers, 4.5 mM/L $MgCl_2$ and 1.25 U Taq Polymerase (Promega, Madison, USA). The cycling profile for the PCR reaction was initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 55 seconds, annealing at 60°C for 55 seconds and extension at 72°C for 80 seconds, and a final extension at 72°C for 10 minutes.

The *CYBB* gene of gp91-phox protein was amplified with three pairs of primers (Hui et al., 1996) that generated three overlapping PCR fragments. The primers are listed in Table 3.2. The location of the primers in the *CYBB* gene is shown in Figure 3.2. The first set of primer amplifies region upstream of exon 1 till a portion of exon 6. The

Table 3.1: Primer sequence for the amplification of p22-phox, p47-phox, p67-phox and β-actin mRNA by RT-PCR.

NADPH-oxidase component	Primers	Fragment length
p22-phox	Forward primer: 5' GTTTGTTTTGTGCCTGCTGGAGT 3' Reverse primer: 5' TGGGCGGCTGCTTGATGGT 3'	325bp
p47-phox	Forward primer: 5' ACCCAGCCAGCACTATGTGT 3' Reverse primer: 5' AGTAGCCTGTGACGTCGTCT 3'	767bp
p67-phox	Forward primer: 5' CGAGGGAACCAGCTGATAGA 3' Reverse primer: 5' CATGGGAACACTGAGCTTCA 3'	726bp

Housekeeping protein	Primers	Fragment length
β-actin	Forward primer: 5' AGCGGGAAATCGTGCGTG 3' Reverse primer: 5' CAGGGTACATGGTGGTGCC 3'	300bp

(Source: Banerjee et al., 2000)

Table 3.2: Primer sequence for the amplification of gp91-phox mRNA by RT-PCR.

Primers	Fragment length
Forward primer (**1L):** 5' CCCAGGGCTGCTGTTTTCAT 3' Reverse primer (**1R)**: 5' CAAAGTAAGACCTCCGGATG 3'	694bp
Forward primer (**2L)**: 5' TGTTGGCAGGCATCACTGGAG 3' Reverse primer (**2R)**: 5' TCTTCACTGGCAGTGCCAAAG 3'	673bp
Forward primer (**3L)**: 5' GCTTGTGGCTGTGATAAGCAG 3' Reverse primer **(3R)**: 5' CATTTGGCAGCACAACCCACA 3'	658bp

(Source: Hui, et al., 1996)

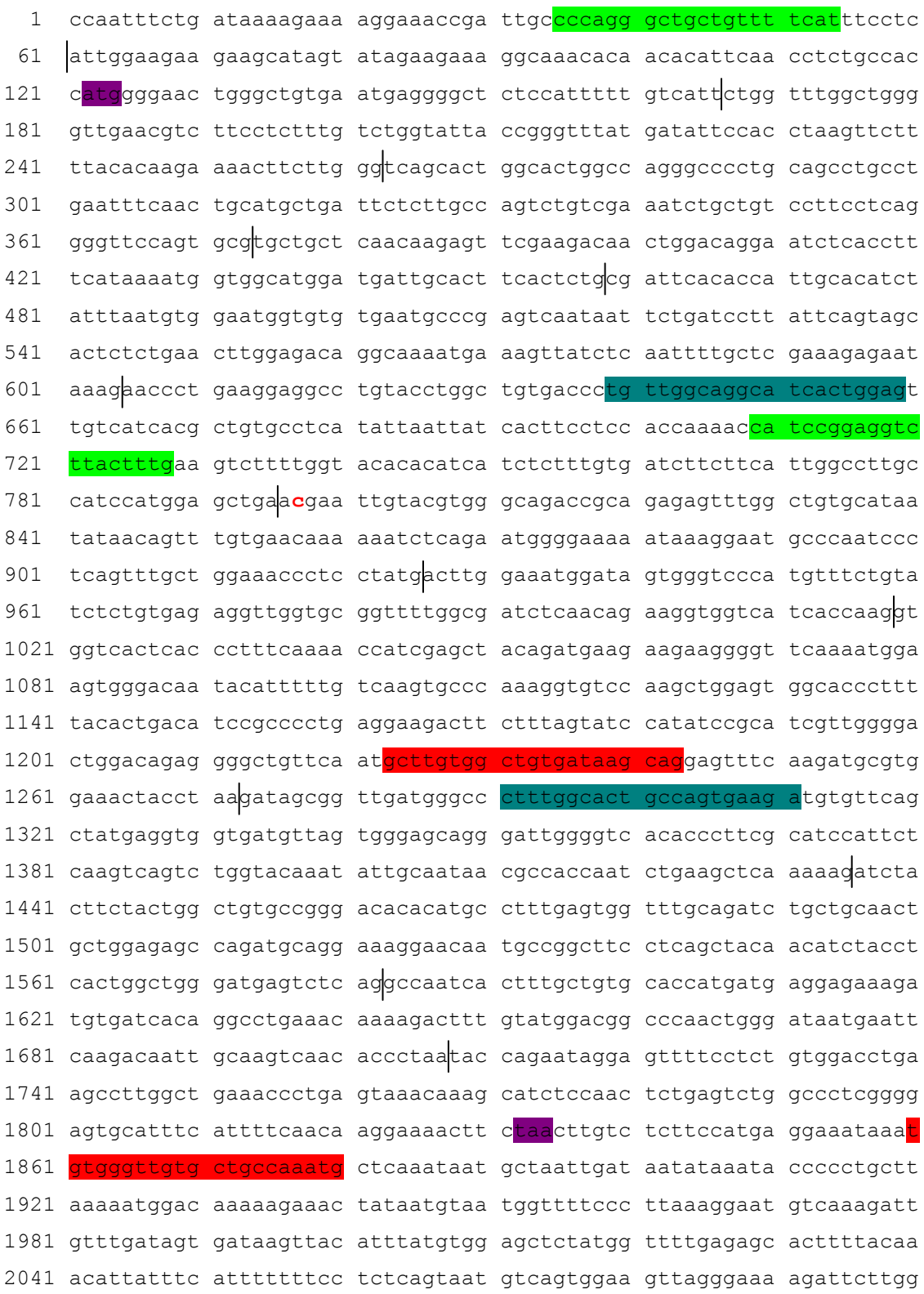

Figure 3.2: Primer sets used for the amplification of *CYBB* mRNA. The straight lines denotes exonic boundary. The nucleotide triplets (codon) highlighted in purple shade represents the start codon and termination codon respectively of the gp91-phox protein.

second fragment comprises exon 6 to exon 10. On the other hand, the third fragment includes a section of exon 9 up to exon 13. cDNA was amplified in a 50 µL PCR mix consisting 400 µM/L dNTPs, 300 nM/L forward and reverse primers respectively, 2.5 mM/L $MgCl_2$ and 1.25 U Taq Polymerase (Applied Biosystems, USA). The cycling condition applied was initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 35 seconds, and a final extension at 72°C for 7 minutes.

The amplified fragments were electrophoresized on 2% agarose gel by running at 100 V for 40 minutes. After completion of electrophoresis, the gel was stained with ethidium bromide and visualized under UV light using the AlphaImager HP Gel Documentation System, USA.

3.7 DNA Extraction

The QIAamp DNA Blood Mini Kit was used for DNA extraction, as recommended by the manufacturer. Chilled lymphoprep (3.5 mL) was added to a plain tube. Blood samples were diluted with equal volume of Phosphate Buffer Saline (PBS), layered onto the lymphoprep and spun at 3,000 rpm for 15 minutes. After centrifugation, the erythrocytes will sediment at the bottom of the tube meanwhile the mononuclear cells (such as lymphocytes and monocytes), also called buffy coat, form a layer in between the lymphoprep and plasma. The buffy coat is harvested using Pasteur pipette and transferred to a clean tube. The cells were washed twice with PBS by centrifugation at 2,500 rpm for 5 minutes. The supernatant was discarded and 200 µL of the pelleted mononuclear cells were transferred to a clean microcentrifuge tube.

QIAGEN Protease (20 µL) and 200 µL AL buffer was then added and vortexed briefly before incubation at 56°C for 10 minutes. After incubation, 200 µL absolute

ethanol was added and mixed by pulse-vortexing. The sample was then transferred to mini spin column with collection tube and spun at 6,000 x g for 1 minute. The flow through was discarded and 500 µL AW1 buffer was added to the spin column and centrifuged at 6,000 x g for 1 minute. The filtrate was removed and washed with 500 µL placed in another AW2 buffer at 20,000 x g for 3 minutes. After centrifugation, the spin column was transferred to a clean microcentrifuge and 200 µL AE buffer was added. The tube was incubated at room temperature for 5 minutes and then centrifuged at 6,000 x g for 1 minute. The spin column was removed and the flow through containing DNA was then subjected to denaturation by heating at 95°C for 5 minutes, and another 5 minutes on ice to keep the single stranded DNA from ligating. The concentration and purity of extracted DNA was determined by using the NanoDrop 1000 instrument (Thermo Scientific, USA). Generally, a ratio of ~ 1.8 is considered pure for DNA sample. A lower ratio may be the result of a contaminant absorbing at 280 nm.

3.8 Genomic DNA Polymerase Chain Reaction

PCR of genomic DNA (gDNA) was done to study exon 7 of the *CYBB* gene using primer set 7L/7R as described in Hui et al. (1996). The primer sequences are listed in Table 3.3 and the location of primers are shown in Figure 3.3.

The starting material of about 250 ng DNA was amplified in a 50 µL PCR reaction with 200 µM/L dNTPs, 250 nM/L forward and reverse primers respectively, 2.0 mM/L $MgCl_2$ and 1.25 U Taq Polymerase (Applied Biosystems, USA). The PCR cycling profile employed was initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 45 second, and a final extension at 72°C for 7 minutes. The amplified

Table 3.3: Primer sequence for the gp91-phox gDNA amplification of exon 7 by PCR.

Primers	Fragment length
Forward primer (**7L):** 5' TGATCTGGACTTACATTTTTCA 3' Reverse primer (**7R):** 5' AGTAATGAAACTGTAATAACAACA 3'	196bp

(Source: Hui, et al., 1996)

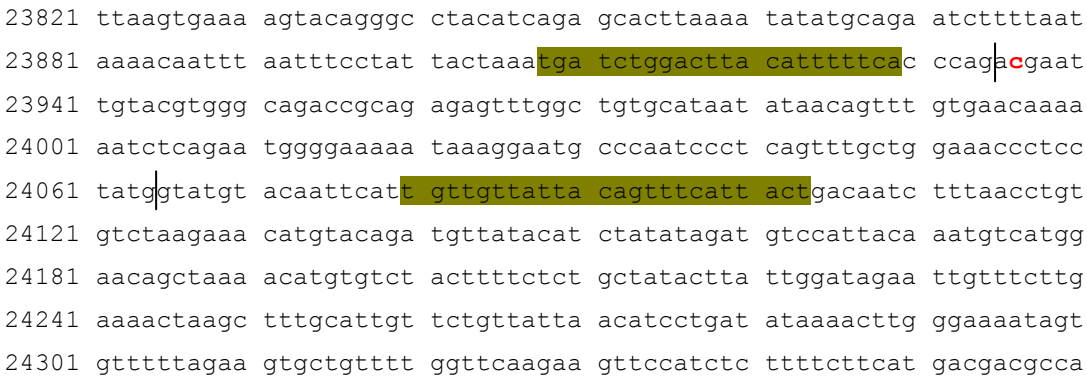

Figure 3.3: Primer sequence used for the genomic DNA amplification of exon 7 of *CYBB* gene. The straight lines represent the beginning and the last nucleotide of exon 7 respectively.

fragments were electrophoresized on 2% agarose gel, stained with ethidium bromide and visualized under UV light to check for the presence of the PCR products.

3.9 Purification of RT-PCR and PCR Product

The RT-PCR and PCR products were purified by the spin column method with Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) according to manufacturer's instructions.

Equal volume of PCR product (i.e. 40 µL) was mixed with the membrane binding solution in a microcentrifuge, transferred to an assembled spin column and incubated at room temperature for 1 minute. The spin column was then centrifuged at 16,000 x g for 1 minute and the flow through was discarded. The column was washed with 700 µL membrane wash solution by centrifuging at 16,000 x g for 1 minute. The flow through was removed and washed again with 500 µL wash solution for 5 minutes. As a final step to elute the DNA, the spin column was transferred to a clean microcentrifuge. Nuclease free water was added without touching the membrane with the pipette tip and incubated at room temperature for 5 minutes. After incubation, the tube was centrifuged at 16,000 x g for 1 minute. The flow through containing eluted DNA was stored and the concentration was determined with NanoDrop 1000 (Thermo Scientific, USA).

3.10 Sequencing

Amplified fragments that had been purified were sent to First Base Laboratories (M) Pte Ltd and sequenced on an automated fluorescent sequencer (ABI 3730XL, Applied Biosystems) using Big Dye Teminator (V3.1) chemistry. The RT-PCR

products were sequenced using forward primers of each reaction set. The PCR products were sequenced using the reverse primer. Sequencing traces were analyzed using Sequence Scanner v1.0 software. GenBank reference sequences NG_009065.1 (DNA) and NM_000397.3 (mRNA) were used for sequence comparison. In the numbering of cDNA, the A of the ATG translation initiation codon was taken as +1, and thus this codon was taken as codon 1.

3.11 X Chromosome Typing

The origin of the X chromosome in the male sibling and patient were determined by X chromosome Short Tandem Repeat (X-STR) analysis using the Investigator Argus X-12 Kit (Qiagen GmbH, D-40724, Hilden). The DNA samples were sent to the Forensic Division, Chemistry Department Malaysia for the X chromosome typing. The results were analyzed using GeneMapper (v3.2.1) software.

4.0 RESULTS

The index patient, EKL first came to our attention of having prolonged fever at 6 months of age. He was then tested for phagocytic function activity by chemiluminescence assay. It was revealed that his phagocytic function was low and indicated impairment in his neutrophil function. This prompted that this patient might be suffering from Chronic Granulomatous Disease, a disease of neutrophil dysfunction. A more specific and robust testing by flow cytometry and also the genetic studies were performed to establish the disease status in this patient.

4.1 Neutrophil Function Analysis

The neutrophil respiratory burst was measured indirectly by the conversion of non-fluorescent dihydrorhodamine 1,2,3 (DHR 1,2,3) to rhodamine 1,2,3 (R 1,2,3) that fluoresces. When the cells were treated with PMA, great amount of superoxides were produced by the enzyme NADPH-oxidase. More potent reactive oxygen intermediates (ROIs) such as hydrogen peroxide (H_2O_2) were derived from superoxide molecules to kill or evade the foreign particles in the cells. The hydrogen peroxide will then oxidize the DHR 1,2,3 to a fluorescent R 1,2,3 that is detectable by flow cytometry. Side scatter versus forward scatter was plotted to characterize the leukocytes based on their granularity and cell volume respectively. The respiratory burst was determined by gating 5000 events of neutrophils. Results were expressed as percentage of neutrophils producing a respiratory burst and in terms of the Stimulation Index (SI) = Geometric Mean Channel Fluorescence Intensity of PMA stimulated neutrophils / Geometric Mean Channel Fluorescence Intensity of unstimulated neutrophils.

Figure 4.1 shows the DHR results of a normal control, sister, mother, brother and the patient. The shaded peaks represent PMA stimulated neutrophils, where else the non-shaded peaks denote the non-stimulated neutrophils. The peak shift to the right indicates production of respiratory burst in the neutrophils. The histogram plot of the normal control and patient's sister show 99.8% and 95.8% of their neutrophils generating a respiratory burst. The mother's histogram shows a bimodal response pattern to PMA stimulation with 51.3% of neutrophils unable to produce a respiratory burst, and the other 45.3% producing a respiratory burst. Interestingly, the brother's histogram also showed a bimodal response pattern with 58.4% of the neutrophils producing respiratory burst and 39.5% of neutrophils fail to generate a respiratory burst. However, the patient's neutrophils were unable to produce a respiratory burst upon PMA stimulation with 97% of his cell population remains on the left side of the histogram. The geometric mean channel fluorescence intensity of stimulated and non-stimulated neutrophils as well as the SI values is shown in Table 4.1. The patient displays a very low SI value in contrast to the normal control and sister. The dysfunctional neutrophil population in the mother and brother exhibit a low SI value, while the functional neutrophil population shows comparable SI value to the normal control. This finding indicates that the patient has a very low respiratory burst and he is suffering from neutrophil dysfunction.

The gp91-phox, a protein of the NADPH-oxidase complex is an important component for the oxidative respiratory burst system to eliminate pathogen. It is a transmembrane protein expressed mainly on neutrophils and other cells such as B lymphocytes and macrophages. Side scatter versus CD45 PerCP was plotted to determine the cell population distribution. The results are represented by histogram plot showing the expression of gp91-phox protein in 5000 events of neutrophils.

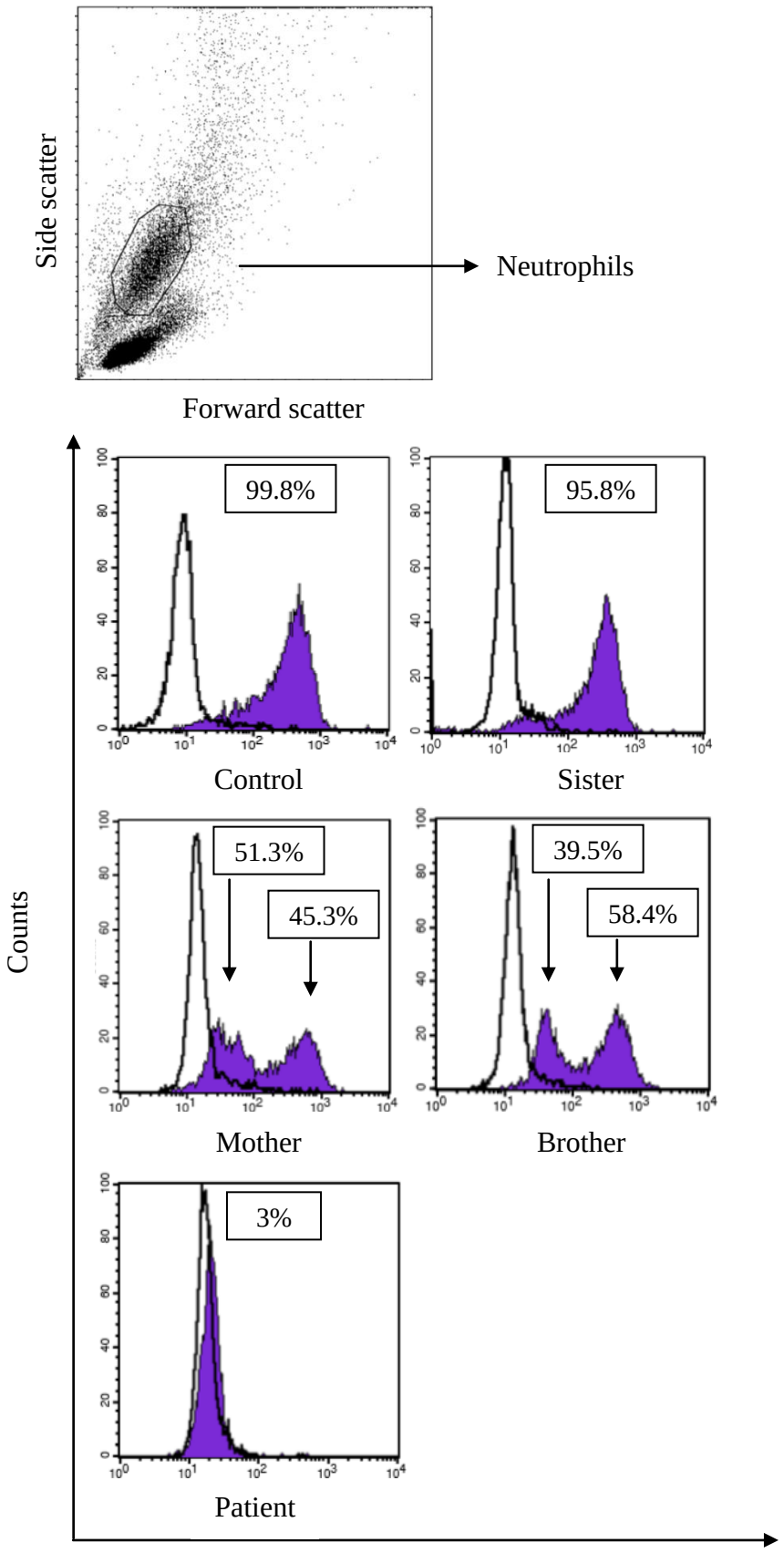

Figure 4.1: Respiratory burst profile of patient EKL and his family members.

Table 4.1: Statistical analysis of the DHR assay. (* Two populations of neutrophil with difference fluorescence intensity were observed in patient's mother and brother)

Subject	**Geometric Mean Channel Fluorescence Intensity of PMA stimulated neutrophils**		**Geometric Mean Channel Fluorescence Intensity of non-stimulated neutrophils**	**Stimulation Index (SI)**	
Control	495.75		10.18	48.7	
Sister	252.29		11.21	22.5	
Mother	45.4	465.52	13.14	3.5	35.4
Brother	41.4	444.89	8.31	5.0	53.5
Patient	37.67		16.05	2.3	

Figure 4.2 shows the neutrophils gp91-phox expression in study subjects. The shaded peaks depict the gp91-phox stained neutrophils and the non-shaded peaks depict the unstained neutrophils. Flow cytometric assessment of gp91-phox protein expression in neutrophils as shown in Figure 4.2 closely resembles the fluorescence pattern seen in the neutrophil function DHR 1,2,3 assay. The histogram of the control and the sister depicts that 98.5% and 99.8% of their neutrophils expresses gp91-phox respectively.

The mother's histogram showed two populations of neutrophils with 43.7% expressing gp91-phox, while 56.3% were negative. Supporting the findings in the DHR 1,2,3 assay, the brother's histogram also displayed two neutrophil populations with 69.6% positive for gp91-phox and 30.4% negative for gp91-phox. The patient's histogram on the other hand showed that 99.8% of his neutrophils were negative for gp91-phox.

The patient's mother was shown to have a bimodal response in her neutrophil activity against PMA and in the gp91-phox expression. Autosomal CGD are caused by recessive alleles. Hence, these observations concluded that the inheritance of neutrophil dysfunction is X-linked and the mother is a carrier of the disease. The sister however turned out not to have inherited the disease. The patient's brother also showed the same neutrophil activity against PMA and the gp91-phox expression as the mother. Since it is atypical for a male to be a carrier of X-linked disease, further investigations was carried out to establish the brother's status. The inability of the patient's neutrophils to mount an immune response upon stimulation with foreign particle (i.e. PMA) shows that his neutrophils are functionally defective and the gp91-phox expression analysis indicates that he suffers from X-linked CGD.

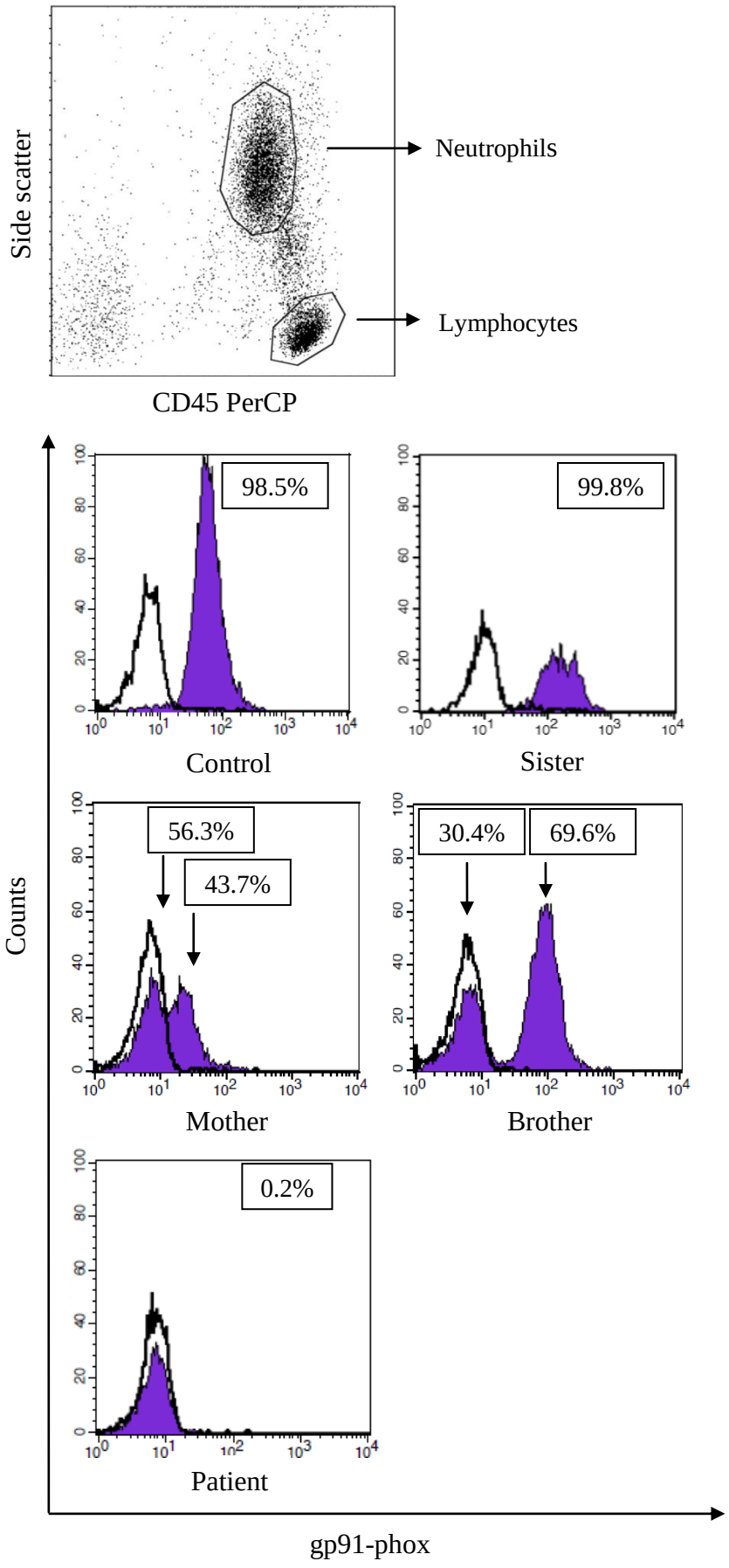

Figure 4.2: gp91-phox protein expression in patient EKL and his family members.

4.2 RT- PCR and Sequencing of the RT-PCR Product

The mRNA of NADPH-oxidase components namely p22-phox, p47-phox, p67-phox and gp91-phox were amplified to study the mutation involved. The autosomal component genes were also analyzed to confirm whether the brother was affected by autosomal or X-linked CGD.

The agarose gel photographs of the amplified autosomal fragments are depicted in Figure 4.3. The autosomal component proteins i.e. p22-phox, p47-phox and p67-phox did not exhibit any changes in their respective genes upon sequencing. The RT-PCR for *CYBB* gene of gp91-phox protein was performed using three sets of primers to yield three overlapping fragments that spanned the entire coding region of the gene. The amplified fragments are of size 694bp, 673bp and 658bp. Figure 4.4 illustrates the agarose gel photograph of the amplified fragments.

The amplified fragments were sequenced using the forward primer (Figure 3.2) of the respective primer sets to detect for abnormality or mutation in the *CYBB* gene. The first fragment and the third fragment of sizes 694bp and 658bp respectively yielded normal length nucleotides for all individuals. The cDNA sequencing results showed that the nucleotide sequences are comparable to the reference sequence. In the case of the second fragment, although the RT-PCR product size was same for all individuals, the result of cDNA sequencing of the patient revealed a point mutation. A single nucleotide base change from C to T was identified at position 676 in the patient (c.676C>T). A part of the second fragment is shown in Figure 4.5. The position of the mutation can be referred in Figure 3.2 whereby the substituted nucleotide position is highlighted in red. In the cDNA of the control, sister, mother and the brother, the position 676 is occupied by a C. As shown in Figure 4.5, the mutated mRNA is not expressed in the carriers.

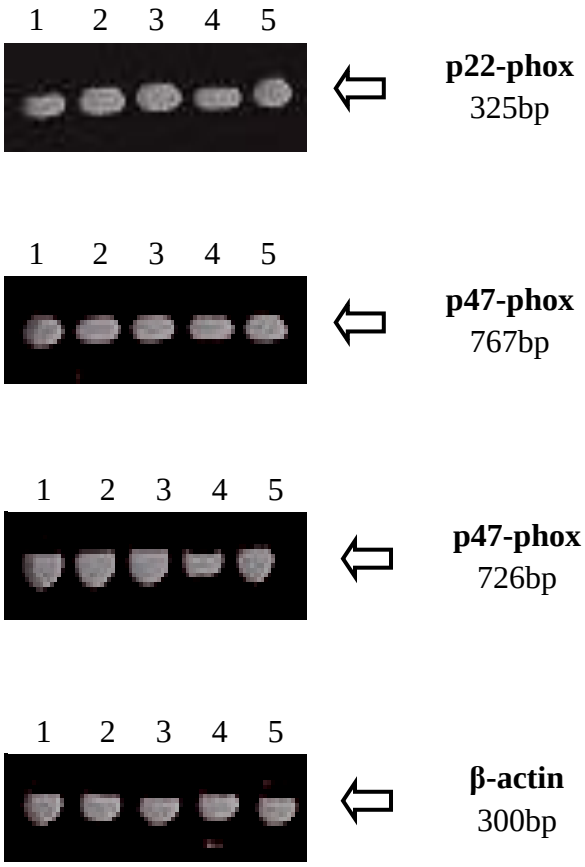

Figure 4.3: Agarose gel picture of amplified fragments of p22-phox, p47-phox, p67-phox and actin mRNA by RT-PCR.

Lane 1: Control

Lane 2: Sister

Lane 3: Mother

Lane 4: Brother

Lane 5: Patient

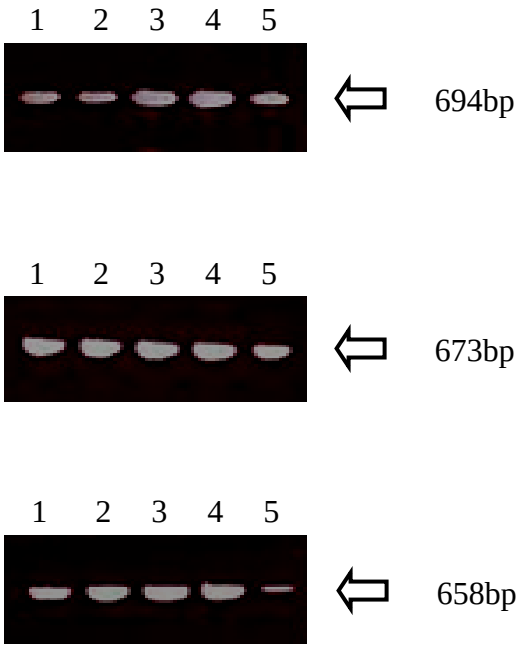

Figure 4.4: Agarose gel picture of the three amplified fragments of *CYBB* gene by RT-PCR.

Lane 1: Control

Lane 2: Sister

Lane 3: Mother

Lane 4: Brother

Lane 5: Patient

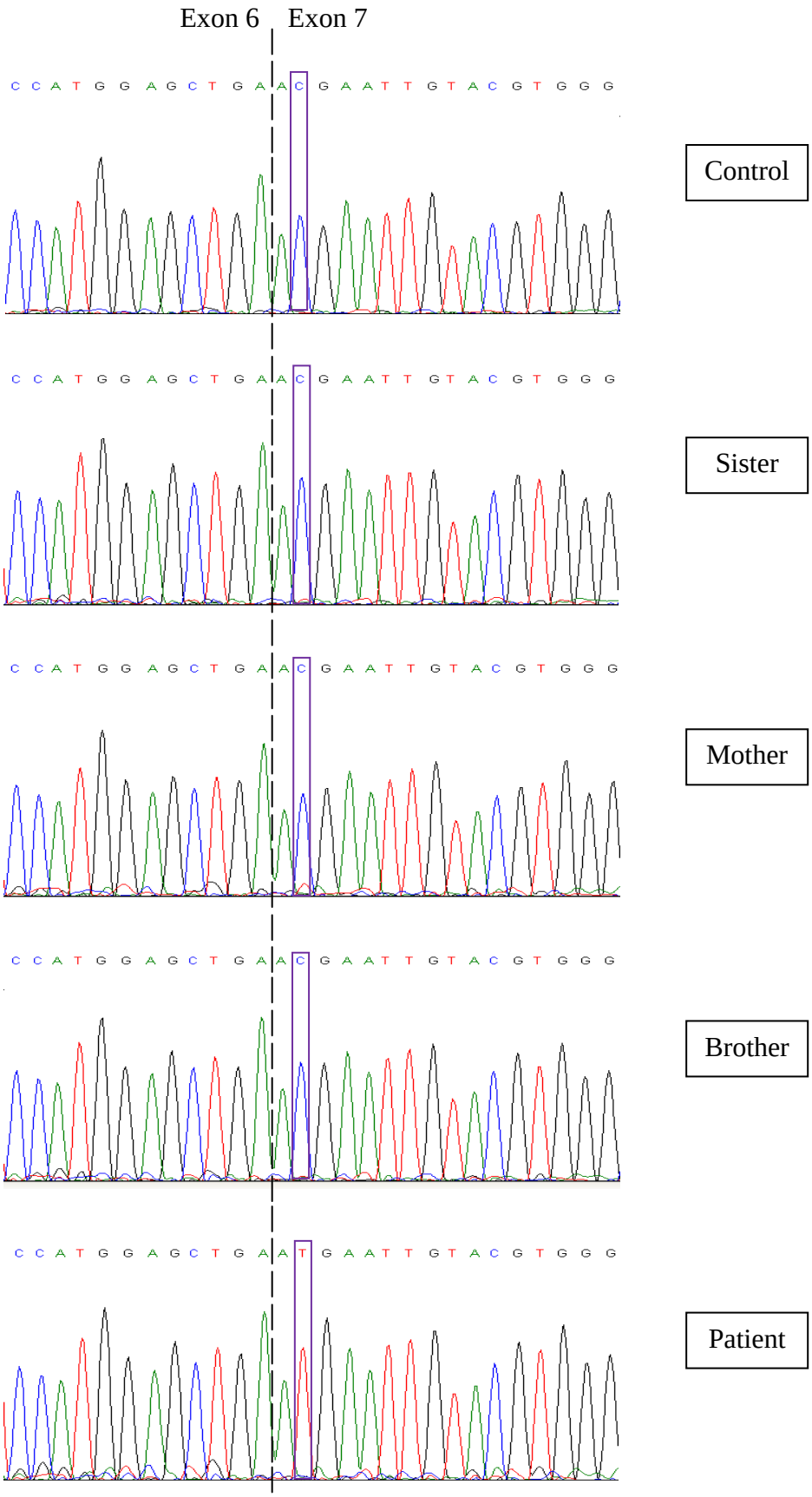

Figure 4.5: Sequencing traces of second fragment of the *CYBB* mRNA. The broken line denotes the exonic boundary.

4.3 PCR and Sequencing of PCR Product

Genomic DNA was amplified to confirm the findings seen in the cDNA of the study subjects in the *CYBB* gene. Exon 7 was amplified as the nucleotide change was observed in that particular exon at mRNA level. The agarose gel picture in Figure 4.6 shows that the control, sister, mother, brother and the patient display band of the same size which is 196bp.

The amplified fragment was sequenced with the reverse primer used (Figure 3.3) in PCR. The reverse primer was used as the sequencing primer because the mutation point was adjacent to the forward primer. A segment of the sequencing results shown in Figure 4.7 illustrates that the patient had G that corresponds to C (Figure 3.3, the changed nucleotide is highlighted in red) in position 23936 of genomic DNA, while the control and sister had A that corresponds to T. The carrier mother and brother displayed two peaks at this position, having G and A. Both of them are heterozygous at this locus of the gene and hence are carriers for the mutation. The findings in the genomic DNA confirms the mutation seen in the cDNA of the patient which is c.676C>T.

4.4 X Chromosome Typing

X chromosome typing was carried out as the brother was suspected of having Klinefelter syndrome, indicated by the presence of two neutrophil populations in the DHR assay and gp91-phox protein expression assay. Hence, the presence of the extra X chromosome and its origin had to be determined. The typing of X chromosome is done by studying the short tandem repeats (STRs) located on the X chromosome. STRs are unique to each individual and are inheritable. There are 30 forensic STR markers at the

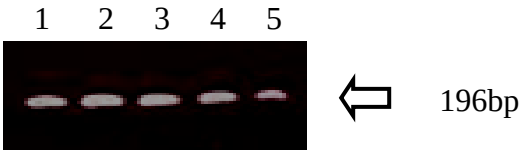

Figure 4.6: Agarose gel picture of the PCR amplified exon 7 of *CYBB* gDNA.

Lane 1: Control

Lane 2: Sister

Lane 3: Mother

Lane 4: Brother

Lane 5: Patient

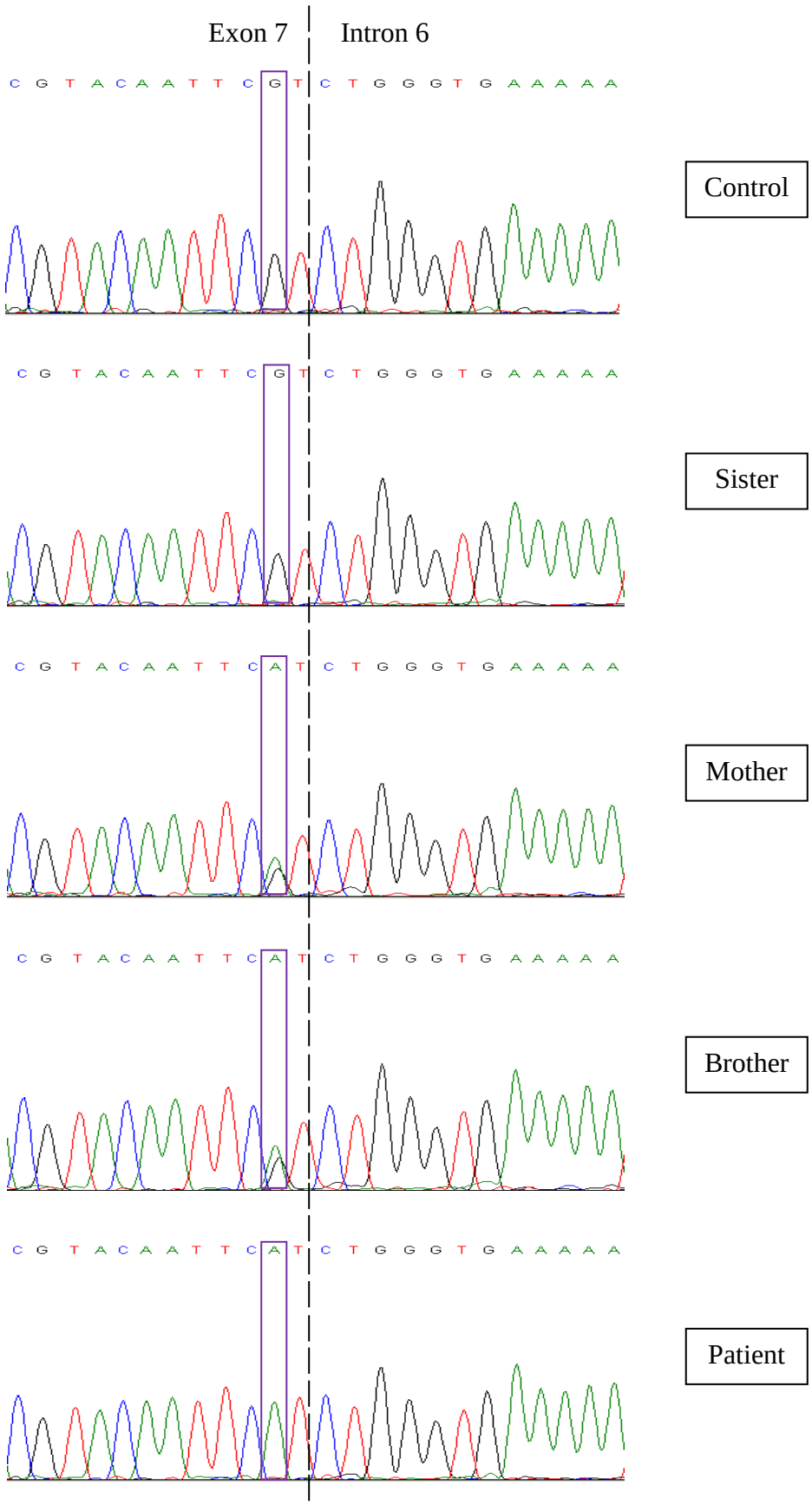

Figure 4.7: Sequencing traces of exon 7 of *CYBB* gene (genomic DNA).The broken line denotes the intronic-exonic boundary.

X chromosome and of that, 12 loci were selected to characterize the repeats. Figure 4.8 depicts the location of the 12 STRs on the X chromosome.

Figure 4.9 illustrates the X-STRs analysis performed on the father, mother, brother and the patient. The numbers shown for each locus represents the number of repeats. The sister's X-STRs were predicted based on the allelic contribution in the brother and patient. The X-STR data corroborated the findings in flow cytometric assay and revealed that the carrier brother had two X chromosomes and inherited both from his mother. Furthermore, one of his maternal X chromosomes that is normal appeared to have undergone recombination in the Linkage II group X-STRs i.e. DXS7132, DXS10079 and DXS10074, so that both X chromosomes were homozygous at these loci. The homozygous loci are underlined in Figure 4.9. The patient had inherited the defective X chromosome from the mother. Meanwhile, the sister had inherited the only X chromosome from her father and a normal X chromosome from her mother. Hence, the sister is not a carrier of the disease, but rather a normal individual.

Figure 4.10 shows the complete family pedigree. It can be concluded that the index patient in this study has X-linked CGD. The mother and the brother are carriers of X-linked CGD. On the other hand, the father and the sister are normal individuals.

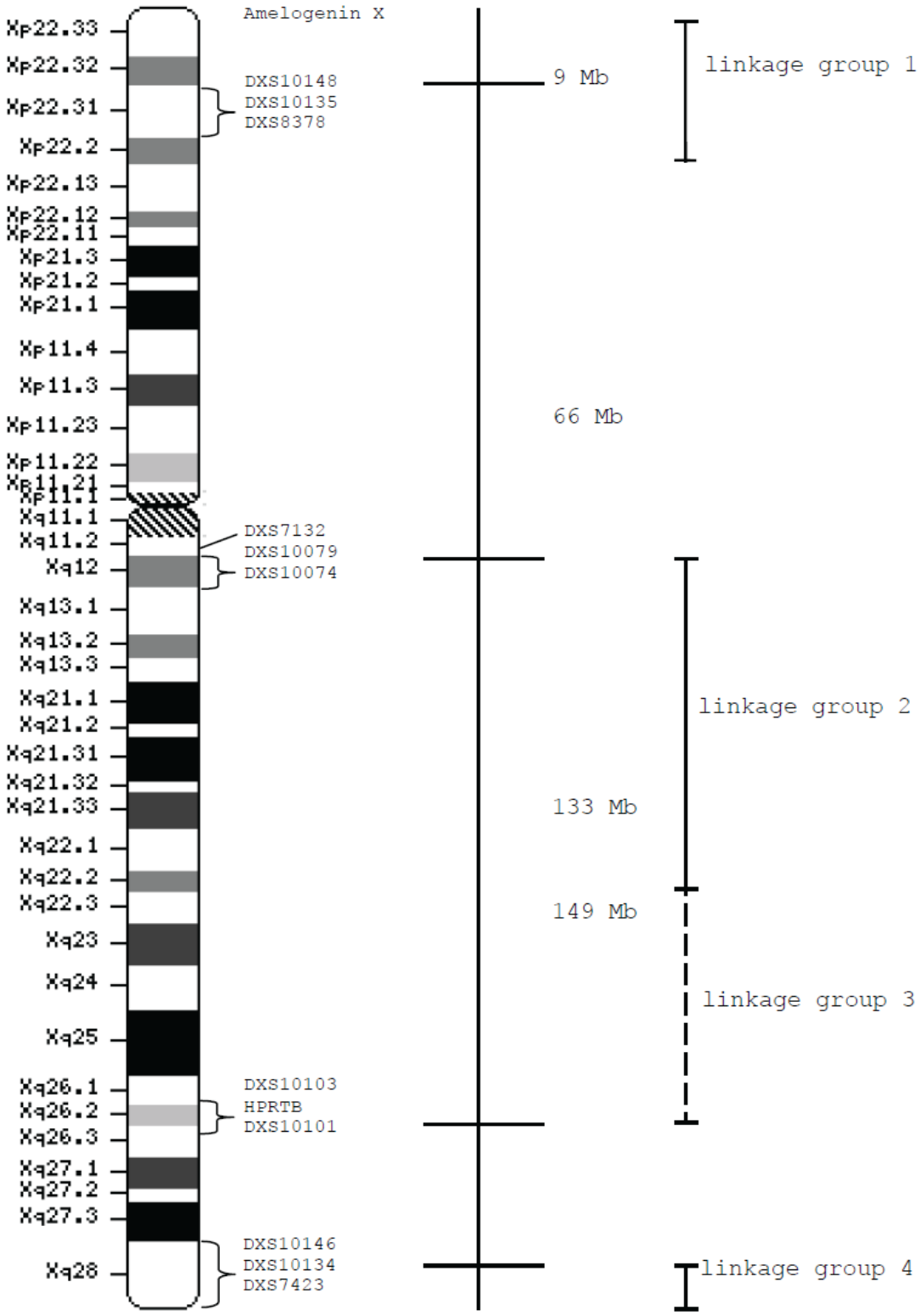

Figure 4.8: Position of the Short Tandem Repeats (STRs) in the X chromosome.

(Source: www.ncbi.nlm.nih.gov/genome/guide/human)

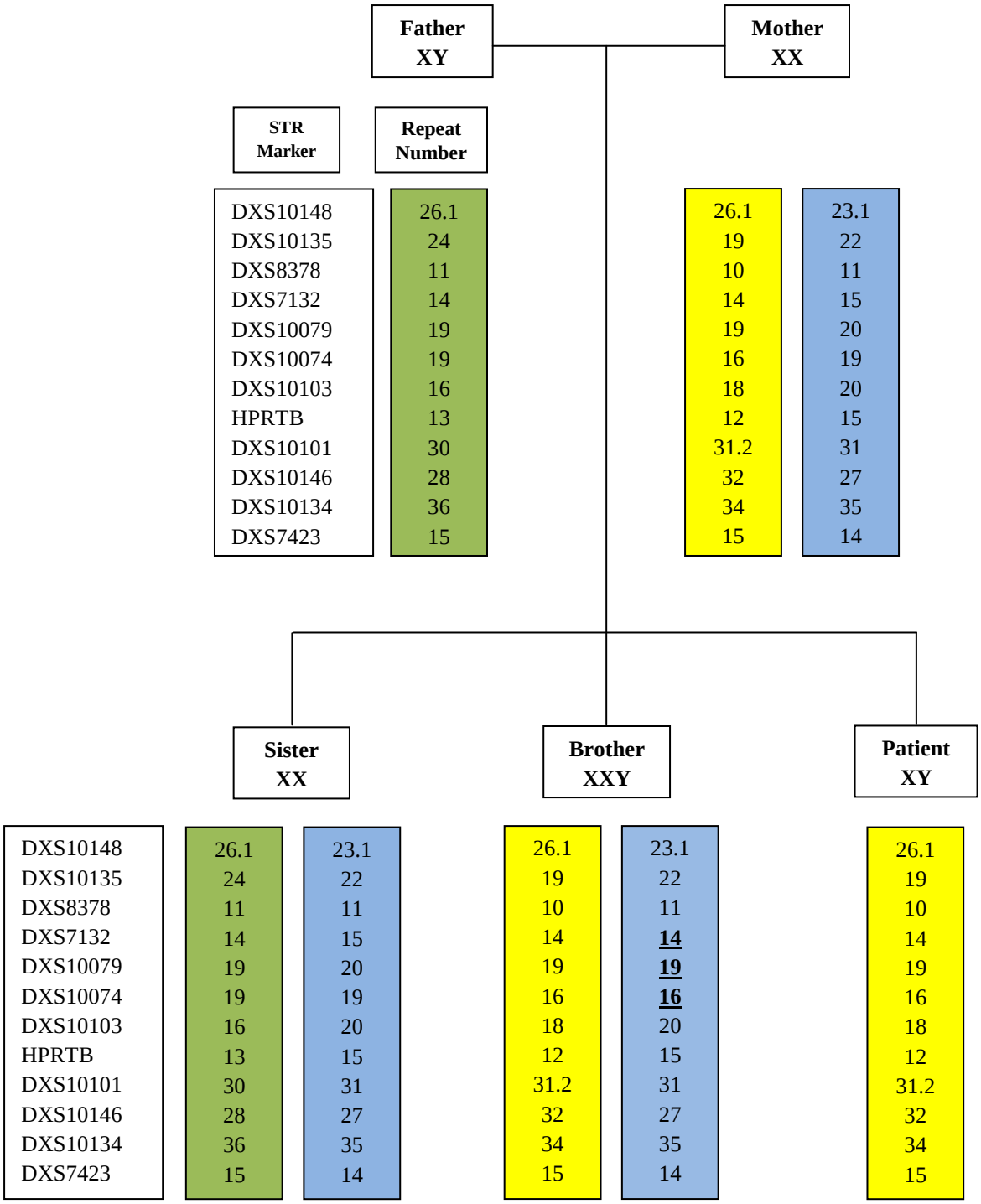

Figure 4.9: X Chromosome Short Tandem Repeats (X-STRs) analysis. The underlined numbers represent the loci that had undergone recombination. The numbers in colored box indicate the number of repeats for each STR markers. Green box is the X chromosome marker of the father, while the yellow and blue are of the mother. Both X chromosomes in the patient's brother are inherited from the mother with three loci identified to have undergone recombination (underlined).

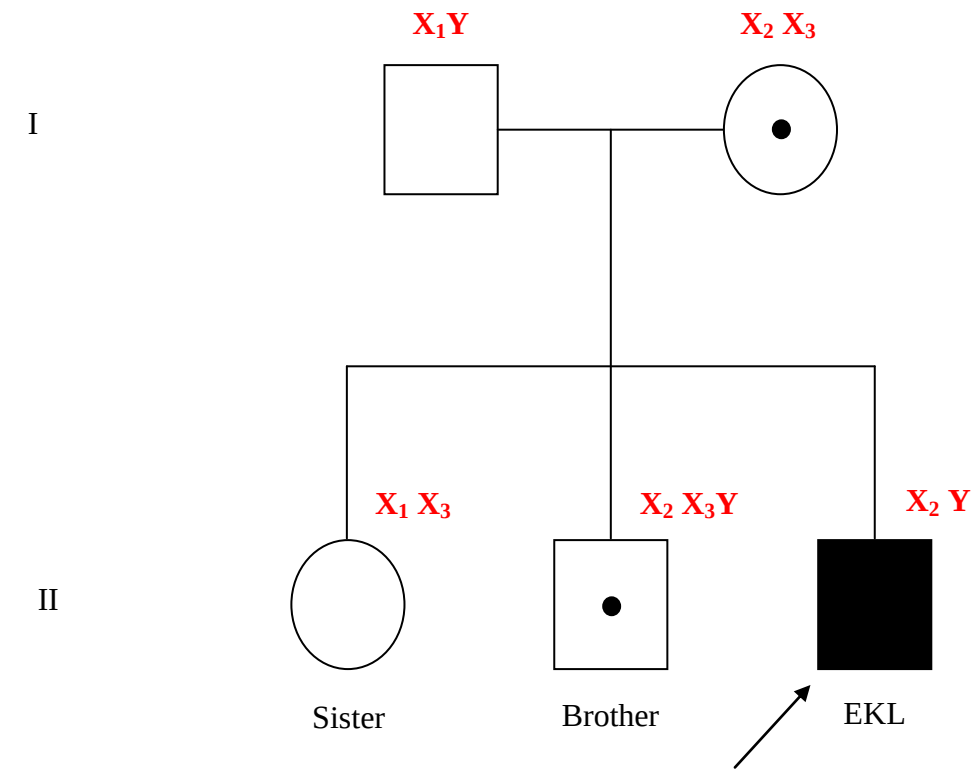

Figure 4.10: Family pedigree of patient EKL.

5.0 DISCUSSION

Chronic Granulomatous Disease is a heterogeneous disease that affects individuals without any ethnicity preference. To the best of our knowledge, there are fourteen known CGD cases that have affected all three major races in Malaysia. Of these cases, there are five patients who were diagnosed with X-linked CGD and nine patients who suffer from autosomal recessive p47-deficient CGD. Though X-linked CGD is the most prevalent type of CGD known to occur, there are more autosomal recessive cases particularly p47-defective CGD, observed in Malaysian population. The reason for these observations is still unclear. This report describes a comprehensive molecular and genetic study of a 2 years old Chinese ethnic Malaysian male suffering from X-linked CGD since he was diagnosed at 6 months old.

The protein that is affected by X-linked CGD is known as gp91-phox which is coded by the *CYBB* gene. Normal gp91-phox nucleotide sequence translates into a 570 amino acid chain. The genetic abnormality observed in the index case, c.676C>T is a nonsense mutation that results in the amino acid change p.Arg226X, the premature termination of the gp91-phox protein at amino acid 226. The translated nucleotide sequence is shown in Figure 5.1. The mutation had caused the gp91-phox protein to be truncated and hence not functional. Thus, it can be concluded that the truncated *CYBB* mRNA is expressed in patient's neutrophils (Figure 4.5) but lacks the gp91-phox protein expression as depicted in the flow cytometric assay (Figure 4.2). The c.676C>T mutation has been reported in 17 cases with X-linked CGD (Roos et al., 2010). All these cases do not express the gp91-phox protein, which generally indicates a poor prognosis (Kuhns et al., 2010). The absence of a functional component of NADPH-oxidase complex diminishes the oxygen reducing capacity of the enzyme. Hence, ROIs needed for the killing of microorganism are not produced. Therefore, patients with

Control

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  1 MGNWAVNEGL SIFVILVWLG LNVFLFVWYY RVYDIPPKFF YTRKLLGSAL ALARAPAACL
 61 NFNCMLILLP VCRNLLSFLR GSSACCSTRV RRQLDRNLTF HKMVAWMIAL HSAIHTIAHL
121 FNVEWCVNAR VNNSDPYSVA LSELGDRQNE SYLNFARKRI KNPEGGLYLA VTLLAGITGV
181 VITLCLILII TSSTKTIRRS YFEVFWYTHH LFVIFFIGLA IHGAERIVRG QTAESLAVHN
241 ITVCEQKISE WGKIKECPIP QFAGNPPMTW KWIVGPMFLY LCERLVRFWR SQQKVVITKV
301 VTHPFKTIEL QMKKKGFKME VGQYIFVKCP KVSKLEWHPF TLTSAPEEDF FSIHIRIVGD
361 WTEGLFNACG CDKQEFQDAW KLPKIAVDGP FGTASEDVFS YEVVMLVGAG IGVTPFASIL
421 KSVWYKYCNN ATNLKLKKIY FYWLCRDTHA FEWFADLLQL LESQMQERNN AGFLSYNIYL
481 TGWDESQANH FAVHHDEEKD VITGLKQKTL YGRPNWDNEF KTIASQHPNT RIGVFLCGPE
541 ALAETLSKQS ISNSESGPRG VHFIFNKENF Stop
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Patient

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  1 MGNWAVNEGL SIFVILVWLG LNVFLFVWYY RVYDIPPKFF YTRKLLGSAL ALARAPAACL
 61 NFNCMLILLP VCRNLLSFLR GSSACCSTRV RRQLDRNLTF HKMVAWMIAL HSAIHTIAHL
121 FNVEWCVNAR VNNSDPYSVA LSELGDRQNE SYLNFARKRI KNPEGGLYLA VTLLAGITGV
181 VITLCLILII TSSTKTIRRS YFEVFWYTHH LFVIFFIGLA IHGAE Stop
```

Figure 5.1: Amino acid translation of the gp91-phox protein for control and patient.

nonfunctional NADPH-oxidase enzyme are unable to mount an immune response when there is an infection event. Similarly, in this study the patient had shown to have almost null (0.2%) gp91-phox expression in his neutrophils (Figure 4.2). This stands to reason for his severe clinical condition at a very tender age.

The mother was found to be a carrier of X-linked CGD. The sequencing of the *CYBB* gene had clarified the carrier status of the patient's mother. The presence of functional and mutant allele in her *CYBB* gene explains the bimodal pattern of neutrophil response observed in the flow cytometry assay. The mRNA RT-PCR showed that the mutation is caused by recessive allele since the mother does not suffer from clinical symptoms though she is a carrier. The bimodal expression pattern of neutrophils when stimulated with PMA could be the result of random X-inactivation. This is substantiated by the presence of two distinct neutrophil populations in the DHR assay. The findings also were corroborated with the gp91-phox protein expression assay in which only half of her neutrophils express gp91-phox. The short tandem analysis in the X chromosome had shown that she had passed the defective X chromosome to the index patient in this study.

Interestingly, the patient's brother also exhibited similar bimodal pattern of neutrophil expression in the DHR assay as the mother. He demonstrated similar findings in the gp91-phox protein expression assay whereby half of his neutrophils do not express the gp91-phox protein and the other half does. Usually, only females are known to be carriers of X-linked diseases. Hence, this observation had prompted further studies to determine the brother status. The X-STR analysis showed that the brother was a Klinefelter and he had inherited both of his mother's X chromosomes. The presence of an extra X chromosome in the brother had protected him from manifesting CGD symptoms. However, one of his inherited functional maternal allele had undergone recombination event in the Linkage II group X-STRs i.e.DXS7132, DXS10079 and

DXS10074 (Figure 4.9). The X-STR result had strengthened the evidence that he is indeed a carrier of X-linked CGD. To the best of our knowledge, this is the first report of a child who is a carrier of X-linked CGD with Klinefelter's syndrome.

There are ten PIDs that had resulted from mutations in X chromosome genes (Pessach & Notarangelo, 2009). Table 5.1 shows the X-linked primary immunodeficiencies and the affected genes. The concurrence of Klinefelter's syndrome (XXY) with other X-linked syndrome has been observed at a low frequency (Pueschel et al., 1987). One of such syndrome is Incontinentia pigmenti, a condition that affects particularly the skin and central nervous system. It is a distinctive in that it is normally fatal in males and hence very few males are born with this condition (Buinauskaite et al., 2010). However, seventy-two male Incontinentia pigmenti patients have been reported in a study and their survival mechanism has been attributed to the concurrence of Klinefelter's syndrome in eight cases. The other proposed survival mechanisms in males with Incontinentia pigmenti are hypomorphic mutations and somatic mosaicism (Buinauskaite et al., 2010). The XXY karyotype is believed to protect the affected males from such diseases. The inheritance of a lethal mutation as in Incontinentia pigmenti and our index case's sibling does not cause disease as the XXY karyotype establishes heterozygote genotype that promotes survival.

The prevalence of Klinefelter's syndrome in male population ranges from 1/500 to 1/1000. It is proposed that Klinefelter's syndrome occur at higher frequency i.e. 1/10 in the group of disease carriers (Forti et al., 2010). This further suggests that Klinefelter's syndrome is a minor pathway of mitigation against the disease. Approximately 2/3 of Klinefelter patients are undiagnosed till the evaluation of couple infertility (Pueschel et al., 1987). This is due to the phenotype variability and the main symptoms of this syndrome are androgen deficiency and infertility (Forti, et al., 2010). Similarly, the older brother who was 4 years old when the index patient was diagnosed

Table 5.1: X-linked primary immunodeficiencies.

Disease	X Chromosome Location	Gene
Chronic Granulomatous Disease (CGD)	Xp21.1	*CYBB*
Wiskott-Aldrich syndrome (WAS)	Xp11.23-p11.22	*WASP*
Severe Combined Immunodeficiency (SCIDX1)	Xq13	*IL2RG*
X-linked Agammaglobulinemia (XLA)	Xq21.3-q22	*BTK*
Hyper IgM syndrome type 1 (HIGM1)	Xq26	*CD40L*
X-linked Lymphoproliferative disease (XLP) Type 1	Xq25	*SH2D1A*
X-linked Lymphoproliferative disease (XLP) Type 2	Xq25	*XIAP*
Immune deficiency with ectodermal dystrophy (EDA-ID)	Xq28	*IKBKG*
Immune dysregulation-polyendocrinopathy-enteropathy-X-Linked syndrome (IPEX)	Xp11.23-q13.3	*FOXP3*
Properdin deficiency	Xp11.4-p11.23	*PFC*

(Source: Pessach & Notarangelo, 2009)

with CGD, was normal in appearance and did not exhibit any symptoms. The timely detection of this condition in patient's brother had prompted to early intervention and management. This will enable the doctors to start appropriate treatment and allow the parents to be better prepared for the possible challenges that might be imposed due to this condition.

The only limitation in this study is that the sample size obtained for analysis is small (n=1). This is a single subject study involving the immediate family members. The small sample size for the study is justified by the prevalence of CGD; 1 in 250, 000 live births. Since this is a very rare disease, it was challenging to recruit more patients to be included in the study. Some true CGD cases might have gone unnoticed due to early death and hence investigations could not be done. Result interpretation, particularly confidence intervals and p-values is the main drawback with small sample size studies. However, since this study is mainly aimed to define the genetic abnormality that caused the disease phenotype in the patient, it does not require statistical analysis for result interpretation.

The future direction of this study would be to determine residual oxygen intermediates production in chronic granulomatous disease patients. Survival of patients with CGD was strongly associated with residual ROI generation as a continuous variable, independently of the specific gene affected. It is postulated that patients with CGD and modest residual ROI production confer significantly less severe illness and a greater likelihood of long-term survival than patients with little residual ROI production. Superoxide-dependent ferricytochrome *c* reduction stimulated by phorbol myristate acetate assay is usually performed to evaluate the residual ROI production. Another potential method that can be employed in future study of CGD is the matrix-assisted laser desorption ionization: time of flight mass spectrometry (MALDI-TOF-MS) analyses for the identification of NADPH-oxidase proteins.

6.0 SUMMARY

This study brings the number of reported cases where Klinefelter's syndrome is associated with X-linked primary immunodeficiencies to five (Sanders, et al., 1974; Harris & Docherty, 1988; Schejbel, et al., 2009; Balci et al., 2008). These cases argue for family members to be included in investigations of primary immunodeficiency patients, for two main reasons. Firstly, as these studies suggest other concurrent and potentially modifying, genetic abnormality may surface. Secondly, the screening of entire families also identifies a) carriers with varying levels of functional capacity as in the family study of properdin deficiency (Schejbel, et al., 2009), b) cases before they become clinically apparent as illustrated by the Roos, et al.study of p47-phox deficient CGD (Roos et al., 2006) and c) conditions that may affect the selection of a potential bone marrow donor.

All the three stated objectives of the study have been accomplished. The genetic abnormality in the patient had been defined by the use of reverse transcription PCR of RNA and PCR of gDNA. The nucleotide substitution in the mRNA at position 676 from C to T (c.676C>T) was identified as the genetic mutation that had caused CGD in the affected patient. The mother and brother are known to be carriers as they possess both C and T nucleotide based on the genomic DNA findings.

The nucleotide substitution had resulted in the premature termination of the gp91-phox protein in the index patient. Hence, he had no gp91-phox protein in his neutrophils and this had cause loss of function of the NADPH-oxidase enzyme to generate reactive oxygen intermediates. The inability of the enzyme to generate ROIs had caused the patient to have serious infections at a very young age. This explains the clinical features suffered by the patient were due to the genetic abnormality in the *CYBB* gene.

The study had led to the setting up of diagnostic test for Chronic Granulomatous Disease for the clinical usage in the Institute for Medical Research. The neutrophil function assay protocol was adopted from the manufacturer's instruction with some minor modifications in the sample processing method. The gp91-phox protein expression assay was modified from the manufacturer's instruction to fit the local laboratory settings. The genetic study carried out on *CYBB* gene by reverse transcription PCR on mRNA and PCR on gDNA was adapted from Hui et al., (1996) for detecting mutation in the patient. The cycling conditions for both reactions were slightly modified according to the instrument settings. Hence, the establishment of these tests led to the setting up of confirmatory diagnostic test for CGD.

The findings of this study were used in the publication of a paper in the Asian Pacific Journal of Allergy and Immunology entitled "X-linked chronic granulomatous disease in a male child with an X-CGD carrier, Klinefelter brother". This paper was accepted for publication on 18th September 2012 and the citation for this paper is *Asian Pac J Allergy Immunol 2013;31*(2)*:167-72.*

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