IDENTIFICATION OF A NOVEL INVARIANT SPLICE SITE MUTATION OF BRUTON'S TYROSINE KINASE (*BTK*) GENE IN A MALAYSIAN FAMILY WITH X-LINKED AGAMMAGLOBULINEMIA

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ABSTRACT

X-linked agammaglobulinemia (XLA) is a rare genetic disorder caused by mutations in the Bruton's tyrosine kinase (*BTK*) gene. These mutations cause defects in early B cell development. A patient with no circulating B cells and low serum immunoglobulin isotypes was studied as were his mother and sister. Flow cytometry showed the patient lacked BTK protein expression in his monocytes while the mother and sister had 62% and 40% of the monocytes showing BTK protein expressions, respectively. Results from genetic studies revealed that the patient had a novel base substitution in the first nucleotide of intron 9 in the *BTK* gene, and the mutation was IVS9+1G>C. This mutation resulted in exon 9 skipping, and a loss of 21 amino acids. This defect rendered the patient susceptible to recurrent pyogenic infections, otitis media, bronchopneumonia, asthma, and failure to thrive. Genetic study revealed that both mother and sister have heterozygous alleles at the similar mutational point as in the patient, confirming that both were carriers. This study supports the necessity of combining flow cytometry and genetic study in the diagnosis of XLA and the information obtained would be useful for subsequent genetic counseling, carrier detection and prenatal diagnosis.

Key words: Bruton's tyrosine kinase, *BTK* gene, splice site mutation, X-linked agammaglobulinemia, exon skipping, BTK protein

ABSTRAK

'X-linked agammaglobulinemia' (XLA) adalah penyakit genetik yang jarang berlaku, disebabkan oleh mutasi dalam gen Bruton's tyrosine kinase (*BTK*). Mutasi tersebut mengakibatkan kecacatan dalam perkembangan awal sel B. Seorang pesakit yang tidak mempunyai sel B dan kekurangan semua 'isotype' serum immunoglobulin diuji bersama dengan ibu dan kakaknya. Keputusan 'flow cytometry' menunjukkan pesakit tersebut tidak mempunyai ekspresi protin BTK dalam monositnya manakala ibu dan kakaknya mempunyai 62% dan 40% monosit menunjukkan ekspresi BTK masing-masing. Keputusan ujian genetik menunjukkan bahawa pesakit tersebut mempunyai gantian bes yang baru pada nukleotida pertama 'intron' 9 dalam *BTK* gen, dan mutasi tersebut adalah IVS9+1G>C. Mutasi tersebut mengakibatkan loncatan 'exon' 9, dan kehilangan 21 asid amino. Kecacatan tersebut mengakibatkan pesakit tersebut cenderung kepada jangkitan bakteria berulangan, keradangan telinga, radang paru-paru, asma, dan kegagalan membesar. Ujian genetik menunujuk bahawa ibu dan kakak adalah 'heterozygous alelle' dalam titik mutasi yang sama dengan pesakit tersebut, kedua-dua mereka dikelaskan sebagai pembawa kepada penyakit tersebut. Kajian ini menyokong kepentingan untuk menggabungjalinkan keputusan 'flow cytometry' dan ujian genetik dalam diagnosi penyakit XLA. Maklumat yang didapati dari kedua-dua kaedah ini dapat diguna untuk kaunseling genetik, pengesahan pembawa penyakit dan diagnosi pra-natal.

Kata-kata kunci: Bruton's tyrosine kinase, gen *BTK*, mutasi mencapah, 'X-linked agammaglobulinemia', locatan 'exon', protin BTK

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µHC	immunoglobulin mu heavy chain
BCR	B cell receptor
BLNK	B cell linker protein
bp	base pair
BTK	Bruton's tyrosine kinase gene
BTK	Bruton's tyrosine kinase protein
CD	Cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CIDs	combined Immunodeficiencies
CRS	class-switch recombination
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylene-diamine tetra acetic acid
FITC	fluorescein Isothiocyanate
g	gravity
gDNA	genomic deoxyribonucleic acid
Grb2	growth factor receptor-bound protein 2
Ig	immunoglobulin
ITAM	immunoreceptor tyrosine-based activation motif
MFI	mean fluorescence intensity
MgCl2	magnesium chloride
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PID	primary immunodeficiency
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SHM	somatic hypermutation
U	unit
XLA	X-linked agammaglobulinemia

1.0 INTRODUCTION

Primary immunodeficiencies (PIDs) are disorders in which particular component of the body's immune system is absent or does not function properly. PIDs are caused by mutation in a particular gene. The molecular defect may result in the defects involving humoral, cellular, or innate immunity. The main characteristic of PID patients is an enhanced susceptibility to infections (Notarangelo, 2010).

B cell defects account for around 50% of PIDs (Eley, 2008). Children suffering PIDs due to B cell defects tend to present at around 4 to 6 months of age when mother's passively transferred immunity starts to decline (Tóth et al., 2009). Patients with B cell defects may have low amount of antibody-producing B cells or non-functional B cells leaving them susceptible to infections. They frequently present with recurrent ear, nose, throat and airway infections caused mainly by extracellular encapsulated bacteria such as *Streptococcus pneumonia*, *Haemophilus influenza* or *Pseudomonas* species (Basile et al., 2008).

X-linked agammaglobulinemia (XLA) is a classic example of B cell defects. XLA was reported with an estimated prevalence of 1 in 200,000 live births, in a BTK database (Valiaho, Smith, & Vihinen, 2006). XLA is caused by mutations in the Bruton's tyrosine kinase (*BTK*) gene (Vetrie et al., 1993). Generally, a clinical diagnosis of XLA is made if the patient has very low or no circulating B cells, low serum immunoglobulin levels and a history of recurrent bacterial infections at early age with or without a positive family history. However, only 85% of the patients who comply with these clinical criteria have *BTK* gene mutations, the remaining 15% patients are caused by other genes (Conley et al., 2005). Hence, a definitive laboratory testing is needed to confirm the diagnosis. Characterization of the *BTK* gene mutation in the patient enables the identification of other family members who carry the similar

mutation. Hence, affected male siblings of the patient who confirmed with *BTK* gene mutations can be tested before the onset of any symptoms and given early treatment. Early treatment could avoid unnecessary disease complication in those patient's male siblings. For example, delay in the treatment of some infections and conditions can lead to complication such as bronchiectasis. This complication can affect the patient's quality of life (QOL) and expectancy.

Besides that, information on XLA incidence in Malaysia is very scarce. To date, there are very few reports describing the clinical features and laboratory findings of Malaysian XLA patients (Noh et al., 2013; Okoh et al, 2002). This reflects the needs for more confirmatory laboratory tests to be set up to confirm the diagnosis. A definitive diagnosis will undoubtedly enable the clinicians to treat the patients appropriately. As a result, the patients will be able to live a more normal life and avoid excessive hospitalization.

1.1 Objectives

The objectives of this study were:

- To investigate X-linked agammaglobulinemia and the carrier status in a suspected patient and his family members.
- To investigate the monocyte Bruton's tyrosine kinase protein expression by flow cytometry.
- To investigate the genetic mutations involved in cDNA and genomic DNA level in the patient and the carriers.
- To set up a reliable XLA diagnosis system using small sample volume of whole blood for routine clinical usage in the Institute for Medical Research (IMR), Malaysia.

1.2 Thesis organization

This thesis consists of six chapters, which are introduction, literature review, methodology, results, discussion and lastly the summary. Chapter one contains the general introduction to the research concerned and the objectives of the study. Secondly, in the literature review chapter, a general introduction of Primary Immunodeficiency diseases, B cell development and followed by the description of X-linked agammaglobulinemia, the roles of Bruton's tyrosine kinase protein and so on, are discussed. Methodology chapter describes and explains the research methodology used in this study. In this study, a Malay boy who was clinically diagnosed as X-linked agammaglobulinemia was recruited. The clinical, immunological and genetic investigations of the patient are described in the result part of this thesis. Besides that, the patient's mother and sister were recruited in this study to determine the carrier status in the family. Discussion chapter contains the interpretation of the results and also research finding comparison between this study and previous studies in the literature. Last but not least, all findings are summarized in the summary part.

2.0 LITERATURE REVIEW

2.1 Primary Immunodeficiency diseases

Primary immunodeficiencies (PIDs) are inherited disorders that affect the development, function, or both of the body's immune system (Notarangelo, 2010). In most cases of PIDs, the disorders are caused by single gene mutation; however, some PIDs involve more than one gene mutations (McCusker & Warrington, 2011; Schroeder, Schroeder, & Sheikh, 2004). PIDs are caused by underlying genetic defects whereas secondary immunodeficiencies are caused by other causes, such as viral or bacterial infections, malnutrition, immunosuppressive drugs, environment stress, age extremity such as prematurity or aging, surgery or metabolic diseases (Chinen & Shearer, 2008).

PIDs are rare diseases with an overall estimated prevalence of about 2.3 in 100,000 people, as reported in a Japanese series (Ishimura et al., 2011). A much higher prevalence is observed among populations with high consanguinity rates especially if the disease is caused by autosomal recessive inheritance (Hamamy, Masri, Al-Hadidy, & Ajlouni, 2007). Besides that, ethnicity difference may result in different prevalence for the similar disease. For instance, selective IgA deficiency is common PIDs in Caucasians, accounting for 1 in 500 individuals, whereas selective IgA deficiency is rare among Japanese population, accounting for approximately 1 in 18,000 individuals (Cunningham-Rundles & Ponda, 2005).

Traditionally, the classification of PIDs are based on the component of the immune system which is affected (Notarangelo et al., 2009). However, in some cases, defects in the same gene can result in different clinical phenotypes, and defects in different genes can lead to the same clinical phenotypes (Maggina & Gennery, 2013). This makes the classification difficult to fit all needs. To date, more than 180 different

forms of PIDs were characterized and classified into eight groups (Al-Herz et al., 2011; Maggina & Gennery, 2013). This classification includes combined immunodeficiencies (CIDs), well-defined syndromes with immunodeficiency, predominantly antibody deficiencies, diseases of immune dysregulation, congenital defects of phagocytes, defects in innate immunity, autoinflammatory disorders, and complement deficiencies (Al-Herz et al., 2011), as listed in Table 2.1.

The main characteristic of PID patients is an enhanced susceptibility to particular infectious pathogens. The type of pathogen correlates to the type of immunological defect that is present. For example, patients with combined immunodeficiencies tend to suffer from recurrent infections caused by opportunistic pathogens such as *Candida albicans*, *Pneumocystis jiroveci* or cytomegalovirus, whereas, patients with phagocytes disorders tend to have severe pyogenic (pus-like) bacterial and fungal infections of the skin, respiratory tract, internal organs, and mouth (McCusker & Warrington, 2011).

Antibody deficiencies are the most common type of primary immunodeficiencies. A recent paper described the spectrum of PIDs in Malaysia, as shown in Figure 2.1. Predominant antibody deficiency was the commonest among other PIDs, accounting for 40.38%, followed by phagocytic defect, 17.3%; combined immunodeficiency, 15.38%; other cellular immunodeficiency, 11.5%; other deficiencies, 9.61%; immune deficiency with secondary or other diseases, 3.8%; and immune deficiency associated lymphoproliferation, 1.92% (Noh et al., 2013).

Other than Malaysia, few papers from Asian countries reported that the frequency of antibody deficiencies also accounts for nearly half of the PIDs population. For instance, it has been reported that the frequency of antibody deficiencies is approximately 40% in Japan (Ishimura et al., 2011), 43% in Hong Kong (Lam, Lee, Chan, Ho, & Lau, 2005), 46.2% in Singapore (Lim et al., 2003), 48.2% in China

Table 2.1: The classification of primary immunodeficiencies (PIDs).

	Type of PIDs
1	Combined immunodeficiencies
2	Well-defined syndromes with immunodeficiency
3	Predominantly antibody deficiencies
4	Diseases of immune dysregulation
5	Congenital defects of phagocyte number, function, or both
6	Defects in innate immunity
7	Autoinflammatory disorders
8	Complement deficiencies

(Source: Al-Herz et al., 2011)

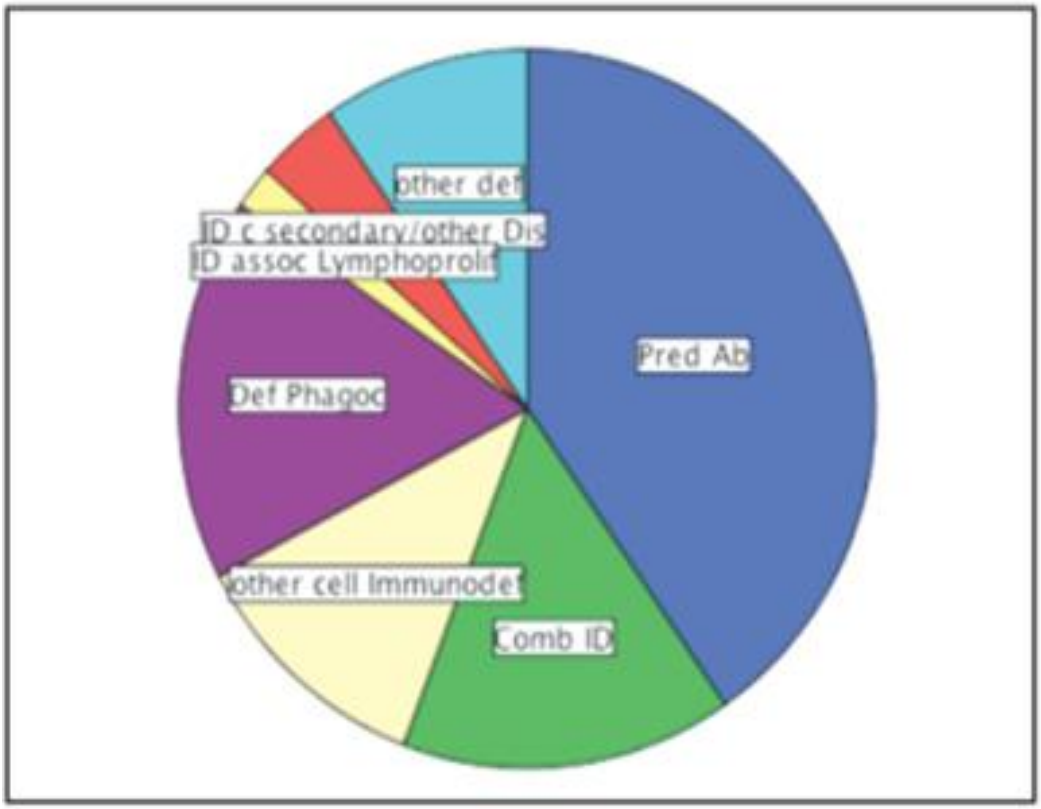

Figure 2.1: Spectrum of Primary Immunodeficiency in Malaysia.

Note: 'Pred Ab' represents predominant antibody defect; 'comb ID' represents combined immunodeficiency; 'other cell immunodef' represents other cellular immunodeficiency; 'Def Phagoc' represents defective phagocytosis; 'ID assoc lymphopro/ other dis' represents immunodeficiency associated lymphoproliferation; 'ID c secondary/ other dis' represents immunodeficiency with secondary/ other disease; and 'other def' represents other deficiencies.

(Source: Noh et al., 2013)

(Wang et al., 2011), 52.2% in Thailand (Benjasupattananan et al., 2009) and 55.9% in Korea (Rhim et al., 2012). However, a higher frequency of antibody deficiencies was observed in Caucasian PIDs. For example, antibody deficiencies accounts for 78% of PIDs in the United States (Joshi, Iyer, Hagan, Sauver, & Boyce, 2009). More than 20 different types of antibody deficiencies have been described to date (Al-Herz et al., 2011) (Table 2.2). Typical categories of antibody deficiencies include X-linked agammaglobulinemia (XLA), common variable immunodeficiencies, hyper IgM syndrome and selective IgA deficiency (Driessen & Burg, 2011).

Children suffering PIDs due to B cell defects tend to present at around 4 to 12 months of age when mother's passively transferred immunity is on the wane (Lim & Elenitoba-Johnson, 2004; McCusker & Warrington, 2011). Patients with B cell defects may have too few antibody-producing B cells or non- functional B cells leaving them susceptible to a wide range of infections. They frequently present with recurrent respiratory tract infections caused mainly by extracellular encapsulated bacteria such as *Streptococcus pneumoniae* or *Haemophilus influenzae* (McCusker & Warrington, 2011). In addition to bacterial infections, patients with antibody deficiencies are susceptible to enteroviral infections and *Gardia lamblia* protozoa infection (Notarangelo, 2010).

2.2 B cell development

During gestation, B cells develop from committed precursors in the fetal liver. After birth, B cells generation takes place in the bone marrow (Abbas, Lichtman, & Pillai, 2010). Early B cell development includes the development from pro-B cell to pre-B cells. This process does not require antigen contact and takes place in the bone marrow (Figure 2.2). Further development of pre-B cells to immature B cells occurs in the bone marrow. However, maturation of B cells in the periphery is antigen-dependent.

Table 2.2: Classification of predominantly antibody deficiencies.

	Diseases
1	Severe reduction in all serum immunoglobulin isotypes with profoundly decreased or absent B cells (a) BTK deficiency (X-linked agammaglobulinemia) (b) μ heavy chain deficiency (c) λ5 deficiency (d) Igα deficiency (e) Igβ deficiency (f) BLNK deficiency (g) Thymoma with immunodeficiency (h) Myelodysplasia with hypogammaglobulinemia
2	Severe reduction in at least 2 serum immunoglobulin isotypes with normal or low number of B cells (a) Common variable immunodeficiency (b) ICOS deficiency (c) CD19 deficiency (d) CD81 deficiency (e) CD20 deficiency (f) TACI deficiency (g) BAFF receptor deficiency
3	Severe reduction in serum IgG and IgA with normal/ elevated IgM and normal numbers of B cells (a) CD40L deficiency (b) CD40 deficiency (c) AID deficiency (d) UNG deficiency
4	Isotype or light chain deficiencies with normal numbers of B cells (a) Ig heavy chain mutations and deletions (b) K chain deficiency (c) Isolated IgG subclass deficiency (d) IgA with IgG subclass deficiency (e) Selective IgA deficiency
5	Specific antibody deficiency with normal Ig concentrations and normal numbers of B cells
6	Transient hypogammaglobulinemia of infancy with normal numbers of B cells

(Source: Al-Herz et al., 2011)

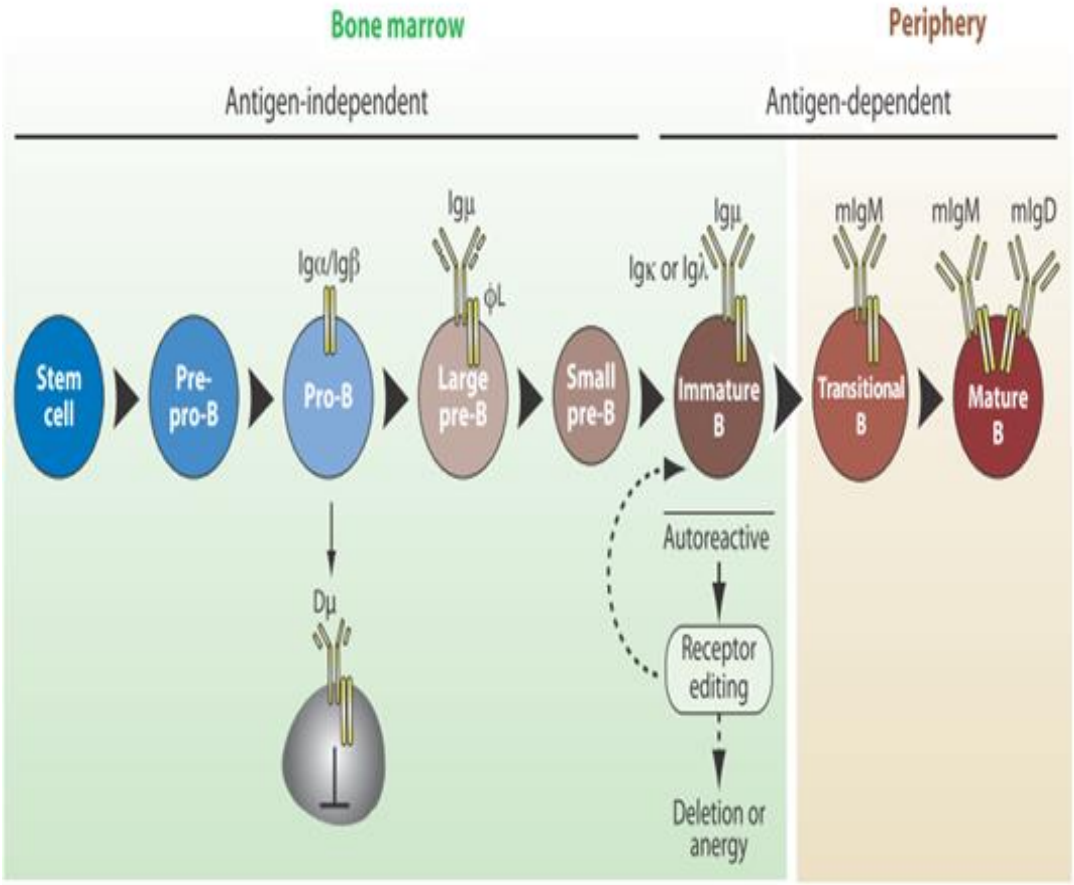

Figure 2.2: Overview of B cell development.

(Source: Kurosaki, Shinohara, & Baba, 2009)

During early B cell development, pro-B cells undergo VDJ rearrangement of the immunoglobulin heavy chain (μHC) loci (Zhang, Srivastava, & Lu, 2004). Pre-B cell receptor (pre-BCR) complex is expressed after the completion of μHC gene rearrangement (Geier & Schlissel, 2006). Expression of μHC leads to pre-B cell expansion.

Pre-B cell receptor (pre-BCR) is expressed on the surface of pre-B cell. Pre-BCR complex consists of two μ heavy chains and two surrogate light chains (Figure 2.3). The surrogate light chains are composed of VpreB protein and λ5 protein. Vpre B protein is homologous to a light chain V domain, whereas λ5 protein is covalently attached to the μ heavy chain by disulfide bond. The pre-BCR is also associated with the signal transducing protein Igα (CD79a) and Igβ (CD79b) which are expressed on all B cells (Fried & Bonilla, 2009).

Figure 2.4 shows the proximal B-cell receptor-mediated signaling pathways. Upon the binding of extracellular antigen with BCR, a series of signal transduction will be initiated. The cytoplasmic domains of Igα and Igβ contain immunoreceptor tyrosine-based activation motifs (ITAMs) provides a docking site for the Syk kinase, Src family kinases (Fyn, Lyn, and Blk), the Tec family kinase (Bruton tyrosine kinase), the adaptor proteins Grb2 and B cell linker protein (BLNK) (Geier & Schlissel, 2006). Once Syk binds to the Igα ITAM, and the B-cell linker protein (BLNK) will activate Btk signaling pathway, which then activates phospholipase C (PLC-γ2) and leads to calcium flux (Wang & Clark, 2003). In the presence of calcium, protein kinase C (PKC-β) will be activated, which then results in the activation of nuclear factor-KB (NF-KB) in the antigen-stimulated B cells. These signaling cascades eventually lead to the activation of transcription factors which triggers B cell proliferation and differentiation (Abbas et al., 2010). Mutation in the *BTK* gene causes early arrest of B cell development, resulting in X-linked agammaglobulinemia (XLA). Apart from *BTK* gene mutation, mutations in

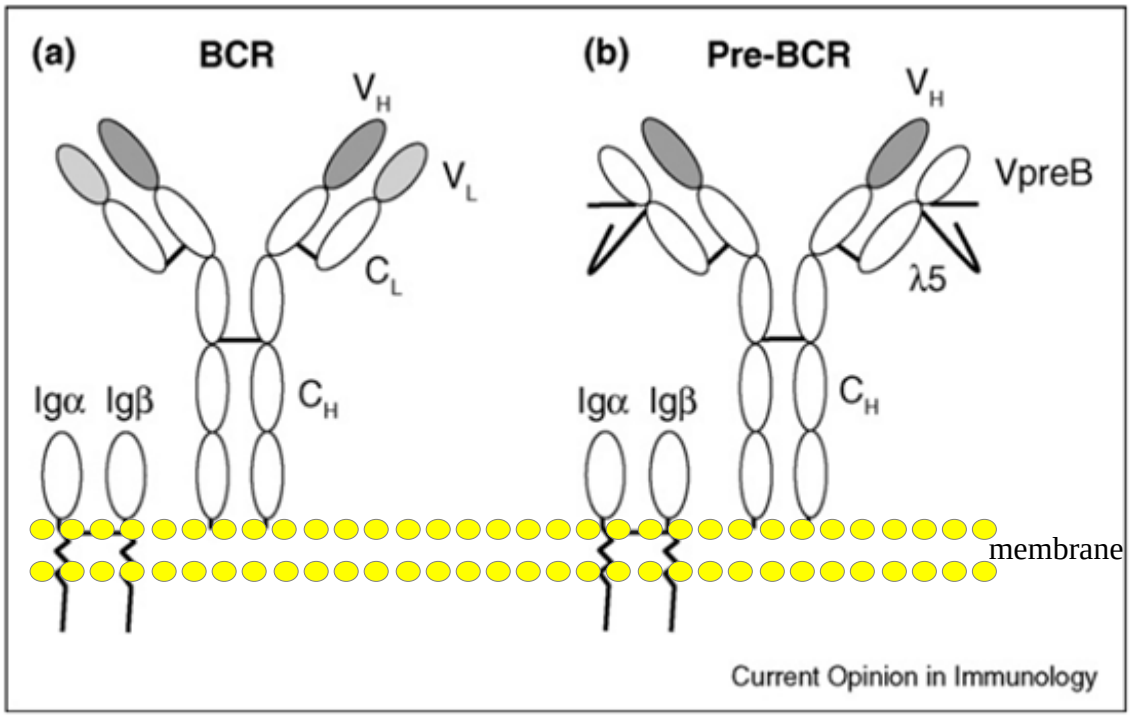

Figure 2.3: Schematic representation of (a) the BCR and (b) the pre-BCR. The signaling molecules, Igα/β, associated with both the pre-BCR and BCR are shown. Both BCR and pre-BCR are membrane-bound receptors.

(Source: modified from Mårtensson, Keenan, & Licence, 2007)

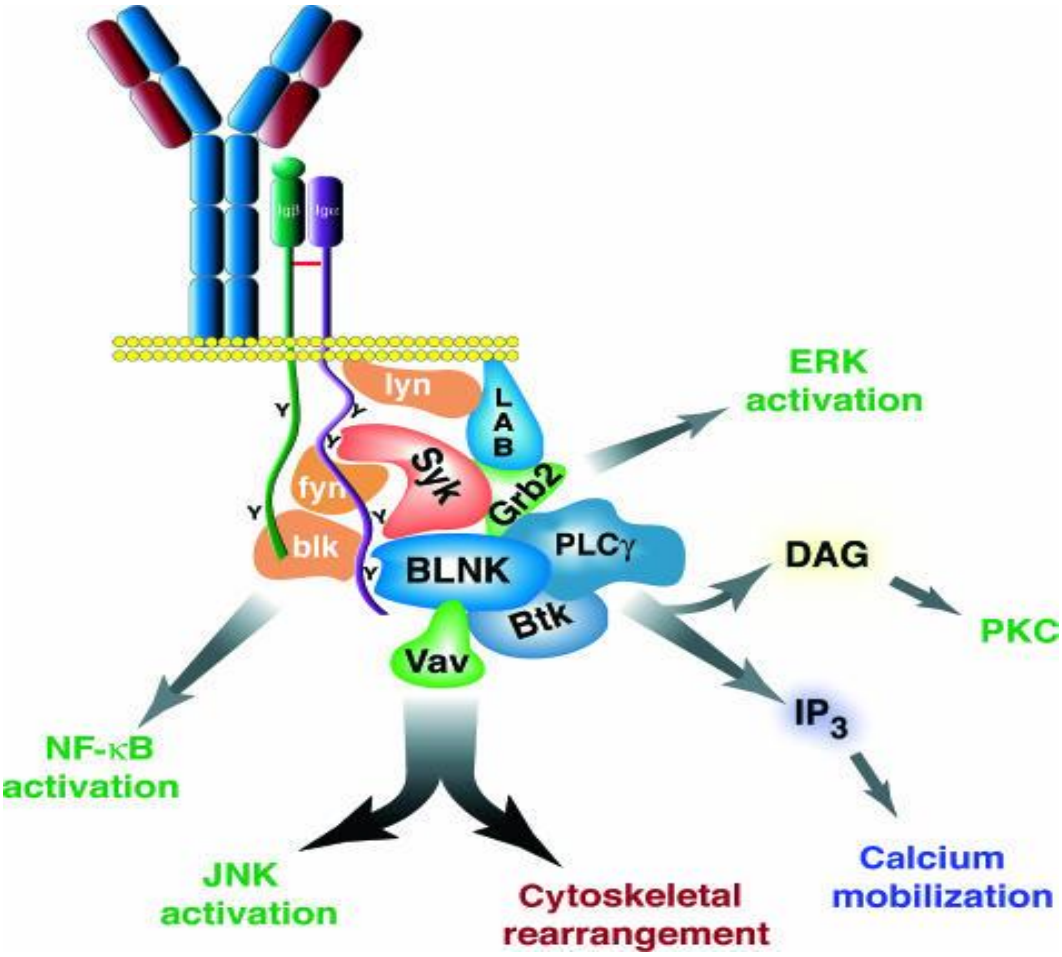

Figure 2.4: Proximal B-cell receptor-mediated signaling pathways. After binding to antigen, the immunoglobulin Igα and Igβ cytoplasmic tails are phosphorylated on the immunoreceptor tyrosine-based activation motif (ITAM) tyrosines by Src-family tyrosine kinases (SFTKs) and/ or Syk. Syk then binds to the Igα ITAM, and the B-cell linker protein (BLNK) binds to tyrosine 204 of Igα. This activates multiple signaling pathways, including: Btk, which activates phospholipase C (PLC-γ2 and leads to calcium flux (blue) and protein kinase C (PKC) activation (green); Grb2, which activates the Ras/ Raf/ mitogen-activated protein kinase (MEK) extracellular signal-regulated kinase (ERK) pathway (green); and Vav, which activates the Rac/Rho/Cdc42 pathway and results both in cytoskeletal rearrangement (maroon) and c-Jun N-terminal protein kinase (JNK) activation (green). The SFTKs themselves activate nuclear factor-KB (NF- KB) (green).

(Source: Wang & Clark, 2003)

μ-heavy chain (Lopez Granados et al., 2002), *λ5* (Miyazaki et al., 1999), Igα (Minegishi et al., 1999; Wang et al., 2002), Igβ (Ferrari et al., 2007) and *BLNK* (Pappu et al., 1999) gene have been reported in patients with agammaglobulinemia. In bone marrow, upon light chain locus rearrangement, pre-B cells develop into immature B cells where heavy and light chain are co-expressed on the cell surface, in association with Igα and Igβ, to form antigen-specific surface receptor (Wang & Clark, 2003). BCR consists of the μ heavy chains and light chain. The μ heavy chains are composed of the variable region (V_H) and constant region (C_H1-3), whereas the light K or λ chains are composed of a variable region (V_L) and a constant region (C_L) (Figure 2.3). These variable regions are binding site for specific antigens.

In bone marrow, the immature B cells may encounter high avidity antigens, such as multivalent self-antigens. However, they do not proliferate and differentiate in the response to self-antigens. Instead, a process called negative selection occurs. During negative selection, immature B cells which recognize and react with self-antigens (auto-reactive immature B cells) may undergo receptor editing, cell death, or functional unresponsiveness instead of activation (Abbas et al., 2010). Therefore, only those immature B cells that are non-reactive to self-antigens leave the bone marrow and further differentiate to mature B cells in the periphery (Pillai & Cariappa, 2009).

In periphery, immature B cells will develop into either one of three different B-cell subsets, which are B-1 B cells, marginal zone B-2 B cells or follicular B-2 B cells (Figure 2.5). B-1 B cells are derived from fetal liver stem cells. B-1 cells undergo renewal and reside in the periphery such as peritoneum and mucosal sites. They are able to elicit early T-independent humoral immune responses. B-1 cells are able to secrete natural antibodies which act against microbial polysaccharides and lipids in the gut, peritoneum or mucosal sites (Abbas et al., 2010; LeBien & Tedder, 2008).

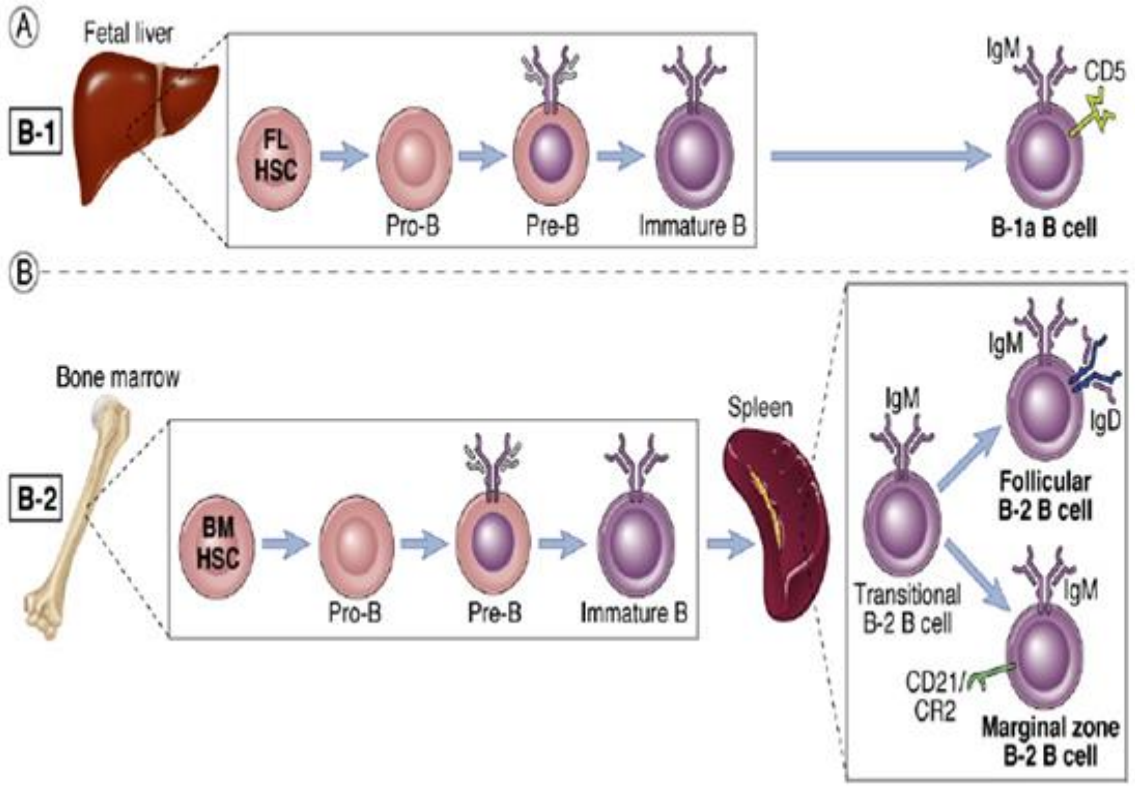

Figure 2.5: B cell subsets. (A) The B-1 lineage is derived from fetal liver-derived stem cells. (B) B-2 lineage is derived from bone marrow precursors. Follicular B cells are circulating lymphocytes, while marginal zone B cells reside primarily in the spleen.

(Source: Abbas et al., 2010)

On the other hands, B-2 B cells are derived from the bone marrow precursors, and developed into either follicular B-2 B cells or marginal zone B-2 B cells in the spleen (LeBien & Tedder, 2008). Majority of the circulating mature B cells are follicular B cells due to its ability to migrate from one lymphoid organ to the next lymphoid organ. Follicular B cells respond to protein antigens, which require helper T-cells collaboration, undergo germinal centre reactions, and develop to memory cells. Whereas, marginal zone B cells reside primarily in the marginal zone of the spleen (Pillai & Cariappa, 2009). Marginal zone B cells act against blood-borne polysaccharides, in T-cell-independent manner, which occurs in the marginal zone of the spleen (Martin, Oliver, & Kearney, 2001).

During a primary immune response, a number of naive B cell, such as follicular B cells, are activated and experience clonal expansion followed by the generation of short-lived plasma cells which secrete low affinity antibodies, IgM. These antibodies are the first defense against the antigen (Wols, 2006). Meanwhile, a number of naive B cells experience a further diversification of its antibody repertoire through class switch recombination (CSR) or somatic hypermutation (SHM), where these processes are T-cell dependent, in the peripheral lymphoid organs. The outcomes of these events are generation of different immunoglobulin isotypes depending on the type of antigens encountered and an increase in high-affinity antibodies. These antibodies are able to eliminate microbes more efficiently (Fried & Bonilla, 2009).

In the meantime, a number of naive mature B cells differentiate into memory cells in the germinal center. During a repeat infection by the same antigen, these memory B cells are able to differentiate into antibody-secreting plasma cells rapidly. These plasma cells are able to produce high-affinity, isotype-switched antibody (IgG, IgM, IgA, or IgE) in a faster and greater magnitude.

2.3 X-linked agammaglobulinemia (XLA)

XLA was the firstly described immunodeficiencies by Bruton in 1952 (Bruton, 1952). Bruton reported an eight year-old boy suffered from recurrent infections and sepsis which were caused by pneumococcus, had no gammaglobulin fraction in his serum when tested with protein electrophoresis. The boy was then treated with monthly intramuscular injections of human gamma globulin and he demonstrated a significant clinical improvement. There was no family history of other affected males in his family. However, in the next few years, patients with the similar clinical phenotypes were studied and noted that most affected patients were males. This finding suggested that Bruton's agammaglobulinemia was inherited in an X-linked pattern (Elphinstone, Wickes, & Anderson, 1956; O'Brien & Sereda, 1956)

The causative gene of XLA, *BTK* gene, was identified in 1993 (Vetrie et al., 1993). The mutation in *BTK* gene causes an arrest in the early B cell differentiation where it blocks the differentiation of pre-B cells into circulating, mature B cells and plasma cells. Affected males have normal number of pre-B cells in the bone marrow but reduced in pre-B-II cells, immature B and mature B cells stages (Nomura et al., 2000; Noordzij, et al., 2002).

Since the patients with XLA have low or no circulating B cells and markedly reduced immunoglobulin isotypes in the serum (López-Granados, Pérez de Diego, Ferreira Cerdán, Fontán Casariego, & García Rodríguez, 2005), they are susceptible to recurrent pyogenic bacterial infections (Cunningham-Rundles & Ponda, 2005). Pneumonia, upper respiratory tract infections, sinusitis and otitis media are the most common clinical presentations in XLA patients (Noh et al., 2013; Wang et al., 2009). Other prevalent clinical problems present among patients with XLA are diarrhea, arthritis, skin infections, meningitis/ encephalitis, conjunctivitis, osteomyelitis, sepsis,

hepatitis, and enteroviral infections (Gaspar & Kinnon, 2001; Lee et al., 2009; Zhang et al., 2010). Vaccine-associated polio has been reported in few XLA patients (Lee et al., 2009; Winkelstein et al., 2006). Hence, immunoglobulin replacement therapy via intravenous or subcutaneous route must be given to patients with XLA to protect them from severe recurrent infections (Maarschalk-Ellerbroek, Hoepelman, & Ellerbroek, 2011).

According to the Pan-American Group for Immunodeficiency (PAGID) and the European Society for Immunodeficiencies (ESID), the definitive diagnostic criteria for XLA are male patient with less than 2% circulating $CD19^+$ B cells along with either a mutation in *BTK* gene; absent *BTK* mRNA in neutrophils or monocytes; absent BTK protein in monocytes or platelets; or a positive family history (Conley, Notarangelo, & Etzioni, 1999). Probable diagnostic criteria of XLA are early onset of recurrent bacterial infections, hypogammaglobulinemia, absent isohemagglutinins and/or poor response to vaccines (Conley et al., 1999).

The prevalence of XLA varies from country to country. X-linked agammaglobulinemia (XLA) was reported with an estimated prevalence of 1:200,000 live births, in a BTK database (Valiaho, Smith & Vihinen, 2006). Recently, Rhim et al. (2012) reported that the estimated prevalence of X-linked agammaglobulinemia (XLA) was 1.06 per one million of Korean populations.

2.3.1 Molecular basis of XLA

The *BTK* gene is located at the long arm of chromosome X, Xq21.3-Xq22 and codes for a 659 amino-acid protein (Vetrie et al., 1993). This gene comprises 19 exons which span over 37.5kb of genomic DNA (Gaspar & Kinnon, 2001).

Up to 2007, 620 unique mutations from 974 unrelated families have been recorded in a BTK database (Valiaho et al., 2006). These mutations vary in type and

are scattered throughout all domains of the BTK protein. They can be categorized as 32% missense mutations, 12% nonsense mutations, 27% deletions, 8% insertions, 19% splice site mutations, 1% multiple mutations, and 1% upstream mutation (Valiaho et al., 2006).

Besides BTKbase, another web-based database on primary immunodeficiency diseases has been set up by Japanese group in 2006, namely Resource of Asian Primary Immunodeficiency Diseases (RAPID). This database enables users access to gene-specific PID and protein information (Keerthikumar et al., 2009).

2.3.2 Cell biology of BTK protein

Bruton's tyrosine kinase *(BTK)* gene encodes for BTK protein. BTK protein is expressed in all the stages of B cells except for plasma cells (Gaspar & Kinnon, 2001). BTK is also expressed in monocytes (Futatani et al., 1998) and platelets (Futatani, Watanabe, Baba, Tsukada, & Ochs, 2001). However, BTK protein is not expressed in T cells, NK cells and neutrophils (Futatani et al., 1998).

BTK protein belongs to a family of cytoplasmic tyrosine kinases, but it is different from the Src family kinases as it lacks the amino-terminal myristylation signal crucial for post-translational modification and membrane localization (Gaspar & Kinnon, 2001). The BTK protein is involved in signal transduction which is important for B cell differentiation, development and signaling (Mohamed et al., 2009). It consists of five distinct structural domains, which include a pleckstrin homology (PH) domain, followed by a Tec homology (TH) domain, a Src homology 3 (SH3) domain, a SH2 domain, and a catalytic kinase (SH1) domain (Gaspar & Kinnon, 2001). The PH, TH, SH3 and SH2 domains are important for protein-protein interactions while the kinase domain is necessary for catalytic activity (Gaspar & Kinnon, 2001).

2.3.3 Roles of Bruton's tyrosine kinase protein in B cell development

Mutations in *BTK* gene lead to the defects in the early B cell development. The roles of BTK protein were studied in a mice model with *BTK* gene mutation, E41K strain (Maas, Dingjan, Grosveld, & Hendriks, 1999). It was demonstrated that BTK was not required in the development of pre-BCR, but it may be involved in the immunoglobulin light chain expression signaling in the pre-B cells (Figure 2.6). BTK was also involved in the central tolerance in which the auto-reactive immature B cells were removed in the bone marrow.

Furthermore, in periphery, BTK might mediate signaling involved in follicular entry where auto-reactive periphery immature B cells would be inhibited from follicles entry. In addition, BTK played an important role in the survival and development of immature B cells into long-lived recirculating B cells. Last but not least, BTK activation may induce terminal differentiation of plasma cells (Maas & Hendriks, 2001).

2.3.4 Diagnostic tests for XLA

Clinical history, physical examination and family history are useful in identifying PIDs, and further laboratory investigations are important to confirm the diagnosis. The initial investigation of patients with suspected antibody deficiencies involves the measurement of serum IgA, IgG and IgM. Patients with very low serum immunoglobulin levels may be an indication of antibody deficiencies. Enumeration of lymphocyte subsets using flow cytometry measures $CD19^+$ B cells in the peripheral blood. Patients with classical XLA have markedly reduced B cells numbers, with less than 2% of $CD19^+$ B cells in the peripheral blood (Lee et al., 2009). However, a recent study showed that patients with specific mutation in *BTK* gene could have normal or near-normal B cell numbers or absent of peripheral B cell numbers (Tao, Boyd, Gonye, Malone, & Schwaber, 2000). Therefore, enumeration of B cell alone could not be the

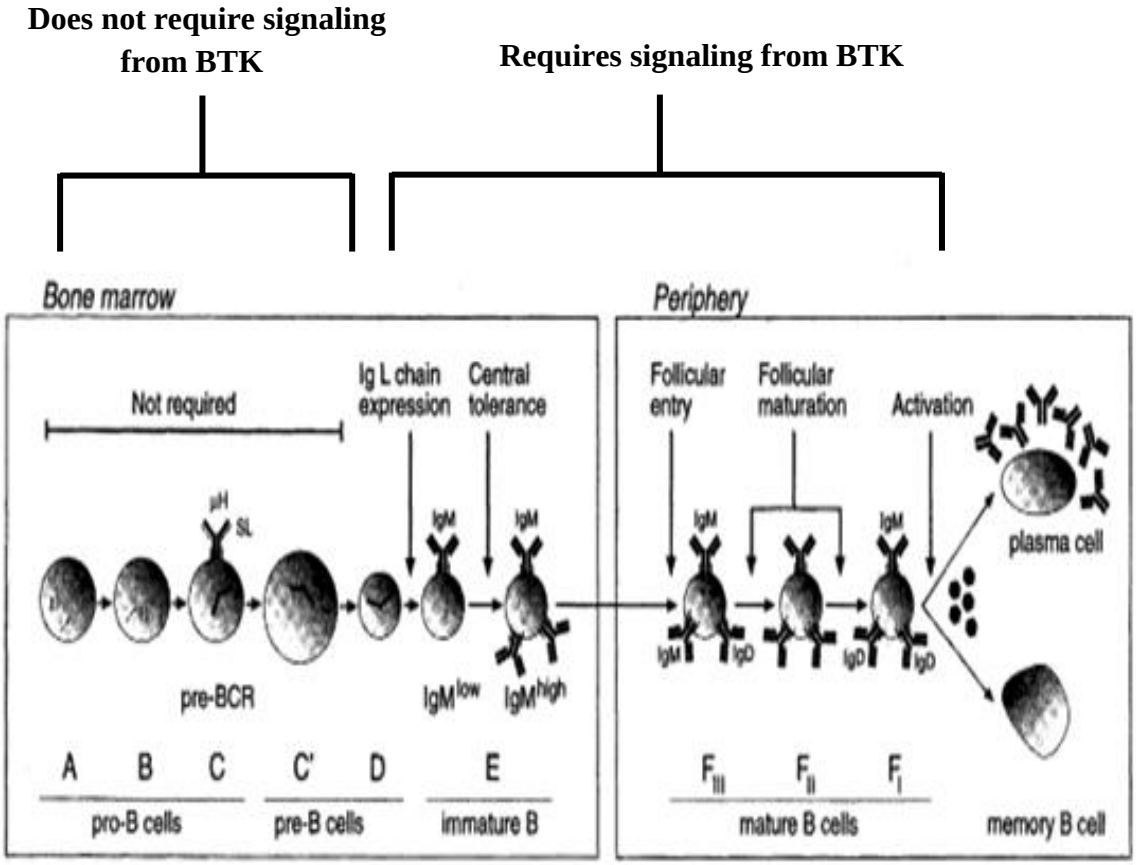

Figure 2.6: Roles of BTK protein in B cell development.

(Source: modified from Maas & Hendriks, 2001)

definitive diagnostic tool for XLA.

Low number or absence of B cell in XLA patient is caused by deficient BTK protein expression. Few studies showed that BTK protein is also expressed in monocytes and platelets (Futatani et al., 1998, 2001). Since XLA patients are deficient in B cell number but have normal monocytes and platelet numbers, BTK expression can be analyzed from monocytes or platelets. In 1998, a rapid flow cytometric analysis of monocyte BTK protein expression was introduced by Futatani et al. Since then, this test has been then widely used for diagnosing XLA. Most patients with XLA have deficient BTK expression in their monocytes and B cells (Futatani et al., 1998; Kanegane et al., 2001; López-Granados et al., 2005). However, some patients with missense mutations in the *BTK* gene has been reported to express BTK protein in their B cells, but the function was at fault (Perez de Diego et al., 2008). Therefore, XLA should not be excluded in antibody deficiency suspected with positive BTK expression until a genetic analysis of *BTK* gene is carried out. In other words, the definitive diagnosis of XLA remains genetic analysis of *BTK* gene (Ameratunga, Woon, Neas, & Love, 2010; Hashimoto et al., 1996; Vorechovsky et al., 1995; Zhang et al., 2010).

2.3.5 Clinical management of XLA

Antibody deficiency as well as X-linked agammaglobulinemia requires immunoglobulin replacement therapy via intravenous or subcutaneous route (Gaspar & Kinnon, 2001). Both intravenous and subcutaneous route appear to be safe and have comparable efficacy on the prevention and treatment of infections and also earlier monitoring for disease complications (Maarschalk-Ellerbroek et al., 2011; Notarangelo, 2010). A recent study showed that some patients were still susceptible to respiratory tract infections especially pneumonia despite immunoglobulin replacement therapy

(Plebani et al., 2002). This may be due to insufficient residual serum IgG or lack of transport of IgG to the mucosal surface site to provide immunity.

The combination of immunoglobulin replacement therapy and antibiotic prophylaxis is effective in treating chronic infections such as chronic sinusitis or bronchiectasis (Fried & Bonilla, 2009). However, these current treatments are not only life-long treatments but, they are also not curative. Other alternative such as lentiviral-mediated gene therapy (Conley, Rohrer, Rapalus, Boylin, & Minegishi, 2000; Hendriks, Bredius, Pike-Overzet, & Staal, 2011) and hematopoietic stem cell transplantation (Conley et al., 2005) has been introduced to treat XLA, but these procedures comes with different types of risks that could affect the patients' quality of life. Therefore, it is crucial that an accurate diagnosis of XLA be made prior to these risky procedures.

3.0 METHODOLOGY

3.1 Study subjects

The index case was a seven year old Malay boy, who was referred from Paediatrics Institute, Kuala Lumpur General Hospital. The patient had a history of recurrent pyogenic infections since he was one year old. He had recurrent otitis media and was admitted almost every year for recurrent bronchopneumonia that usually had slow response to antibiotics treatment. Moreover, he was also admitted once for *influenza A* viral infection.

At six years and eight months of age, he was admitted to the hospital with another episode of bronchopneumonia, serial chest X-ray tests showed recurrent middle lobe consolidation. Besides that, he had episodes of wheezing, suggestive of asthma, therefore was started on metered dose inhaler prophylaxis. His asthma was well controlled after started on inhalers. After the treatment, his symptom was more of prolonged productive cough especially in the morning which is the symptom of bronchiectasis rather than asthma.

Physical examination showed a failure to thrive without clubbing but pectus carinatum was observed. Respiratory examination revealed crepitations with minimal rhonchi. No hepatosplenomegaly or enlarged lymph nodes were observed.

High resolution computed tomography of the thorax showed features of early bronchiectasis. Based on his clinical and laboratory findings (as described in chapter 4), clinical diagnosis of XLA with bronchiectasis was made.

He was then started on three-weekly IVIg therapy at the age of six years and nine months old. Since then, he remained well and suffered no further recurrent episode of pneumonia.

3.2 Blood samples

Blood samples in EDTA-coagulant (9 ml) were collected from the patient, his mother, his sister and a normal control with informed consent. This study was approved by the institutional review board of Institute for Medical Research and the Medical Research Ethics Committee, Ministry of Health, Malaysia.

3.3 Flow cytometric assay

This assay was set up to investigate monocyte Bruton's tyrosine kinase (BTK) protein expression in study subjects. Cell surface staining of monocytes was performed prior to intracellular BTK staining. Intracellular staining of BTK protein was performed with minor modifications according to the manufacturer's protocol (BD Biosciences, San Diego, CA, USA). Firstly, Blood (2 ml) from patient, mother, sister and a normal control was collected in a tube with ETDA anticoagulant. A volume of 10 µl of surface marker PE-conjugated anti-human CD14 (BD Pharmingen™, USA) was added into each tube with 100 µl of whole blood and mixed briefly. These tubes were then incubated for 15 minutes at room temperature, in the dark. After incubation, 2 ml of pre-warmed Lyse/fix buffer was added into each tube. Cells was mixed by vortexing and then incubated at 37°C water bath for 10 minutes. After that, cells were centrifuged at 500 g for 3 minutes and the supernatant was removed. Pellets were washed with 2 ml PBS and centrifuged at 500 g for 3 minutes. Supernatant was then removed. The tubes were vortexed to loosen the pellet. The cells were then permeabilized by adding 1 ml of BD phosflow Perm buffer II and incubated for 20 minutes on ice, in the dark. Cells were washed twice with 2 ml of PBS. Cells were centrifuged at 500 g for 3 minutes and supernatant was removed. Cells were resuspended in 100 µl BD Pharmingen™ stain

buffer. A volume of 10 µl of AlexaFluor® 647 conjugated mouse IgG2a, K isotype control (BD Phosflow™, USA) or AlexaFluor® 647 conjugated BTK-antibodies (clone 53/BTK) (BD Phosflow™, USA) were added to each tube, respectively. The monoclonal antibody BTK clone used in this experiment was designed to target N-terminal of the BTK protein, as stated by the manufacturer. The tubes were vortexed gently and then incubated at room temperature for 30 minutes in the dark. Cells were centrifuged at 500 g for 3 minutes. Then, cells were washed once with 2 ml PBS, centrifuged at 500 g for 3 minutes and then supernatant was removed. BD Pharmingen™ stain buffer (500 µl) was then added into the tube with pellet prior to flow cytometric assay. The monocyte BTK expression was analyzed on flow cytometer BD FACSCalibur using BD CellQuest™ Pro software. Two thousand monocytes were gated from side scatter versus CD14-PE plot. The expression of BTK protein was further analysed from the gated monocyte population.

3.4 RNA extraction

RNA was isolated from whole blood using the QIAamp RNA blood mini kit according to manufacturer's protocol (Qiagen GmbH, Hilden, Germany). One mililitre (ml) of human whole blood with EDTA coagulant was mixed with 5 ml of EL Buffer in a falcon tube. The tube was incubated for 15 minutes on ice. While incubating, the tube was mixed by vortexing briefly for two times. Then, the tube was centrifuged at 400 g for 10 minutes at 4^{o}C, and the supernatant was discarded completely. A pellet was formed after centrifugation. Two ml of EL Buffer was added to the cell pellet. The cells were resuspended by vortexing briefly. The tube was then centrifuge at 450 g for 10 minutes at 4^{o}C, and the supernatant was discarded completely. Seven hundred microliter (µl) RLT Buffer with beta-mercaptoethanol was added to pelleted leukocytes

and vortex to mix. The lysate was pipetted directly into a QIAshredder spin column in a 2 ml collection tube and centrifuged for 2 minutes at maximum speed to homogenize. QIAshredder spin column was discarded and homogenized lysate was saved. A volume of 700 µl of 70% ethanol was added to the homogenized lysate and mixed by pipetting. Sample was pipetted carefully into a new QIAamp spin column in a 2 ml collection tube without moistening the rim. The spin column was then centrifuged for 15 seconds at 8,000 g. The QIAamp spin column was transferred into a new 2 ml collection tube. RW1 Buffer 700 µl was applied to the QIAamp spin column and centrifuged for 15 seconds at 8,000 g to wash. QIAamp spin column was placed in a new 2 ml collection tube. RPE Buffer 500 µl was pipetted into the QIAamp spin column and centrifuged for 15 seconds at 8,000 g. The QIAamp spin column was opened carefully and 500 µl of RPE buffer was added. The cap was closed and centrifuged at 15,000 g for 3 minutes. The QIAamp spin column was placed in a new 2 ml collection tube and the old collection tube with the filtrate was discarded. The spin column was centrifuged at full speed for 1 minute. QIAamp spin column was transferred into a 1.5 ml microcentrifuge tube and 50 µl of RNase-free water was pipetted directly onto the QIAamp membrane and centrifuged for 1 minute at 8,000 g to elute the RNA. Total RNA concentration was quantitated by using NanoDrop N1000 spectrophotometer.

3.5 First-Strand cDNA synthesis

In order to reverse transcribe total RNA (1 µg) into its complementary DNA strand (cDNA) using SuperScript® II Reverse Transcriptase kit (Invitrogen, USA), a total volume of 15 µl consisting of 1 µl of random primers (500 µg/ml) and 1 µg of RNA was prepared. Sterile nuclease free water was added to make final volume up to

15 µl. The mixture was then incubated in a thermal cycler at 70^{o}C for 5 minutes and quick chilled at 4^{o}C.

While waiting for the incubation, a master mix cocktail was prepared by adding the following reagents to the final volume of 24 µl; 8 µl of 5X First-Strand buffer, 4 µl of 0.1M DTT, 4 µl of 10 mM dNTP,1 µl of recombinant RNasin® ribonclease inhibitor (40 U/µl), and 7 µl of sterile nuclease free water. The tubes were collected after a quick chill. One microliter of 200 U/µl of superscript® II Reverse Transcriptase and 24 µl of master mix cocktail was then added into each incubated tube to make the final volume to 40 µl. The reaction tubes were then heated at 42^{o}C for 1 hour, followed by 90^{o}C for 5 minutes and 4^{o}C until the tubes were collected. The tubes were stored at -20^{o}C until future use.

3.6 PCR of cDNA

A set of primer covering *actin* gene was used as a housekeeping gene to show presence of cDNA template before proceeding with amplification of *BTK* gene. The sequence of primer set used to amplify *actin* gene is shown in Table 3.1.

When there was presence of PCR products as amplified by the *actin* primer set, the similar cDNA template was used for *BTK* gene amplification. The *BTK* gene was amplified from the cDNA with seven overlapping PCR primer sets which covered all the 19 exons of *BTK* gene (as shown in Table 3.2) using PCR conditions described previously (Hashimoto et al., 1996). These seven sets of primers were used to screen for *BTK* gene mutations at the cDNA level. The normal sequence of 19 exons of human *BTK* gene with highlighted primer sets is shown in Figure 3.1.

The PCR was performed in a volume of 50 µl consisting 1 µl cDNA, 4.5 µl of 25 mmol/L $MgCl_2$, 4 µl of 10x PCR buffer, 1.25 µl (10 µM) of forward primer, 1.25 µl

Table 3.1: Primers used for *actin* gene amplification from cDNA.

	Primer name	**5'-3' primer sequence**	**Fragment length**
Forward primer	**ActinF:**	AGCGGGAAATCGTGCGTG	307 bp
Reverse primer	**ActinR**	CAGGGTACATGGTGGTGCC	

(Source: Thomas, Samanta, & Fikrig, 2008)

Table 3.2: Primers used for *BTK* gene amplification from cDNA.

Primer set	Forward primer/Sense primer (5'-3') / Reverse primer/ Antisense Primer(5'-3')	Fragment position*	Fragment length
1	**BTK1F:** AGCTACCTGCATTAAGTCAG **BTK3R:** CTTCTCGGAATCTGTTTTC	137-438	302 bp
2	**BTK3F:** CACTTGTGTTGAAACAGTGG **BTK7R:** TCCGGTGAGAACTCCCAGGT	376-740	365 bp
3	**BTK6F:** ATGCTATGGGCTGCCAAATT **BTK10R:** TTTAGCAGTTGCTCAGCCTG	675-1080	406 bp
4	**BTK9F:** GTATGAGTGGTATTCCAAAC **BTK14R:** GGTCCTTTGGATCAATTTCC	1029-1397	369 bp
5	**BTK13F:** GCAGGCCTGGGATACGGATC **BTK15R:** GGTGAAGGAACTGCTTTGAC	1355-1751	397 bp
6	**BTK15F:** ATGGCTGCCTCCTGAACTAC **BTK17R:** TGTCAGATTTGCTGCTGAAC	1629-1931	303 bp
7	**BTK17F:** CGGAAGTCCTGATGTATAGC **BTK19R:** CAAGAAGCTTATTGGCGAGC	1890-2193	304 bp

* The numbering of the nucleotide position follows the nucleotide numbering of the reference cDNA sequence with Genebank reference NM_000061.2 (mRNA)

(Source: Hashimoto et al., 1996)

Figure 3.1: The normal sequence of 19 exons of human *BTK* gene with highlighted primer sets. The sequence shown was obtained from Genbank reference NM_000061.2 (cDNA). Single line denotes the boundaries of each exon.

(10 µM) of reverse primer, 2 µl of 10 mM dNTP mixture, 0.25 µl of GoTaq® Flexi DNA polymerase (5 U/µl) (Promega, USA) and sterile nuclease free water added to make final volume up to 50 µl. The mixture was denatured at 94^{o}C for 3 minutes, followed by 33 cycles at 94^{o}C for 30 seconds, at 54^{o}C for 30 seconds, and at 72^{o}C for 40 seconds, then with a final extension at 72^{o}C for 8 minutes. The samples were kept at 4^{o}C prior to gel electrophoresis.

3.7 Leukocyte isolation and DNA extraction

Leukocytes were separated from 5 ml of EDTA blood using Ficoll Hypaque (Lymphoprep^{TM,} Axis-shield, Norway) and the DNA was then extracted using the QIAamp DNA Blood mini kit (Qiagen GmbH, Hilden, Germany).

3.7.1 Leukocyte isolation

A volume of 5 ml of EDTA anti-coagulated whole blood was diluted with 5 ml of PBS and mixed well. A volume of 3.5 ml of lymphoprep^{TM} solution (Axis-shield, Norway) was pipetted into two plain tubes, respectively. The diluted blood (5 ml) was layered on the lymphoprep^{TM} solution carefully in each tube and spun at 600 g for 15 minutes. The buffy coat layer which was rich with leukocytes was harvested and then washed with PBS at 400 g for 5 minutes. The supernatant was discarded. The pellet was broken by vortexing. Then, the pellet was washed with PBS at 400 g for 5 minutes. The supernatant was aspirated until 200 µl PBS left in the tube. The tube was vortexed. Approximately 100 µl of PBS was added to the tube to rinse the tube. The tube containing 300 µl of PBS which was rich with leukocytes could be kept in 4^{o}C overnight or proceed with DNA extraction directly.

3.7.2 DNA extraction

Genomic DNA was isolated from the leukocytes of the patient, mother, sister and a normal control, respectively, using QIAamp DNA Blood mini kit, according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). The microfuge containing 300 µl of PBS with leukocytes was spun at 1,000 g for 2 minutes. Approximately 200 µl of the pellet was transferred into a new microfuge tube. A volume of 20 µl protease was added and mixed well with pipette. A volume of 200 µl of AL Buffer was added and vortexed briefly, then incubated at 56^{o}C for 10 minutes. After incubation, 200 µl absolute ethanol was added and vortexed briefly. The sample was transferred into a mini spin column with collection tube and spun at 7,000 g for 1 minute. The flow through was discarded, 500 µl AW1 buffer was added and the column was spun at 7,000 g for 1 minute. Again, the flow through was discarded, then, 500 µl AW2 buffer was added and the column was spun at 20,000 g for 3 minutes. The flow through was discarded, and the spin column was then transferred into a clean microfuge tube. AE Buffer 200 µl was added, followed by incubation at room temperature for 5 minutes and spun at 7,000 g for 1 minute. The spin column was then discarded. The microfuge tube was placed in a dry-bath at 96^{o}C (Eppendorf, USA) for 5 minutes to denature the DNA. Then, the denatured DNA samples were kept on ice prior to DNA concentration and purity estimation. The DNA concentration and purity was determined using NanoDrop N1000 spectrophotometer. The DNA samples were then kept in -20^{o}C for future use.

3.8 PCR of genomic DNA

In order to confirm the mutation found at the cDNA level, genomic DNA was amplified with respective primers which flanking intron-exon boundaries as listed in Table 3.3 using PCR conditions as described previously (Hashimoto et al., 1996).

Table 3.3: Primers used for amplification of the *BTK* gene from genomic DNA.

	Primer name	**5'-3' primer sequence**	***Primer Position**	**Fragment length**
Forward primer	**BTKg9F**	gggaggtgctggatgaactg	31035-31054	138 bp
Reverse primer	**BTKg9R**	cagtcaggtgttagaaggtcc	31152-31172	

* The numbering of the nucleotide position follows the nucleotide numbering of the reference gDNA sequence with Genebank reference NG_009616.1 (gDNA)

(Source: Vorechovsky et al., 1995)

The primers used were positioned in the BTK introns which were just upstream and downstream of the exon-intron boundaries in order to investigate mutation involving invariant splice sites.

The PCR was performed in a volume of 50 µl consisting 200 ng of gDNA template, 4.5 µl of 25 mmol/l $MgCl_2$, 4 µl of 10x PCR buffer, 1.25 µl (10 µM) of forward primer, 1.25 µl (10 µM) of reverse primer, 2 µl of 10 mM dNTP mixture, and 0.25 µl of GoTaq® Flexi DNA polymerase (5 U/µl) (Promega, USA) and sterile nuclease free water added to make final volume up to 50 µl. The mixture was denatured at 94^{o}C for 3 minutes, followed by 33 cycles at 94^{o}C for 30 seconds, at 54^{o}C for 30 seconds, and at 72^{o}C for 40 seconds, then with a final extension at 72^{o}C for 8 minutes. The samples were kept at 4^{o}C prior to gel electrophoresis.

3.9 PCR product purification

Following electrophoresis, PCR product was purified using Wizard® SV gel and PCR clean-up system kit according to the manufacturer's protocol (Promega, USA). Equal volume of PCR product (40 µl) was added to 40 µl membrane binding solution respectively. SV Minicolumn (provided by the kit) was inserted into a collection tube. Prepared PCR product was transferred to the Minicolumn assembly and incubated at room temperature for 1 minute. It was then spun at 16,000 g for 1 minute. The flow through was discarded and Minicolumn was reinserted to the collection tube. Membrane Wash Solution containing ethanol (700 µl) was added and the column was spun at 16,000 g for 1 minute. The flow through was discarded and Minicolumn was reinserted to the collection tube. Membrane Wash Solution containing ethanol (500 µl) was added and the column was spun at 16,000 g for 5 minutes. The collection tube was emptied and the column assembly was centrifuged for 1 minute. This time, the

microcentrifuge lid was open to allow evaporation of any residual ethanol. Minicolumn was then transferred carefully to a clean 1.5 ml microcentrifuge tube. Nuclease-Free Water (50 µl) was added to the Minicolumn followed by incubation at room temperature for 5 minutes. It was then centrifuged at 16,000 g for 1 minute. The minicolumn was discarded. The concentration and purity of the purified PCR products were quantitated using NanoDrop-1000 spectrophotometer. An aliquot of purified products were then sent for DNA sequencing. The remaining purified PCR products were stored at 4^{o}C.

3.10 DNA Sequencing

The purified PCR products were sent to First Base Laboratories (M) Pte Ltd where sequencing was carried out using BigDye Terminator v.3 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). GenBank reference sequence with accession number NM_000061.2 (mRNA) and NG_009616.1 (gDNA) were used as reference for sequence comparison.

3.11 Protein sequence prediction

The cDNA sequences of each study subject were translated into amino acid codon respectively using Expasy translation tool (http://web.expasy.org/translate/). The BTK protein sequences of each study subject were then compared to the reference BTK protein sequences, as described in GenBank reference sequence with accession number AAB64205.1 (protein). Both study subject and the reference protein sequences were aligned using SDSC Biology workbench online application (http*://workbench.sdsc.edu/)* to facilitate the interpretation of the mutation noted in protein level.

4.0 RESULTS

4.1 Patient's serum immunoglobulin measurement history

Patients with antibody deficiency have very low serum immunoglobulin levels, making them susceptible to recurrent infections. In this study, the patient's serum immunoglobulin measurement history was traced back from the test record book of Allergy and Immunology Research Centre, Institute for Medical Research. Table 4.1 shows the history of the patient's serum immunoglobulin levels before and after receiving intravenous immunoglobulin replacement therapy. Few Immunoglobulin levels data were not available because the samples were sent to different laboratories for measurement or the patient did not turn up. Serum samples that were collected and tested prior to the next IVIg infusion were labelled as 'pre-IVIg' level; whereas serum samples collected and tested 24-hour or 48-hour after the IVIg infusion were labelled as 'post-IVIg' level.

Before receiving IVIg treatment, laboratory finding revealed that the patient's serum immunoglobulin levels were lower as compared to that of an age matched child (Table 4.1). He was started on three-weekly IVIg therapy at the age of six years and nine months old. He had low serum IgG level prior to the next infusion during six years and nine months old, 47mg/dL. The serum IgG level started to increase and remained within the normal limit of the reference range. The serum IgG level prior to the next infusion (pre-IVIg) was slightly lower than the respective post-IVIg IgG level. However, his serum IgA and IgM levels before and after receiving IVIg therapy remained lower than lower limit of the reference range.

Table 4.1: History of the patient's serum immunoglobulin levels before and after receiving intravenous immunoglobulin replacement therapy.

Age / Ig level (mg/dL)	6y9m	6y9m		6y11m		7y2m		7y2m		7y3m			**Reference range (mg/dl) 6-9 years old**
Status	Before receiving IVIg therapy	Pre	Post	Pre	Post	Pre	Post (24H)	Pre	Post (24H)	Pre	Post (24H)	Post (48H)	
IgG	41	47	N.D	483	N.D	N.D	1107	754	1244	783	1170	1237	**550-1200**
IgA	48	38	N.D	31	N.D	N.D	39	37	39	41	19	39	**60-170**
IgM	<12	28	N.D	15	N.D	N.D	22	12	19	34	35	25	**40-95**

Note: 'y' represents years; 'm' represents months; 'Pre' represents pre-IVIg; 'Post' represents post-IVIg; 24H represents 24 hours after IVIg infusion; 48H represents 48 hours after IVIg infusion; 'N.D.' represents not determined.

4.2 Lymphocyte subset immunophenotyping

Lymphocyte subset immunophenotyping using flow cytometry was able to determine the absolute count and the percentage values of T-cell subsets (CD3, CD4, and CD8), B-cell (CD19) and natural killer cell (CD16 and CD56). This assay is used as a diagnostic and management tool for screening of primary immunodeficiency patients with defective of any lymphocyte subsets and also monitoring of the treatment progression in acquired immunodeficiency patients. Patients with XLA have less than 2% $CD19^+$ B cells in the peripheral blood (Conley, Notarangelo, & Etzioni, 1999).

Tables 4.2 and 4.3 show the patient's lymphocyte subset immunophenotyping results tested when he was six years and eight months old and seven years and five months old, respectively.

Before receiving IVIg therapy, the patient had no circulating B cells (Table 4.2). After receiving IVIg therapy, he was tested again and the results showed that he had negligible number of B cells that was 7 cells/ µl B cells (Table 4.3). Interestingly, the percentage and absolute count of total T cells of the patient were slightly higher than a normal child with the same age. His CD4 T helper cells were also slightly higher than a child with the same age, but NK cells were slightly lower in the patient (Tables 4.2 and 4.3). This phenomenon could be due to compensation of B cell insufficiency.

4.3 Flow cytometric analysis

In general, XLA patients have less than 2% $CD19^+$ B cells. Due to the insufficient amount of B cells in XLA patients, for further molecular biology analysis, monocytes which are known to express BTK protein were used to determine the BTK

Table 4.2: Patient's lymphocyte subset immunophenotyping was determined at six years and eight months old (pre-IVIg treatment).

	Patient		**Reference range (7-17 years old)**	
	Percentage (%)	Absolute count (cells/ µl)	**Percentage (%)**	**Absolute count (cells/ µl)**
Total B cells ($CD19^+$)	0	0	**21-28**	**700-1300**
Total T cells ($CD3^+$)	94	4293	**62-69**	**1800-3000**
T helper cells ($CD3^+CD4^+$)	64	2923	**30-40**	**1000-1800**
T cytotoxic cells ($CD3^+CD8^+$)	26	1188	**25-32**	**800-1500**
Natural killer cells ($CD16^+CD56^+$)	4	183	**8-15**	**200-600**

Table 4.3: Patient's lymphocyte subset immunophenotyping was determined at seven years and five months old (post-IVIg treatment).

	Patient		**Reference range (7-17 years old)**	
	Percentage (%)	Absolute count (cells/ µl)	**Percentage (%)**	**Absolute count (cells/ µl)**
Total B cells ($CD19^+$)	0	7	**21-28**	**700-1300**
Total T cells ($CD3^+$)	93	3110	**62-69**	**1800-3000**
T helper cells ($CD3^+CD4^+$)	58	1974	**30-40**	**1000-1800**
T cytotoxic cells ($CD3^+CD8^+$)	29	992	**25-32**	**800-1500**
Natural killer cells ($CD16^+CD56^+$)	6	184	**8-15**	**200-600**

protein expression. Futatani et al. (1998) reported the use of intracellular staining of monocyte BTK protein expression to confirm the diagnosis of XLA where patients with XLA have deficient monocyte BTK expression. In this study, BTK expression was analyzed from the gated $CD14^{+}$ stained monocyte population. As mentioned in the methodology section, this antibody used in the experiment was designed to target N-terminal of BTK protein which means that any mutation at the N-terminal of BTK protein could abolish the binding affinity of the antibody to the mutated BTK protein. Mouse IgG2a, K isotype control was used to assess the level of background staining for monocyte BTK expression analysis. Monocyte BTK expression was defined based on isotype control expression. Mean fluorescence intensity (MFI) is represented by the geometrical mean of the gated population, as obtained from the flow cytometric histogram statistical analysis. Figure 4.1 shows the overlay histograms of population stained with isotype control and BTK. The overlapping of BTK peak with isotype control peak represents a negative expression. While a positive expression is represented by a separation between isotype control and BTK peak. In the normal control, MFI value of monocyte isotype control expression was 9. When stained with BTK, only one monocyte population was seen and its MFI value was 39. The result indicated that 98% of the monocytes in normal control expressed BTK protein (Figure 4.1). In contrast, in the patient, MFI value of isotype control expression was 9. When stained with BTK, only one monocyte population with MFI value of 11 was seen. The result indicated that 94% of the monocytes in the patient did not express BTK protein. In the mother, MFI value of isotype control expression was 7. When stained with BTK, two distinct populations were seen. One monocyte population, 62%, had BTK expression with MFI value of 29 whereas another monocyte population, 38%, did not express BTK (MFI = 10). In the sister, MFI value of isotype control was 5. When stained with BTK, a continuous population was seen. In term of percentage, 40% of

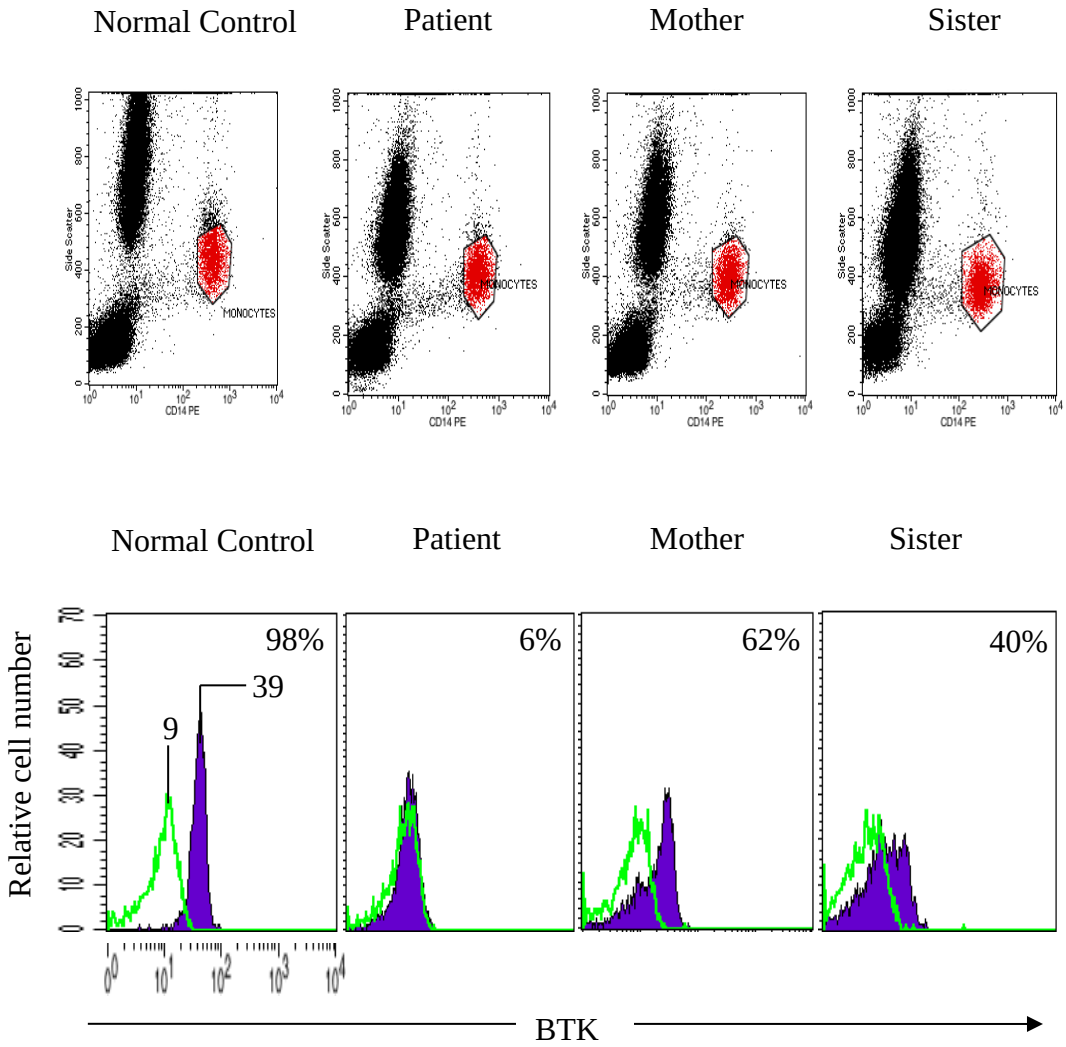

Figure 4.1: Flow cytometric analysis of monocyte BTK expression in a normal control, the patient, his mother and sister. The red gated population represents monocyte population (two thousands events). The green line indicates the monocyte isotype control antibody expression and purple shaded area indicates monocyte BTK expression. MFI values for isotype control expression (9) and BTK expression (39) are shown for normal control's panel. The percentage in the upper right corner indicates the percentage of monocytes expressing BTK protein.

monocytes expressed BTK protein (MFI = 22) and 60% monocytes did not express BTK protein (MFI = 7).

4.4 RT-PCR and sequencing of amplified cDNA products

Mutations in Bruton's tyrosine kinase *(BTK)* gene were known to cause XLA. In order to investigate the gene mutation in the XLA patient, reverse-transcriptase polymerase chain reaction (RT-PCR) of the patient's cDNA with respective *BTK* primers was performed.

Figure 4.2 shows the results of RT-PCR analysis of *BTK* gene using primers listed in Table 3.2. The *BTK* gene was then amplified using seven sets of overlapping primers as described previously (Hashimoto et al., 1996). The PCR reactions using the primer set 1, 2, 4, 5, 6, and 7 (Figure 4.2A) generated fragments that were normal in size as compared to the normal control. All of these products were sequenced and had normal sequence as compared to the normal control. However, PCR products amplified from primer set 3 showed abnormal bands on the 2% agarose gel (Figure 4.2A). Primer set 3 consists of an exon 6 forward primer, BTK6F and an exon 10 reverse primer, BTK10. The patient's cDNA yielded a single shorter product of 343 base pairs (bp), while the normal control's product was 406 bp long. Amplification of the mother and sister's cDNA yielded both products. Sequencing of the cDNA PCR products from the mother and sister confirmed that the 343 bp product was the same as the patient's, while the 406 bp product was the same as the normal control's product. Sequencing showed the patient's cDNA PCR product was shorter due to exon 9 skipping (Figure 4.3). At the meantime, *actin* gene was used as a housekeeping gene to confirm the presence of cDNA template. RT-PCR of cDNA with a set of actin primers generated a 307 bp fragment (Figure 4.2B).

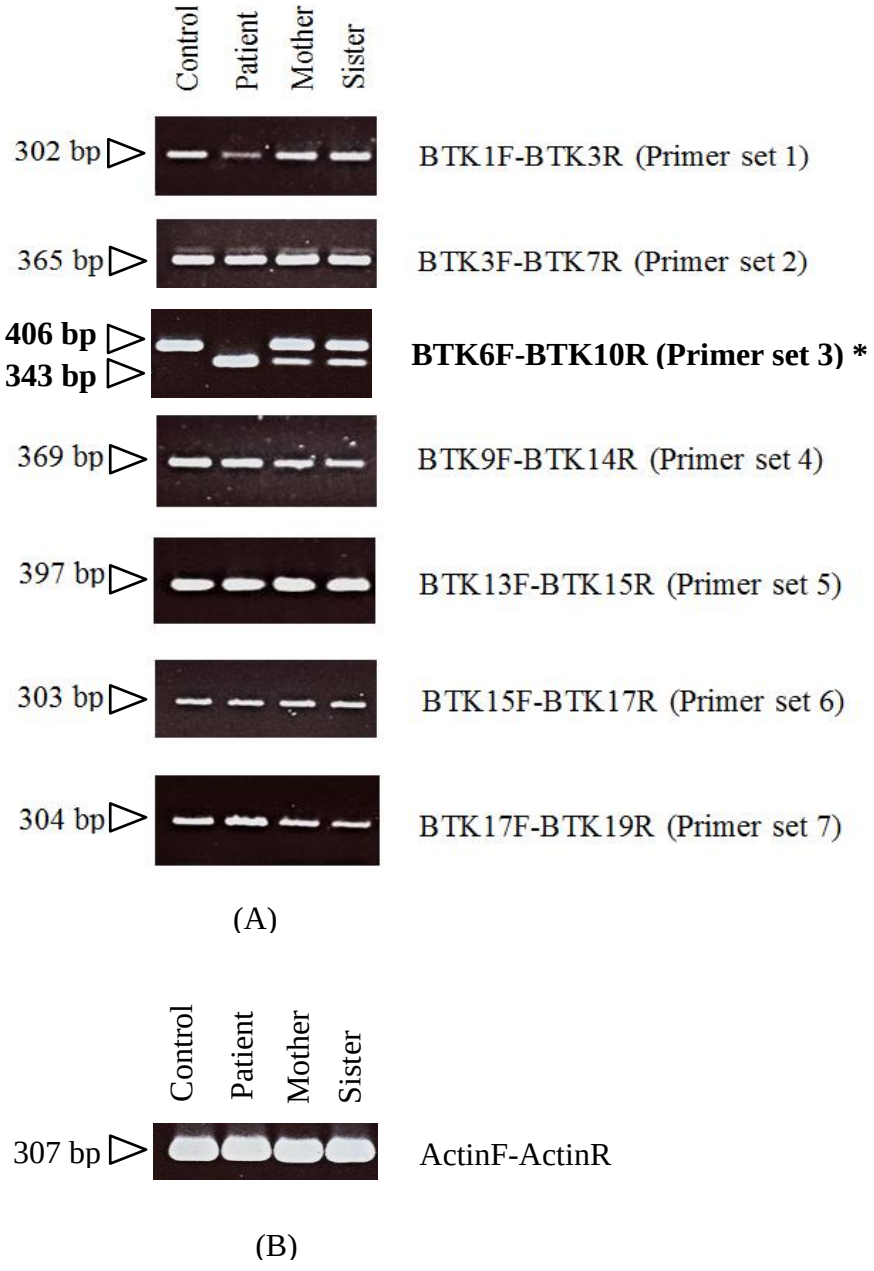

Figure 4.2: Agarose gel electrophoresis of RT-PCR products.
(A) *BTK* gene fragment amplified by seven sets of overlapping primers. Six fragments were normal in size. * Asterisk indicates the fragments amplified by primer set 3, BTK6F-BTK10R, showing products with different length in patient, mother and sister.
(B) *actin* gene fragment.

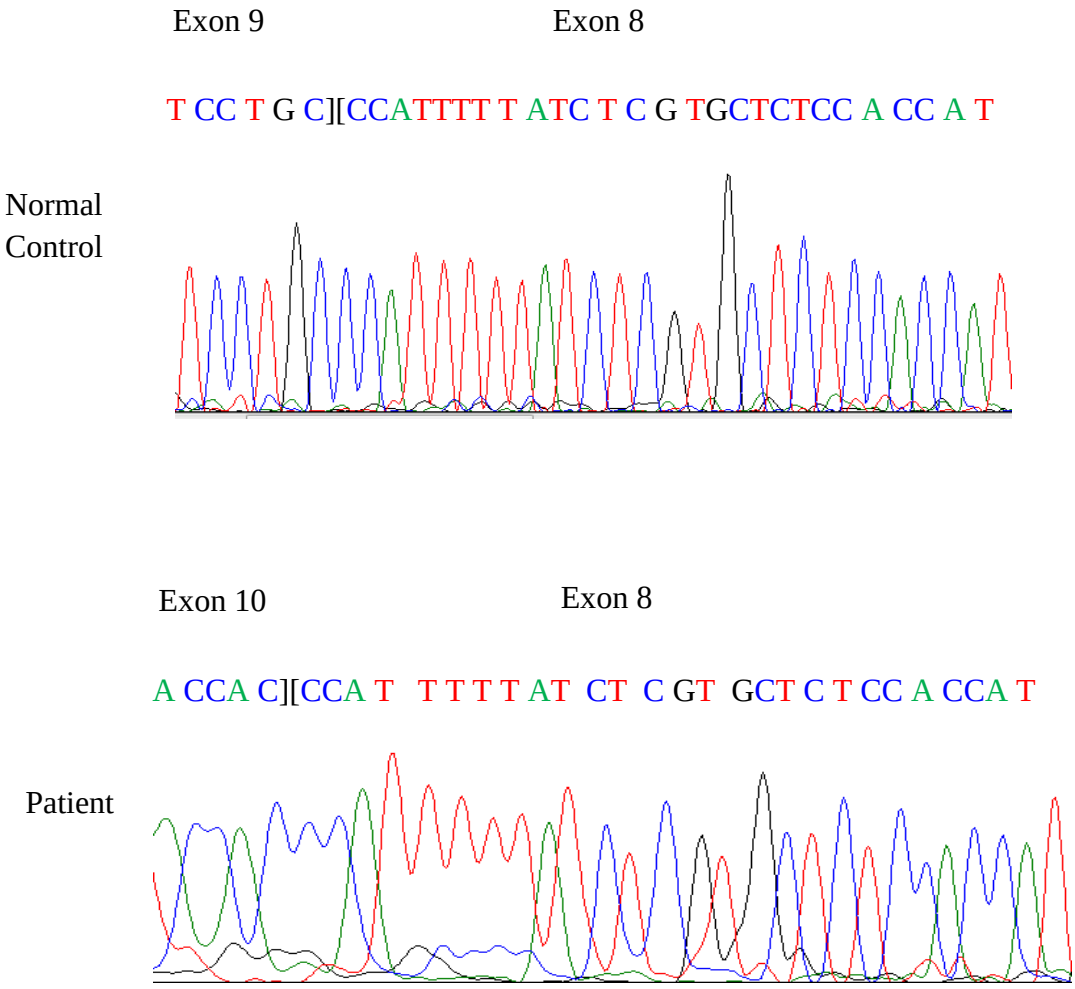

Figure 4.3: Direct sequence analysis of RT-PCR products from a normal control and the patient. Reverse sequence is shown.

4.5 PCR and sequencing of amplified genomic DNA products

The patient had an exon 9 skipping of the *BTK* gene in the cDNA level. In order to confirm the mutation and clarify the mechanism of exon skipping, amplification of genomic DNA from the study subjects was performed using a set of primers covering the relevant exon-intron boundaries. The forward primer used in this assay was located in intron 8 whereas the reverse primer was located in intron 9 respectively, which covered exon 9 and both invariant splice sites (Table 3.3). The direct sequencing of the PCR product amplified by this primer set revealed that the first nucleotide of intron 9 had changed from G>C, in the patient as compared to the normal control (Figure 4.4). However, his mother and sister showed both G and C nucleotides at that position (Figure 4.4), reflecting that they are heterozygous at this locus. This finding confirmed that they were carriers.

4.6 Protein sequence prediction

The sequencing results from the PCR products amplified by seven sets of overlapping primers were compiled and the complete coding sequences were translated into its respective amino acid by online Expasy nucleotide Translate tool (http://web.expasy.org). Both amino acid sequences were aligned using SDSC biology workbench programme (http://workbench.sdsc.edu).

Complete *BTK* gene coding sequences of the normal control coded for a protein of 659 amino acid residues, whereas the patient's complete *BTK* gene coding sequences coded for a protein of 638 amino acid residues. Alignment result shows the patient had a deletion of 21 amino acids, which were from 260^{th} amino acid residue until 280^{th} amino acid residue (Figure 4.5). This twenty one amino acid residues deletion

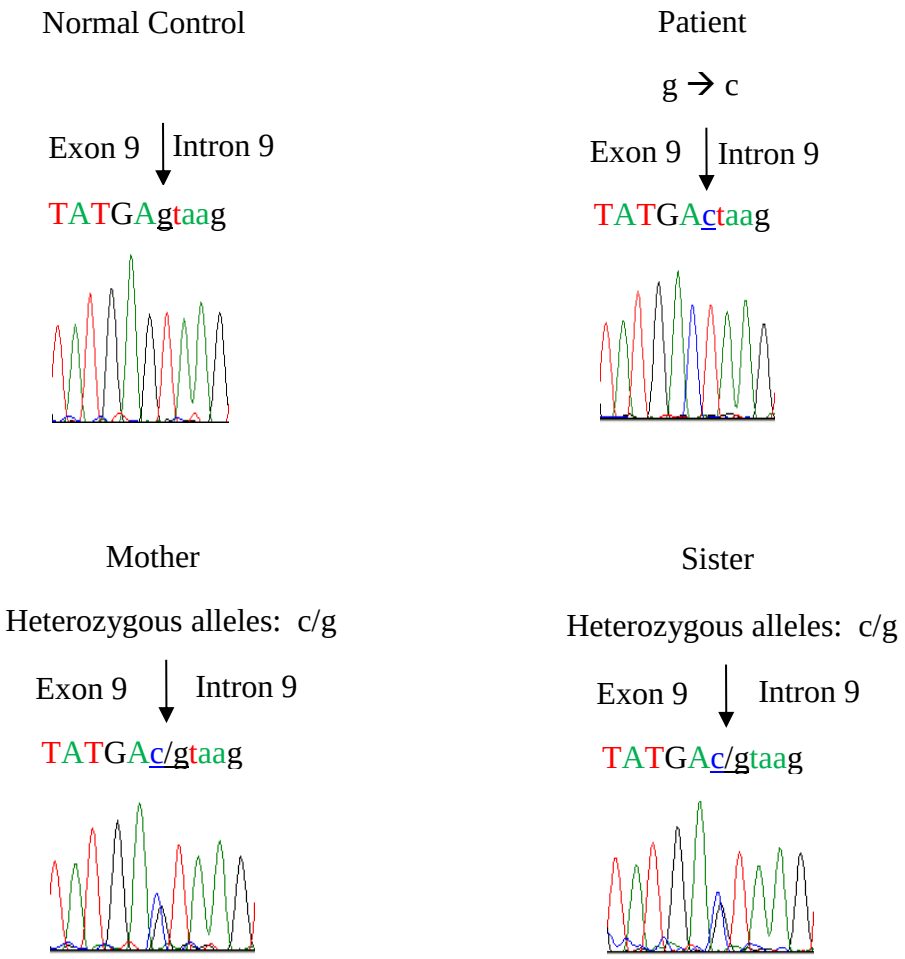

Figure 4.4: Direct sequence analysis of genomic DNA from a normal control, the patient, his mother and sister. Forward sequence is shown. Capital letters represent the sequence of exon 9 and small letters represent the intron 9 sequence. The underlined nucleotides represent the mutation point.

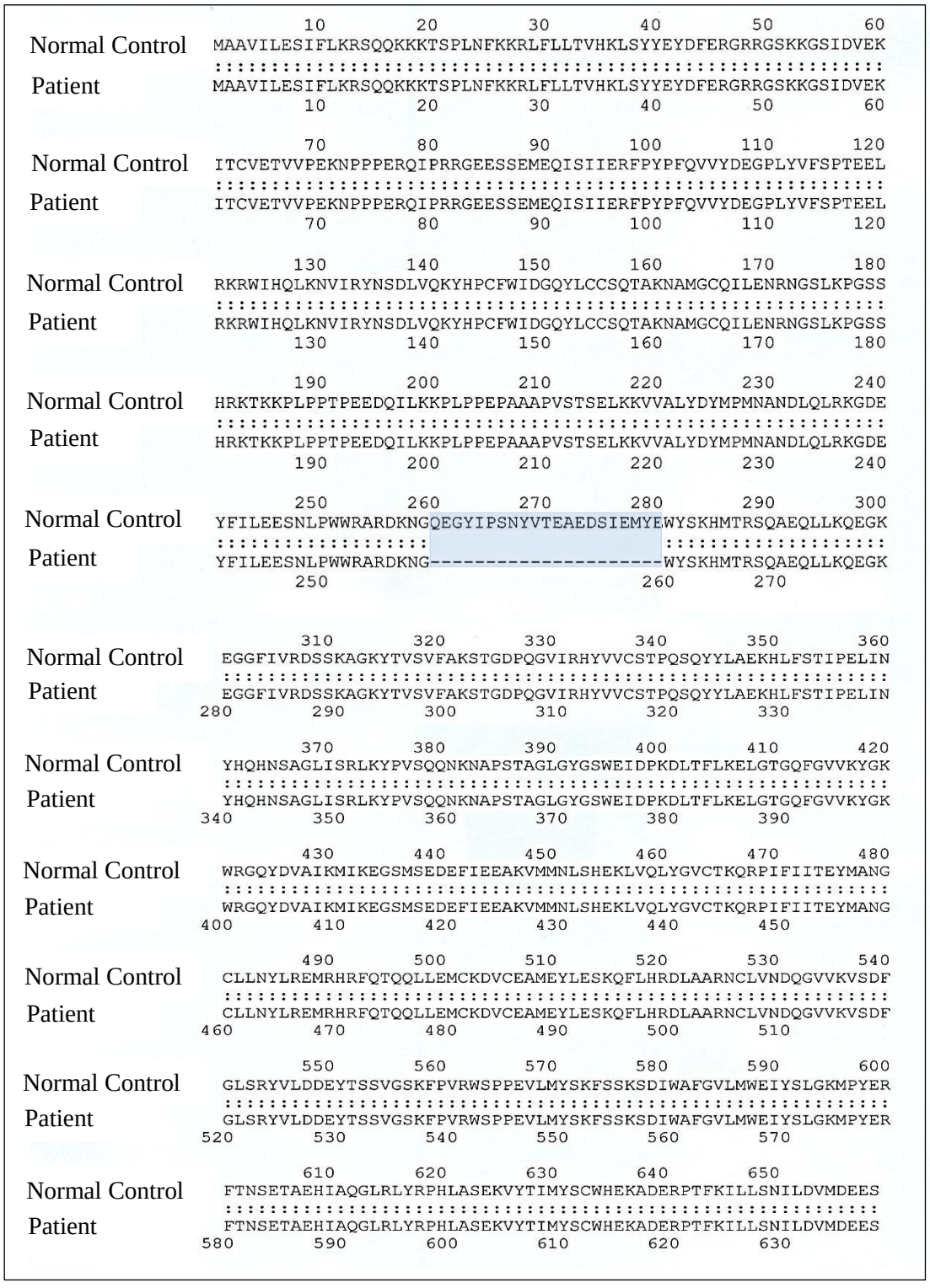

Figure 4.5: Comparison of amino acid sequences between a normal control and the patient. Alignment that is marked by ':' indicates an identical residue in the alignment, while alignment that is unmarked indicates a mismatch or deletion in the alignment.

corresponded to the skipping of exon 9. Among these twenty one amino acid residues, nine of these amino acids are located at SH3 domain of the BTK protein (Figure 4.6).

4.7 Pedigree of the patient

Figure 4.7 shows the pedigree of the patient. The patient was the youngest child of a non-consanguineous marriage. He had an elder sister. His mother and sister appeared healthy; both of them were recruited in this study. Besides that, he had also two maternal aunts who appeared healthy but were inaccessible for this study. However, his mother reported that two maternal uncles died at below two years of age, due to high fever with unknown causes.

The carrier status of the mother (II-3) and sister (III-1) in this family was ruled out and confirmed in this study by the combination of flow cytometric assay (Figure 4.1) and genetic study (Figures 4.2 and 4.4).

Figure 4.6: Human BTK protein sequence from Swiss-Prot database with accession number Q06187. Each domain is boxed and numbered as (I) PH domain, (II) TH domain, (III) SH3 domain, (IV) SH2 domain, and (V) SH1 domain, as defined from Pfam database. The skipped exon 9 which corresponds to 21 amino acid residues is bolded.

(Source: Bateman et al., 2002; Boeckmann et al., 2003)

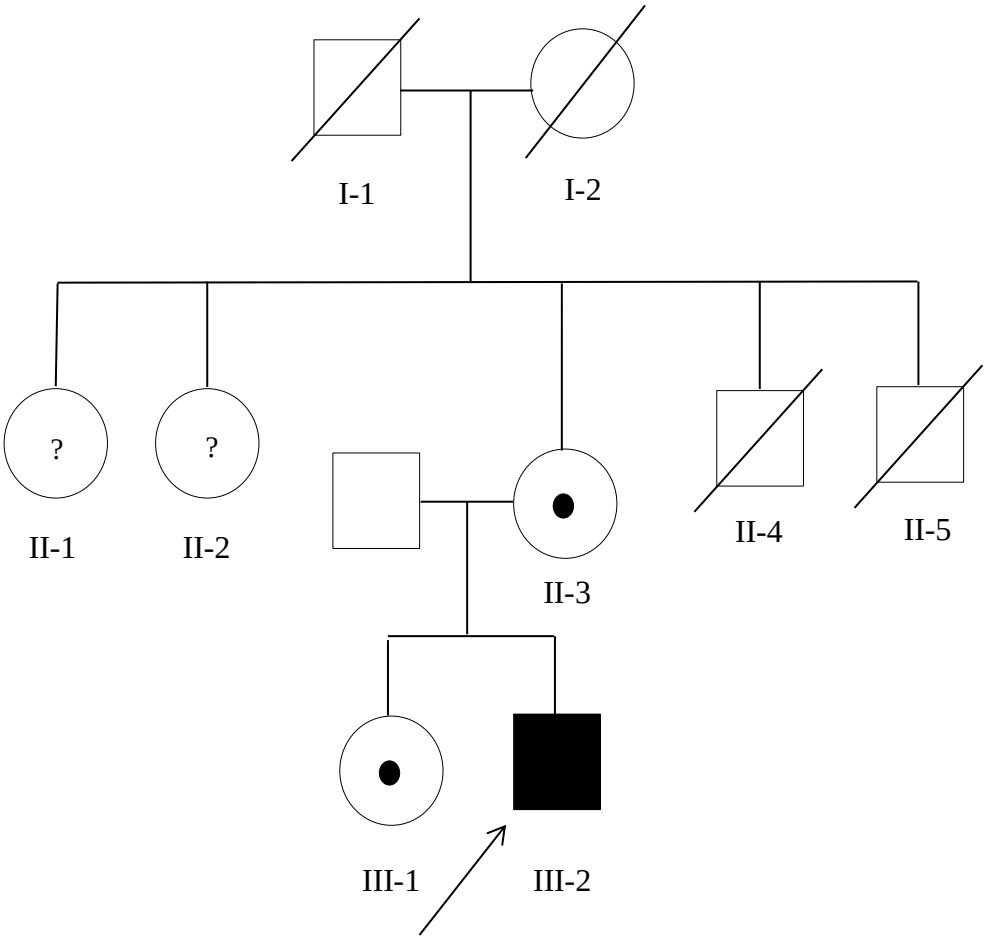

Figure 4.7: The pedigree of the patient with XLA (III-2). Boxes represent men and circles represent women. Shapes with slashes represent deceased individuals. Shapes with a question mark represent individuals not accessible to the study. Open shapes represent healthy individuals. Solid shape represents affected patient. Shapes with a dot represent carriers.

5.0 DISCUSSION

Bruton's agammaglobulinemia was firstly described by Bruton in 1952 (Bruton, 1952). Bruton's agammaglobulinemia is also known as X-linked agammaglobulinemia (XLA). XLA is a classical type of B cell defects. Majority patients with XLA tend to manifest symptoms at the age of 12 months, ranging from six to fourteen months, as the maternally acquired immunoglobulins diminish (Lobo et al., 2003). In this study, the patient had a history of recurrent pyogenic infections since he was one year old.

In view of low serum immunoglobulin levels and recurrent infections, three-weekly IVIG replacement therapy was started in the patient. The main source of immunoglobulin replacement therapy is human IgG obtained from pools of plasma of healthy blood donors (Maarschalk-Ellerbroek et al., 2011). Most products contain no IgM and small amount of IgA. IgM in an intravenous antibody replacement therapy, particularly, may form large complexes resulting in adverse reactions. The purified products, therefore, contains more than 95% IgG. The IgG repertoires are thought to reflect the immunity produced by previous exposure of various pathogens in the donors' blood (Maarschalk-Ellerbroek et al., 2011). Therefore, after immunoglobulin replacement therapy initiation, the patient's serum IgG level was boosted up until achieving normal values, but not for serum IgM and IgA levels.

The patient's pre-IV IgG level was measured immediately prior to IVIg infusion whereas post-IV IgG level was measured after 24 hour or 48 hour of IVIg infusion as described in section 4.1. Based on the patient's serum immunoglobulin measurement history as described in section 4.1, the patient's post-IV IgG level was higher than those prior to the next infusion. As shown in Table 4.1 (section 4.1), after receiving intravenously administrated IgG, the infused IgG enters the bloodstream directly, hence, when measured, the serum IgG level increases drastically. This serum IgG level

measured is denoted by post-IVIg IgG level in this study (Table 4.1). Then, the IgG redistributes rapidly into tissue compartments, and then is catabolized slowly, therefore the serum IgG level is lower at the measuring time (Bonilla, 2008). At this time, the serum IgG level measured is denoted by pre-IVIg IgG level (Table 4.1). When the serum IgG level is lower than lower limit of protective value, the patient are susceptible to infections, therefore, another dose of IgG infusion will be needed. The appropriate dose of immunoglobulin replacement therapy for antibody deficient patient is determined by the IgG trough level, the median half-life of IgG product and the intrinsic metabolism of the patient (Bonilla, 2008). Waldmann, Strober and Blaese (1970), as quoted by Bonilla (2008) reported that the mean physiological catabolism half-life of IgG is about 23 days in healthy adults. Therefore, three weekly of immunoglobulin replacement therapy is sufficient to replenish the IgG and protect the patient from infections. Since receiving IVIg therapy, the patient remained well and suffered no further recurrent infections.

BTK protein is a cytoplasmic tyrosine kinase that plays crucial role in B cell proliferation and differentiation. Other than B cells, BTK is also expressed in monocytes (Futatani et al., 1998) and platelets (Futatani et al., 2001), but it is down-regulated in T cells, natural killer cells and neutrophils (Futatani et al., 1998). In this study, the patient had no circulating B cells. To study the BTK protein expression in this patient, monocyte BTK protein expression was evaluated using flow cytometric analysis. Flow cytometric analysis is a rapid and sensitive method to analyze monocyte BTK protein expression and has been widely used to diagnose XLA patients and to identify female carriers (Futatani et al., 1998; Kanegane et al., 2001; López-Granados et al., 2005).

Intracellular staining of monocyte BTK expression with anti-BTK monoclonal antibody 48-2H by flow cytometry was firstly introduced by Futatani et al. (1998) and

successfully demonstrated deficient BTK protein expression in XLA patients. However, the monoclonal antibody clone used in the study was from a different clone, 53/BTK. This clone was designed to target N-terminal of BTK protein, as stated by the manufacturer. In this study, the genetic results showed that the patient had an invariant splice site mutation which caused a skipping of exon 9. Zhu et al. (1994) reported an invariant splice site mutation at the same locus, but the base substitution was G>A, instead of G>C as identified in this study. Zhu et al. (1994) also reported that the IVS9+1G>A mutation caused exon 9 skipping which resulted in an in-frame deletion of 21 amino acids and a truncated BTK protein. Hence, it is postulated that the point mutation seen in the studied patient would result in a truncated BTK protein with 21 amino acids deletion as well. Nine of these amino acids are located in the SH3 domain of the BTK protein (Figure 4.6). The truncated mRNA expression was identified in the patient (Figure 4.2). However, the truncated protein expression was unable to be identified (Figure 4.1). The antibody used in the experiment was unable to bind to the truncated protein even if N-terminal was assumed to be remained intact to the protein in the patient. The inability of the antibody to bind to truncated protein was reflected by the flow cytometric result, in which the studied patient's monocytes were not stained with the antibody in spite of the truncated BTK protein was expressed as reported by Zhu et al. (1994). The 6% monocytes BTK expression shown by flow cytometry in this study might be a non-specific interaction between the monoclonal antibodies and the targeted antigens.

Monocyte BTK expression was defined based on isotype control using flow cytometry. A carrier usually displays a bimodal BTK expression pattern of positive population and negative population in her monocytes (Futatani et al., 1998; Kanegane et al., 2001), but the ratio of these two populations varies among the female carriers (Futatani et al., 2001). Interestingly, both mother and sister from the studied family

showed variant BTK expression patterns, instead of a classic bimodal BTK expression (Figure 4.1). X-chromosome inactivation could be a possible explanation for the lack of classic bimodal distribution of monocytes' BTK expression in both female carriers in this study. Carrier status detection may be difficult if there is a significant skewing of X chromosome inactivation resulting in the majority of cells being either affected or unaffected. In the case of significant skewing towards normality, carrier detection by sequencing would be essential.

In order to clarify the carrier status, sequencing of *BTK* gene of cDNA and genomic DNA of the patient's mother and sister was performed. Both sequencing results showed that the patient's mother and sister had both normal and mutated alleles. The amplified genomic DNA from the mother and sister showed both G and C nucleotide at the same point, the first nucleotide at the 5' end of intron 9 (Figure 4.4), reflecting that they are heterozygous at this locus. The normal allele with G nucleotide would have normal intron splicing and produced a normal sequence, whereas the mutated allele with C nucleotide would lead to a splicing error and resulted in a skipping of exon 9. In normal phenomenon, one of the X-chromosomes in females will be inactivated randomly, regardless of the parental origin, in order to achieve dosage compensation (Migeon, 2007). In which, some of their cells express the paternal allele which was normal and some express the maternal allele which were mutated allele in this case study. In this study, X-chromosome which carries the G allele, in the mother and the sister, is inherited from their father, whereas, the mutant allele, C allele, is inherited from their mother. This mutant allele is the disease-causing allele, which is inherited from the mother. Random inactivation happened in both mother and sister, whereby the X-chromosome which carried mutant allele was inactivated in some of their cells. And, the other cells expressed X-chromosome which carried normal allele.

This cellular mosaicism resulted from X-chromosome inactivation protects them from suffering from disease. Therefore, they are carriers and would appear healthy.

In this study, the mutation IVS9+1G>C, as seen in the patient, resulted in a skipping of exon 9 (Figure 4.3), which lead to a missing of 21 amino acids. Of all these 21 amino acid residues, 260th residue to 268th residue, were located in the SH3 domain of BTK protein (Figure 4.6). Zhu et al. (1994) predicted that these amino acid residues were crucial for the formation of the putative SH3 ligand-binding pocket. SH3 domain is involved in protein-protein interaction (Gaspar & Kinnon, 2001). Therefore, disruption of this domain would affect the binding ability of BTK to its target. Even if the functional domain remains intact, the alteration in the BTK binding capability to its target leads to the disease in the patient.

In the aspect of immunological findings, the patient had no circulating B cell (Table 4.2), interestingly, he had a very low but detectable level of serum IgA, IgM and IgG before started on IVIg replacement therapy (Table 4.1). This interesting finding was consistent with few studies in the literature, in which patients with XLA had virtually absence of circulating B cell in the peripheral blood, but they had very low serum IgA, IgM and IgG level remained in the blood at diagnosis and before receiving therapy (Lobo et al., 2003; Wang et al., 2009; Zhang et al., 2010). Nonoyama et al. (1998) reported that in XLA patients there was a population of B cells that were able to proliferate, undergo isotype-switching and differentiate into specific antibody producing cells. They termed these B cells as leaky B cells. However, the origin of these leaky B cells is still to be determined. These observations raised a few questions: What would be the origin of these leaky B cells? Can a splice site mutated gene produce wild-type transcript? Interestingly, Noordzij et al. (2002) reported that patients with splice site mutation were able to produce low levels of wildtype BTK transcripts that were enough for a small amount of B cells development in these patients. Similar observation was

found in the studied patient who had an IVS9+1G>C splice site mutation (Figure 4.4), and as such it is postulated that the same phenomenon may occur leading to the development of few leaky B cells that were mature enough to produce serum immunoglobulin. Although the mechanism that leads to the production of wild type transcript from splice site mutation remain to be unveiled, Noordzij et al. (2002) produced evidence of wild type *BTK* transcript existence from splice site mutated *BTK* gene. However, the origin of the leaky B cell is still unknown.

Sequencing result of the genomic DNA revealed that the patient had a base change from nucleotide G to C, in the splice donor site, the first nucleotide at the 5' end of intron 9 in the *BTK* gene (Figure 4.4). The dinucleotide, GT, at the 5' end of the intron and dinucleotide, AG, at the 3' end of the intron are invariant splice sites and critical for a proper splicing of exon and intron. Mutations in any nucleotide in these splice sites may lead to splicing errors, such as exon skipping, intron inclusion or activation of a proximal or distal cryptic splice site (Noordzij et al., 2002). Point mutations affecting nucleotide 'G' of invariant splice donor site of intron 9, IVS9+1G>A, which resulted in exon 9 skipping and severe phenotype, have been described previously (Futatani et al., 2001; Zhu et al., 1994). Compared to these reported mutations, the patient in this study had a different base substitution in the first nucleotide of intron 9, and the mutation was IVS9+1G>C (Figure 4.4), which also resulted in a skipping of exon 9 (Figures 4.2 and 4.3). Apart from the mutation described here, a base change or deletion of nucleotides 3' downstream of the invariant donor splice site of intron 9, c.839+4A>G or c.839+(4_7)delAGTA, has also been described elsewhere, resulting in either a frame-shift mutation (E280fsX281) or a skipping of exon 9 (Lee et al., 2011; Tóth et al., 2009; Velickovic, Prasad, Weston, & Benson, 2004). Other reports described the mutation c.838delGAGT involving exon 9 and intron 9 resulted in either a frame-shift mutation (E280fs) (Danielian et al., 2003) or

a skipping of exon 9 (Mesci et al., 2006). Based on the study and reports as mentioned above, invariant splice donor site of intron 9 which is IVS9+1G could be a hotspot for *BTK* gene mutation.

The common clinical features seen in the XLA patients with invariant splice donor site of intron 9 were recurrent pyogenic infections since young age, recurrent otitis media, bronchiectasis, low level of serum immunoglobulin and low B cells. But, they might have other clinical features that may be related to this type of mutation. The patient with mutation IVS9+1G>A, as described by Zhu et al. (1994), had chronic sinusitis and severe arthritis involving multiple joints; whereas the patient in this study had bronchopneumonia, asthma, failure to thrive and lower serum IgG level. Allergy and asthma are very common in children, accounting for 155 million individuals worldwide (Cookson & Moffatt, 2000), however, asthma is rarely reported in XLA patients. A study demonstrated that helper T cells of six XLA patients predominantly produced type-1 cytokine such as IFN-ɣ upon mitogen or antigen stimulation, instead of type-2 cytokine (Amedei et al., 2001). Th1-type cytokine such as interferon gamma facilitates the production of opsonizing and complement-fixing antibodies by B cells, promotes cell-mediated immunity, macrophage activation and phagocytosis (Amedei et al., 2001; Ghadimi, de Vrese, Heller, & Schrezenmeir, 2010; Romagnani, 2008), while Th2-type cytokines contribute to asthma and allergy. Amedei et al. (2001) described that XLA patients' helper T cells tend to have bias towards Th1 response than Th2 response hence it might protect them from allergy and asthma. In the aspect of genetics, it has been reported that polymorphism or mutations in particular genes located at chromosomes 5, 6, 10, 11, 14 and 16 were associated with asthma phenotype (Hall, 1998). A more recent review paper suggested that the most consistent loci responsible for asthma and allergic disease are located on chromosomes 5, 6, 12 and 13 (Cookson & Moffatt, 2000) and none of the candidate genes is located in X-chromosome. Based on

this information, it would be difficult to correlate asthma and XLA that was observed in this case study. However, there was a report by Shabestari and Rezaei (2008) describing an XLA patient with asthma and allergic rhinitis. They postulated that the T cell responses of that patient might be preferentially skewed to the Th2 pattern. Th-2 type cytokines such as IL-4, IL-5, IL-6, IL-9 and IL-13 are involved in recruitment and survival of eosinophils, growth and degranulation of mast cells and basophils, induction of B cell isotype class-switching to IgE production, and inhibition of several functions of macrophages (Berger, 2000; Lloyd & Hessel, 2010; Robinson, 2010; Romagnani, 2008). Type-2 cytokine, in other words, could contribute to asthma and allergy. However, the mechanism still needs to be elucidated. It may be suggestive to include the investigation of the antigen-induced cytokine profile of T helper cells in Malaysian XLA patients in the future study.

Zhu et al. (1994) demonstrated that a patient with a point mutation, IVS9+1G>A, had unaffected kinase activity even though the mutation truncated the BTK protein. The patient described in this thesis had also a point mutation, IVS9+1G>C, at the similar locus. Therefore, it is interesting to assess the kinase activity.

6.0 SUMMARY

In summary, the patient had a novel base substitution in the first nucleotide of intron 9, and the mutation was IVS9+1G>C. Flow cytometric assay revealed that the patient lacked BTK protein expression in his monocytes, speculating that the absence of BTK expression in the B cells as well. Absence of B cell BTK expression resulted in very low circulating B cells and antibodies, which rendered the patient susceptible to recurrent pyogenic infections, otitis media, bronchopneumonia, asthma, and failure to thrive. Flow cytometric assay revealed that both mother and sister had BTK expression in nearly half of their monocytes. Genetic study revealed that both mother and sister have heterozygous alleles at the similar mutational point as in the patient, confirming that both were carriers. Based on the results obtained from the experiments, objective 1, 2 and 3 that were set out for this study were achieved. This study also determined all the requirements for the setting of a reliable small sample volume from the patient's whole blood to diagnose XLA for clinical usage in the IMR. Overall, this study supports the necessity of the combination of flow cytometry and genetic study in the diagnosis of XLA and the information would be useful for subsequent genetic counselling, carrier detection and prenatal diagnosis.

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APPENDIX

The result of this study was published in an ISI-indexed journal.

The detail of this published research article is as below:

Chear, C. T., Gill, H. K., Ramly, N. H., Dhaliwal, J. S., Bujang, N., Ripen, A. M., & Mohamad, S. B. (2013) A novel Bruton's tyrosine kinase gene (*BTK*) invariant splice site mutation in a Malaysian family with X-linked agammaglobulinemia. *Asian Pacific Journal of Allergy and Immunology*, 31 (4), 320-324.

A novel Bruton's tyrosine kinase gene *(BTK)* invariant splice site mutation in a Malaysian family with X-linked agammaglobulinemia

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Summary

X-linked agammaglobulinemia (XLA) is a rare genetic disorder caused by mutations in the Bruton's tyrosine kinase (*BTK*) gene. These mutations cause defects in early B cell development. A patient with no circulating B cells and low serum immunoglobulin isotypes was studied as were his mother and sister. Monocyte BTK protein expression was evaluated by flow cytometry. The mutation was determined using PCR and followed by sequencing. Flow cytometry showed the patient lacked BTK protein expression in his monocytes while the mother and sister had 62% and 40% of the monocytes showing BTK protein expressions respectively. The patient had a novel base substitution in the first nucleotide of intron 9 in the *BTK* gene, and the mutation was IVS9+1G>C. This mutation resulted in exon 9 skipping. This defect rendered the patient susceptible to asthma, failure to thrive, recurrent pyogenic infections, otitis media and bronchopneumonia. His mother and sister were heterozygous for this mutation. The combination of flow cytometry and genetic study is necessary in the diagnosis of X-linked agammaglobulinemia and may be used for subsequent genetic counseling, carrier detection and prenatal diagnosis. *(Asian Pac J Allergy Immunol 2013;31:320-4)*

Key words: *BTK* gene, splice site mutation, X-linked agammaglobulinemia

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Introduction

X-linked agammaglobulinemia (XLA) is a rare disease with an estimated prevalence of 1 in 200,000 live births.[1] It is caused by mutations in the Bruton's tyrosine kinase (*BTK*) gene[2] which block the differentiation of pre-B cells into circulating, mature B cells and plasma cells. Affected males have normal number of pre-B cells in the bone marrow.[3] However, they have low or absent circulating B cells and markedly reduced immunoglobulin isotypes in the serum,[4] which renders them susceptible to recurrent pyogenic bacterial infections.[3] Hence, immunoglobulin replacement therapy via intravenous or subcutaneous route must be given to patients with XLA to protect them from severe recurrent infections.[5]

The definitive diagnostic criteria for XLA are male patient with less than 2% circulating CD19+ B cells along with either a mutation in *BTK* gene; absent *BTK* mRNA in neutrophils or monocytes; absent BTK protein in monocytes or platelets; or a positive family history.[6] Probable diagnostic criteria of XLA are early onset of recurrent bacterial infections, hypogammaglobulinemia, absent isohemagglutinins and/or poor response to vaccines.[6]

The *BTK* gene is located at Xq21.3-Xq22 and codes for a 659 amino-acid protein.[2] This gene comprises 19 exons which span over 37.5kb of genomic DNA.[7] The BTK protein is cytoplasmic tyrosine kinase which is involved in signal transduction for B cell proliferation and development.[7]

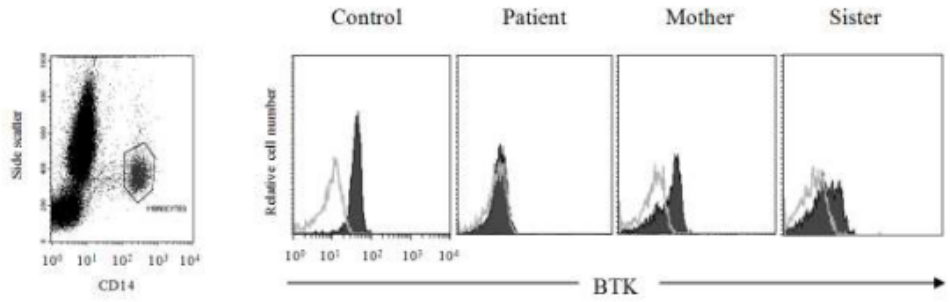

Figure 1. Flow cytometric analysis of monocyte BTK expression in a normal control (Control), the patient (Patient), his mother (Mother) and his sister (Sister). Two thousand events of monocytes were gated from side scatter versus CD14 dot plot. BTK expression was further analyzed in the gated monocytes, as shown in the histograms. The line indicates the isotype control antibody expression and shaded area indicates BTK expression.

Up to 2007, 620 unique mutations from 974 unrelated families have been recorded in a BTK database.[1] These mutations are varied in type and scattered throughout all domains of the BTK protein. They can be categorized as missense mutations, 32%, nonsense mutations, 12%, deletions, 27%, insertions, 8%, splice site mutations, 19%, multiple mutations, 1% and upstream mutation, 1%.[1] However, a genotype-phenotype correlation has not been shown to date for these mutations. Furthermore, there are very few reports describing the clinical features and laboratory findings of Malaysian XLA patients. In this report, we describe a Malaysian XLA patient with a novel *BTK* gene splice site mutation, and the carriers from his family.

Case report

A seven year-old Malay boy with no circulating B cells (CD19+ cells) and low serum immunoglobulin isotypes was studied. He had a history of recurrent pyogenic infections since he was one year old. He had recurrent otitis media and was admitted almost every year for recurrent bronchopneumonia that usually had slow response to antibiotics treatment. At seven years of age, he was admitted to the hospital with another episode of bronchopneumonia, the serial chest X-ray showed recurrent middle lobe consolidation. He also had episodes of wheezing, suggestive of asthma, therefore was started on metered dose inhaler prophylaxis. Physical examination showed a failure to thrive without clubbing but pectus carinatum was observed. Respiratory examination revealed crepitations with minimal rhonchi. No hepatosplenomegaly or enlarged lymph nodes were observed.

Laboratory finding revealed that he had no circulating B cells and his serum immunoglobulin levels (compared to that of an age matched child) were as follows: IgG = 41mg/dL (550-1200mg/dL), IgA = 48mg/dL (60-170mg/dL), IgM = <12mg/dL (40-95mg/dL). Furthermore, high resolution computed tomography of the thorax showed features of early bronchiectasis. Hence, a clinical diagnosis of XLA with bronchiectasis was made. He was then started on three-weekly IVIg therapy. Since then, he remained well and suffered no further recurrent episodes of pneumonia.

The patient's mother and sister appeared healthy. However, two maternal uncles died at below two years of age, due to high fever.

Flow cytometric analysis of monocyte BTK expression in Figure 1 revealed 94% of patient's monocytes did not express BTK protein with a mean fluorescence intensity (MFI) of 11. In contrast, 98% of the normal control's monocytes expressed the BTK protein, MFI=39. Sixty-two percent of the mother's monocytes expressed the BTK protein, MFI= 29, whereas 38% monocytes did not express BTK protein, MFI=10. The sister had 40% of her monocytes expressed BTK protein, MFI = 22, and 60% monocytes did not express BTK protein, MFI = 7.

The *BTK* gene was amplified from the study subjects' cDNA and Figure 2A depicts the RT-PCR products generated with an exon 6 forward primer, Btk 6F, and an exon 10 reverse primer, Btk 10R.[8]

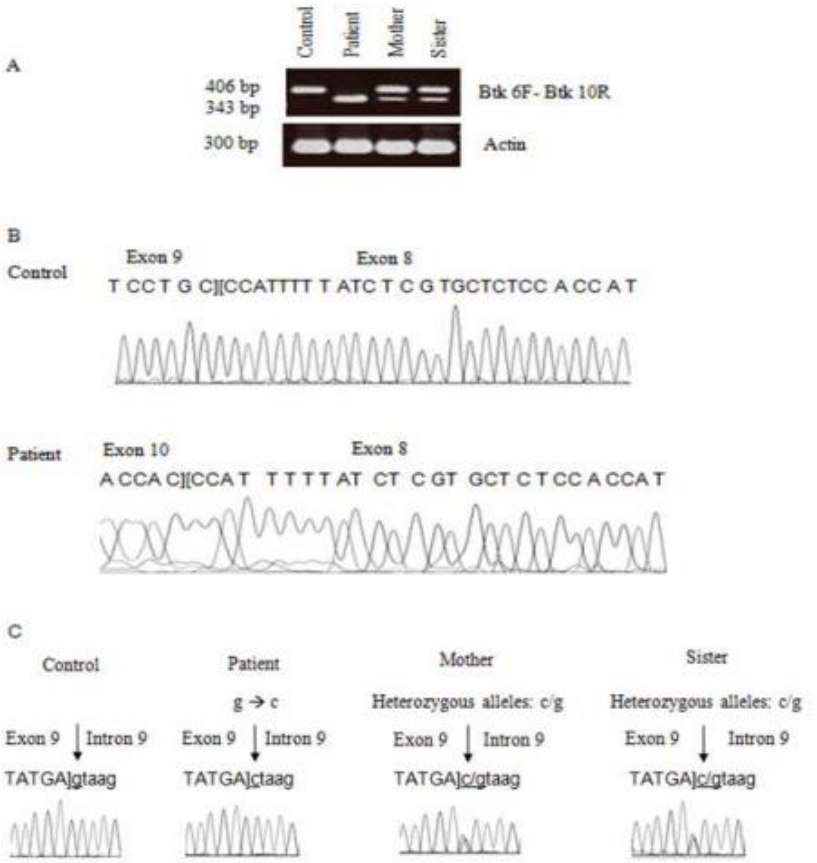

Figure 2. RT-PCR analysis of *BTK* cDNA followed by sequence analysis.
A: RT-PCR products from a normal control, patient, mother, and sister were amplified using a primer set, Btk 6F-Btk 10R, and were visualized on 2% agarose gel. Actin was used as a housekeeping gene.
B: Direct sequence analysis of RT-PCR products from a normal control (Control) and patient (Patient). Reverse sequence is shown.
C: Direct sequence analysis of genomic DNA from a normal control (Control), the patient (Patient), his mother (Mother) and sister (Sister). Forward sequence is shown. Capital letters represent the sequence of exon 9 and small letters represent the intron 9 sequence. The underlined nucleotides represent the mutation point.

The patient's cDNA yielded a single shorter product of 343 base pairs (bp), while the normal control's product was 406 bp long. Amplification of the mother and sister's cDNA yielded both products. Sequencing showed the patient's cDNA PCR product was shorter due to exon 9 skipping (Figure 2B). Sequencing of the cDNA PCR products from the mother and sister confirmed that the 343 bp product was the same as the patient's, while the 406 bp product was the same as the normal control's product. The PCR reactions using the other six primer pairs[8] generated fragments that were normal in size and sequence in all the study subjects.

The mechanism of exon skipping was studied by amplification of genomic DNA from the study subjects using primers located in intron 8 and intron 9 respectively, which covered exon 9 and both invariant splice sites, as described by Vorechovsky et al.[9] Forward sequencing results showed that the first nucleotide of intron 9 had changed from G>C, in the patient as compared to the normal control. However, his mother and sister showed both G and C nucleotides at that position (Figure 2C), indicating they were carriers.

Discussion

BTK is a cytoplasmic tyrosine kinase that plays crucial role in B cell proliferation and differentiation. Other than B cells, BTK is also expressed in monocytes[10] and platelets,[11] but it is down-regulated in T cells, natural killer cells and neutrophils.[10] In this study, our patient had no circulating B cells. To study the BTK protein expression in this patient, monocyte BTK protein expression was evaluated using flow cytometric analysis. Flow cytometric analysis is a rapid and sensitive method to analyze monocyte BTK protein expression and has been widely used to diagnose XLA patients and to identify female carriers.[4,10,12]

Sequencing result of the genomic DNA revealed that our patient had a base change from nucleotide G to C, in the splice donor site, the first nucleotide at the 5' end of intron 9 in the *BTK* gene (Figure 2C). The dinucleotide, GT, at the 5' end of the intron and dinucleotide, AG, at the 3' end of the intron are invariant splice sites and critical for a proper splicing of exon and intron. Mutations in any nucleotide in these splice sites may lead to splicing errors, such as exon skipping, intron inclusion or activation of a proximal or distal cryptic splice site.[13] Point mutations affecting nucleotide 'G' of invariant splice donor site of intron 9, IVS9+1G>A, which resulted in exon 9 skipping and severe phenotype, have been described previously.[11,14] Compared to these reported mutations, our patient had a different base substitution in the first nucleotide of intron 9, and the mutation was IVS9+1G>C (Figure 2C), which also resulted in a skipping of exon 9 (Figure 2B). Apart from the mutation described here, a base change or deletion of nucleotides 3' downstream of the invariant donor splice site of intron 9, c.839+4A>G or c.839+(4_7) delAGTA, has also been described elsewhere, resulting in either a frameshift mutation (E280fsx 281) or skipping of exon 9.[15,16,17] The mutation c.838delGAGT involving exon 9 and intron 9 has been described and also resulted in either a frameshift mutation (E280fs)[18] or a skipping of exon 9.[19] In other words, invariant splice donor site of intron 9 may be a hotspot for *BTK* gene mutation.

The common clinical features of XLA patients with invariant splice donor site of intron 9 are recurrent pyogenic infections since young age, recurrent otitis media, bronchiectasis, low level of serum immunoglobulin and low B cells. They might have different clinical features. The patient with mutation IVS9+1G>A, as described by Zhu et al.,[14] had chronic sinusitis and severe arthritis involving multiple joints; whereas our patient had bronchopneumonia, asthma, failure to thrive and lower serum IgG level. Asthma is uncommon in XLA patients. So far, there is only one report on asthma and allergic rhinitis in XLA patient.[20] It is speculated that patients with IVS9+1G>C might have unusual XLA phenotypes.

In conclusion, our patient had a novel base substitution in the first nucleotide of intron 9, and the mutation was IVS9+1G>C. This mutation resulted in the absence of BTK expression, circulating B cells and antibodies, which rendered the patient susceptible to recurrent infections. The combination of flow cytometry and genetic study is necessary in the diagnosis of XLA and may be used for subsequent genetic counseling, carrier detection and prenatal diagnosis.

Conflict of interest

We declare that we have no conflict of interest.

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