ACIBADEM MEHMET ALI AYDINLAR UNIVERSITY
INSTITUTE OF HEALTH SCIENCES

DEVELOPMENT OF GENE EDITING BASED APPLICATIONS FOR X-LINKED AGAMMAGLOBULINEMIA

MERVE GÜNDOĞDU
MSc. THESIS

DEPARTMENT OF MEDICAL BIOTECHNOLOGY

SUPERVISOR
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DECLARATION

I declare that this thesis work is my own work, I had no unethical behavior at any stages from the planning to the writing of the thesis, I obtained all the information in this thesis in accordance with academic and ethicel rules, I cited all the information and comments that were not obtained with this thesis work, and I provided resources in the list of references. I also declare that there was no violation of any patents and copyrights during the study and writing of this thesis.

25.07.2023

Merve Gündoğdu

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PREFACE AND ACKNOWLEDGEMENT

I would like to dedicate my thesis to my family which are Ali Gündoğdu, Gülizar Gündoğdu and Nur Gündoğdu, who have always supported me financially and morally. Thank you for all your hard work.

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LIST OF ABBREVIATIONS AND SYMBOLS

ARAG	Autosomal Recessive Agammaglobulinemia
bp	Base Pair
BSD	Blasticidin
BTK	*Bruton's tyrosine kinase*
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
ddNTP	Dideoxynucleotidetriphospates
dH_2O	Distilled Water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DSB	Double-Strand Break
ECL	Enhanced Chemiluminescence
EMA	The European Medicines Agency
FBS	Fetal Bovine Serum
FDA	The US Food And Drug Administration
HSC	Hematopoietic Stem Cells
HSPC	Hematopoietic Stem And Progenitor Cells
MART	1α- And B-Chains Of A Melanoma Antigen
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic cid
NHEJ	Non-Homologous DNA End Joining
NSCLC	Non-Small Cell Lung Cancer
P/S	Penicillin-Streptomycin
PAM	Protospacer Adjacent Motif
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pegRNA	Prime Editing Guide RNA
PH	Pleckstrin Homology
PID	Primary Immunodeficiency
PIP3	Phosphatidylinositol 3,4,5-Triphosphate

PVDF	Polyvinylidene Fluoride Membrane
RNA	Ribonucleic Acid
RPMI 1640	Roswell Park Memorial Institute 1640
SCD	Sickle Cell Disease
SCID	Severe Combined Immunodeficiencies
SDM	Site-Directed Mutagenesis
SH2	Src Homology 2
SH3	Src Homology 3
SIN	Self-Inactivating
ssDNA	Single-Stranded DNA
TALEN	Transcription Activator-Like Effector Nuclease
TBS	Tris Buffered Saline
TCR	T Cell Receptor
TH	Tec Homology
TK	Tec Kinase
WPRE	Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element
XLA	X Linked Agammaglobulinemia
ZFN	Zinc Finger Nuclease

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ÖZET

X’e Bağlı Agammaglobulinemi Hastalığına Yönelik Gen Düzenlenmesi Tabanlı Uygulamalar Geliştirilmesi

X'e Bağlı Agammaglobulinemi (XLA), öncelikle erkekleri etkileyen resesif bir hastalıktır. Tekrarlayan enfeksiyonlar, düşük B hücre seviyeleri ve agammaglobulinemi ile karakterizedir. Kesin bir tedavi bulunmamakla birlikte, semptomları kontrol altında tutmak için yaygın olarak immünoglobulin replasman tedavisi kullanılır. Ancak bu tedavi pahalı olabilir ve her zaman etkili olmayabilir. XLA vakalarının yaklaşık %85'ine *Bruton Tyrosine Kinase* (*BTK*) genindeki bir mutasyon neden olur. B hücre proliferasyonu, farklılaşması ve hayatta kalmasında *BTK* rol oynar, ayrıca birincil hümoral bağışıklık savunmasında yer alır. Bu çalışmada, *BTK* mutasyonu nedeniyle oluşan XLA için efektif, yenilikçi ve kişiye özel bir gen tedavisi geliştirilmesi amaçlandı. Buna bağlı olarak klinik bir varyantı 3T3 ve HEK293 hücrelerinde ‘‘site directed mutagenesis’’ ile yeniden oluşturulduktan sonra özgün üçüncü nesil prime editing CRISPR/Cas9 sistemi aracılığıyla değişim tamir edilerek temel bir tedavi uygulaması geliştirilmesi hedeflendi. Transfeksiyon etkinliği düşük olmasına rağmen CRISPR/Cas9 uygulanan hücrelerde küçük bir değişiklik tespit edilebildi. Bu tür nokta mutasyonlarının CRISPR aracılı düzeltilmesinin sadece semptomları iyileştiren standart tedavilerden ziyade kişinin yaşam süresini ve kalitesini artıracağı sonucuna varılabilmektedir. Ancak daha ileri çalışmalar ile bu yaklaşımın gelecekte kullanılacak potansiyel bir tedavi için uygun olup olmadığı değerlendirilmelidir.

Anahtar Sözcükler: Primer Bağışıklık Yetmezliği, X'e bağlı Agammaglobulinemi, Bruton Tirozin Kinaz (*BTK)* CRISPR/Cas9, Gen Düzenleme/Tedavi.

ABSTRACT

Development Of Gene Editing Based Applications For X-Linked Agammaglobulinemia

X-linked agammaglobulinemia (XLA) is a recessive disorder that primarily affects males. It is characterized by recurrent infections, low B-cell counts and agammaglobulinemia. Although there is no definitive cure, immunoglobulin replacement therapy is commonly used to control symptoms. However, this treatment can be expensive and not always effective. About 85% of XLA cases are caused by a mutation in the *Bruton's tyrosine kinase* (*BTK*) gene. *BTK* plays a role in B cell proliferation, differentiation and survival, and is also involved in primary humoral immune defense. The aim of this study was to develop an effective, innovative and personalized gene therapy for XLA caused by a *BTK* mutation. Accordingly, after mimicking a clinical variant in 3T3 and HEK293 cells with site-directed mutagenesis, it was aimed to develop a basic treatment application by repairing the created variant using the third-generation prime-editing CRISPR/Cas9 system specifically designed for the variant. Although the transfection efficiency was low, a slight change in the CRISPR/Cas9 applied cells were determined. It can be concluded that CRISPR-mediated correction of such point mutations may increase the life expectancy and quality of life of the individual, rather than standard treatments that only improve symptoms. However, further studies should evaluate whether this approach is suitable for a potential treatment to be used in the future.

Keywords: Primary Immune Deficiency, X-linked agammaglobulinemia, *Bruton's tyrosine kinase* (*BTK*), CRISPR/Cas9, Gene Editing/Therapy.

1 INTRODUCTION AND AIM

X-Linked Agammaglobulinemia (XLA) is an X-linked recessive disease that usually occurs in males. XLA is characterized by recurrent pyogenic infections, low/immaturity of B cells, and agammaglobulinemia (6). Currently, there is no effective clinical approach for treatment, but there are alternative treatments for symptoms such as immunoglobulin replacement. However, these treatments are very expensive and ineffective (8-10).

Approximately 85% of XLA cases are due to mutations in the *BTK* gene. *BTK* has 19 exons and 659 amino acids and is located on the X chromosome (15). *BTK* is a protein kinase, belongs to the TEC family without its receptor and plays a role in B cell proliferation, differentiation and survival. The *BTK* gene is involved in primary humoral immune defense (17).

CRISPR/Cas9 technology, which has come to the fore in gene therapy studies in recent years, is an important tool in terms of gene editing. CRISPR-Cas9 is the natural defense system of bacteria (50%) and archaea (90%) for editing DNA sequences that they come across with. CRISPR genes are small, palindromic direct repeats separated by non-repeating sequences called spacers (66).

Here we aim to optimize the basis and efficient of a gene editing tool that can be used in the treatment of XLA. Because of there is no efficient treatment and application for XLA, the third generation CRISPR/Cas9 could be the answer.

This study is an important study to see the effect of third generation CRISPR/Cas9 on single base change. In addition, among gene editing studies for XLA treatment, prime editing CRISPR/Cas9 has not been tried before, and the studied variant is a previously unreported variant. It is therefore an innovative potential treatment approach to XLA. Finally, different from the literature (8-11-13-18-65), since it was studied on the 3T3 cell line, it brought different troubleshooting and perspective to the literature.

In summary, in this thesis, a clinical variant causing XLA disease will be mimicked in the 3T3 cells using in vitro mutagenesis. Simultaneously, a CRISPR/Cas9 vector system targeting the *BTK* gene will be designed with bioinformatics tools, and the aforementioned vector system will be co-cultured with cell lines with *BTK* variant created and its therapeutic potential will be evaluated. For this purpose, in vitro analyzes such as flow cytometry, cell culture, western blotting, PCR and real-time PCR will be performed.

2 BACKGROUND

2.1 Immune System

The immune system is the defense system in which the organism can separate the cells that belong to it or not, and according to this distinction, it protects the organism from external and internal pests. Immune system consists of different elements like organs, tissues and cells. All of these elements originate from the bone marrow of the organism and may differentiate in different parts of the body (1). Immune system differentiates from stem cells and proliferate the myeloid or lymphoid cells. Myeloid cells are in innate immunity known as the first line of defense of the immune system. Lymphoid system, which is the second line of defense known as adaptive immunity are divided into B and T cells (Figure 1) (1).

While T cells work as cell contacts, B cells send antibodies to the inflammation area, and the pathogen or antigen there is registered in the immune system memory. Thus, in the second encounter, the immune system of the organism can respond more quickly and effectively (2). Natural killer cells from other immune system elements kill the pathogens directly by sending a death signal, while macrophages digest the cells by phagocytosis. Dendritic cells activate T and B cells by providing communication between the innate and adaptive immune system. Eosinophil, basophil, and neutrophil cells are the cells that help in defense, especially in allergic reactions (2).

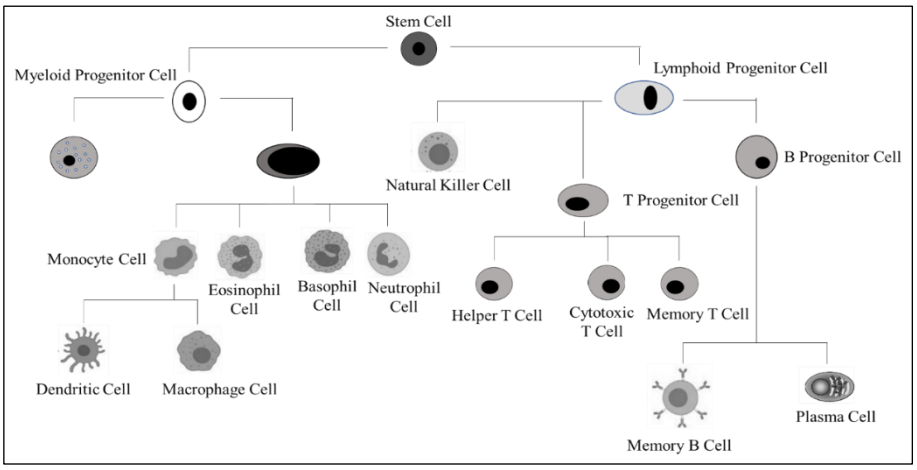

Figure 1 Immune System Classification.

Immune system cells differentiate and mature from hematopoietic stem cells (HSC). HSCs primarily differentiate into myeloid and lymphoid cells, which are more specific precursor cells associated with two main defenses, adaptive and innate immunity. Here, the main immune system cells are classified and schematized (3).

2.1.1 B cells of immune system

B cells are white blood cells responsible for antibody formation in the immune system. Progenitor B cells, mature in the bone marrow in mammals and, when activated, ensure appropriate antibody production. Bone marrow has its own micro-environment and thus completes the proliferation and maturation stages. They are mainly found in the bone marrow and lymph nodes, with a very small amount in the peripheral blood (4).

Antibodies are the main defense against antigens. Various mechanisms are used in antibody production. First, receptors on the surface of B cells, and especially Major Histocompatibility Complexes (MHCs), are effective in recognizing cells that belong to and do not belong to the organism. If the antigen is recognized through these receptors, a B cell produces antibodies specific for that antigen. The main mechanism in antibody formation is the pathway called VDJ recombination. As a result of this interaction, some B cells become memory B lymphocytes, while others differentiate into plasma cells and destroy the pathogen by producing active antibodies (5).

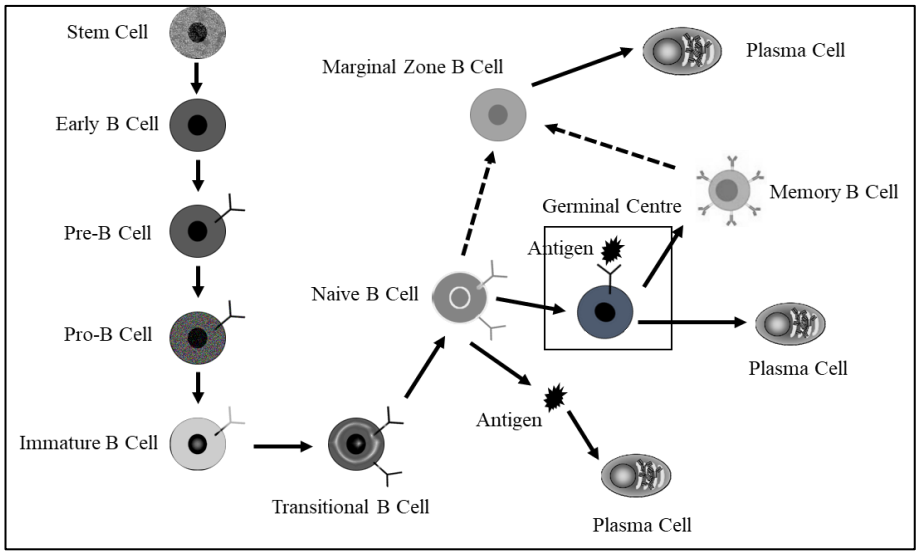

Figure 2 B Cell Development.

B cells differentiate from HSCs and eventually differentiate into plasma cells or memory cells. Development is observed in the bone marrow from HSC to Pre-B cells. Different forms of B cells then develop in the secondary lymphoid organs until they encounter the antigen. It encounters the antigen in the germinal center and transforms into a plasma or memory cell from there. Plasma cells produce antibodies (6).

2.2 Immunodeficiency

Immunodeficiency is caused by the failure or absence of immune system components such as lymphocytes, phagocytes, and the complement system. Immunodeficiency may be primary, such as Bruton's disease, or secondary, such as AIDS (7).

Primary immunodeficiency is divided into many different subtypes. These are groups: as B cell deficiency, T cell deficiency, both T cell and B cell deficiency, phagocyte deficiency, complement deficiency, and immunoglobulin A deficiency (Figure 3) (7).

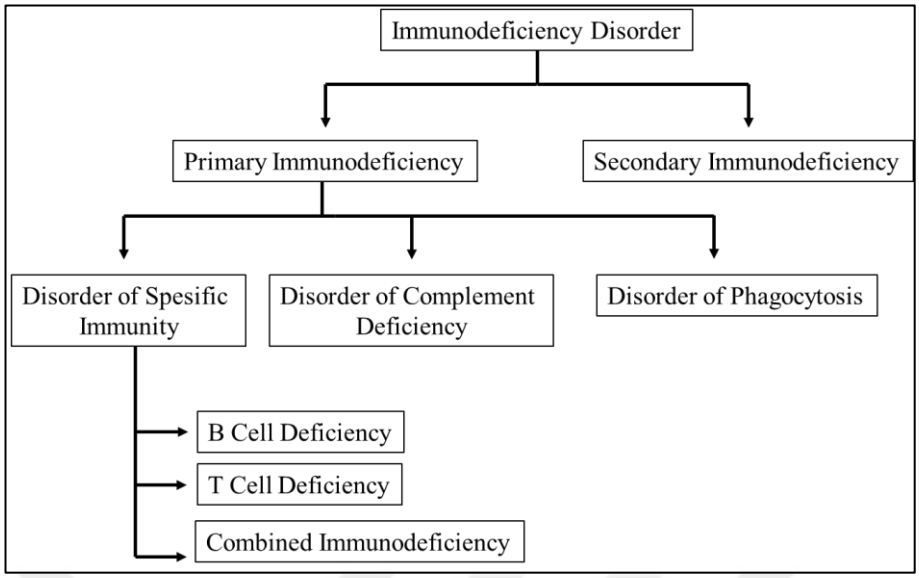

Figure 3 The main classification of immunodeficiency disorders.

Immune system disorders are divided into primary and secondary. Primary immunodeficiencies, classified specific immune disorders such as B cell deficiency, T cell deficiency and combined immunodeficiency; complement system deficiency disorders and phagocytosis disorders. Primary immunodeficiencies are due to inherited and genetic disorders. Secondary immunodeficiencies are acquired later.

Primary Immunodeficiency (PID) consist of more than 130 different disorders that affect the function, development, or both of immune systems (7).

Generally, PIDs are monogenic disorders with a simple Mendelian inheritance; however, some disorders have a more complex polygenic background. Conditions like disease penetration, expression variability and genetic and environmental factors may also contribute to the phenotypic diversity of PIDs. Except for IgA deficiency (IgAD), other PIDs are rare and have an overall incidence of about 1:10.000, although a much higher rate is seen in genetically isolated populations such as consanguineous marriage (7).

Classification			
T Cell Disorders	**B Cell Defects**	**Phagocyte Disorders**	**Complement Disorders**
Severe Combined Immunodeficiency	XL Agammaglobulinemia	Chronic Granulomatous Disease	C1q Deficiency
Wiskott Aldrich Syndrome (Xp11)	Common Variable Immunodeficiency	Leukocyte Adhesion Defect	Factor I Deficiency
Ataxia Telegectiasia (11q)	Selective IgA Deficiency	Chediac Higashi Syndrome	Factor H Deficiency
Digeorge Anomaly	AR Agammaglobulinemia	Myeloperoxidase Deficiency	Factor D Deficiency
	Hyper-IgM Syndromes- XL	Cyclic Neutropenia (electase defect)	Properdin Deficiency

Figure 4 Main disorders and their related diseases.

T-cell immunodeficiency diseases include severe combined immunodeficiencies (SCIDs), Wiskott-Aldrich syndrome, ataxia telangiectasia, DiGeorge syndrome (22q11. 2 deletion syndrome) and etc. B-cell immunodeficiency diseases include XLA, common variable immunodeficiency, selective IgA deficiency, ARA, hyper-IgM syndromes-XL and etc. Phagocyte disorders include chronic granulomatous disease, leukocyte adhesion defect, chediac higashi syndrome, myeloperoxidase deficiency, cyclic neutropenia and etc. Complement disorders include C1q deficiency, factor I/H/D deficienct, properdin deficiency and etc (8).

2.3 X- linked Agammaglobulinemia (XLA)

X- linked agammaglobulinemia (XLA) is an X-linked disorder, and it is the most common pediatric primary immunodeficiency. It is mostly seen in boys and the absence of maternal IgG is observed at 5-6 months of age (9). The diagnosis is made, on average, at the age of 2.5 years and in almost all cases before the age of 5. (10). In fact, not only IgG but all other immunoglobulins (IgA, IgM, IgD, IgE) are seen at low levels. The B-cell development is blocked at the pre-B cell step and B-cells do not develop to mature B lymphocytes. This is related to a mutation in a gene that codes for the protein tyrosine kinase (*Bruton's tyrosine kinase-BTK*) (11). Recurrent bacterial infections affect infants with X-linked agammaglobulinemia, including otitis media, bronchitis, septicemia, pneumonia, and arthritis, while Giardia lamblia causes intestinal malabsorption (12). As a standard, patients are treated in two ways. The first is to inject large amounts of IgG at regular intervals and keep these patients alive, but they can die at an early age in infections caused by antibiotic-resistant bacteria. In addition, the key therapy step is immunoglobulin replacement, nowadays (13). It can be administered in two ways: intravenously (400 - 600 mg/kg every 3 - 4 weeks) (IVIg) and subcutaneously (100 mg/kg every week). However, this treatment, which is very

costly and life long, is not effective. The second is bone marrow transplantation, but this is a very critical operation (13).

Agammaglobulinemia includes the following types (14): X-linked agammaglobulinemia (XLA), X-linked agammaglobulinemia with growth hormone deficiency, and autosomal recessive agammaglobulinemia (ARAG).

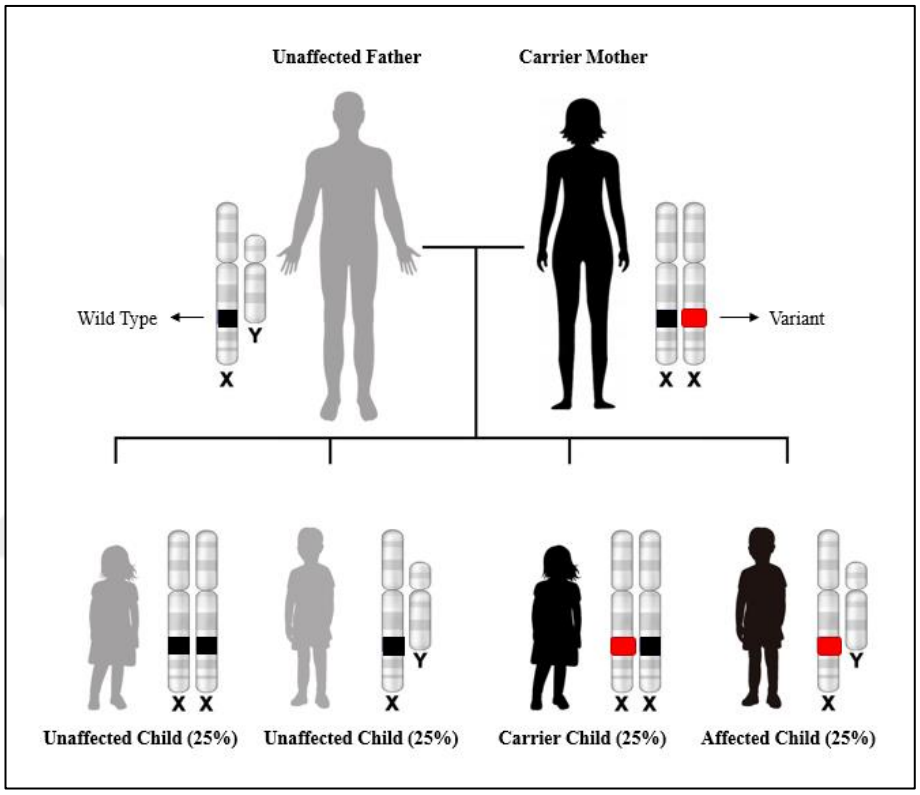

Figure 5 Transmission of X-linked inheritance between generations.

Children of an unaffected father and a carrier mother will be 50% unaffected, 25% carrier and 25% affected. Since the X chromosome is only one in males, it cannot be a carrier, while females may be affected if there are two copies of the variant.

X-linked agammaglobulinemia is described by C. Ogden Bruton in 1952, but the genetic defect of XLA was not discovered until 1992 (15). X-linked agammaglobulinemia is an inherited immunodeficiency caused by variants in the gene encoding *BTK* (16).

X-linked agammaglobulinemia (OMIM #300755) is characterized by recurrent infections, lack of B cells and agammaglobulinemia (17). It occurs due to variations

in the *BTK* gene located on the long arm of the X chromosome [Xq21.33-q22 (contains 19 exons and codes for a 659-amino acid protein) (18). Approximately 80-85% of XLA cases exhibit *BTK* variations, with the remaining instances attributed to de novo mutations. Currently, there is no cure; instead, the primary goal is to prevent infections and symptoms.

X linked agammaglobulinemia is defined by an increased vulnerability to bacterial infections, low serum levels of immunoglobulin isotypes, and a paucity or lack of peripheral blood B cells (usually less than 2%).

In the maintenance of various immunological diseases, such as systemic lupus erythematosus, multiple sclerosis and rheumatoid arthritis, *BTK* plays a significant role (19).

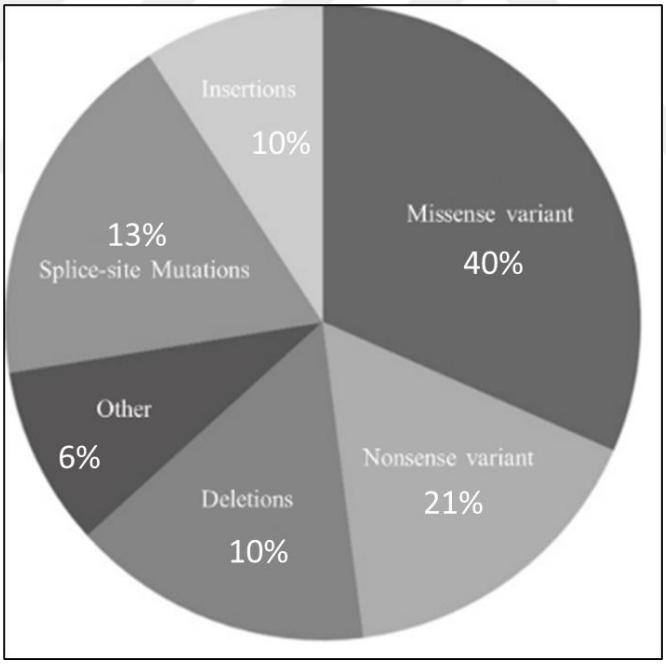

Figure 6 Main genetic disorder types that cause XLA.
Missense variants are most common (20).

2.4 The Cytoplasmic Protein-Tyrosine Kinase (*BTK*)

The TEC family of non-receptor protein kinases includes Brutons' tyrosine kinase (BTK) (21). A cytoplasmic tyrosine kinase with the pleckstrin homology (PH), SH2, Src homology 3 (SH3), tec homology (TH), and tec kinase (TK) functional domains is encoded by the BTK gene. While the TH, SH3, and SH2 domains interact with other proteins, the N-terminal PH domain interacts with membrane phosphatidylinositol (3,4,5)-triphosphate (PIP3) (22).

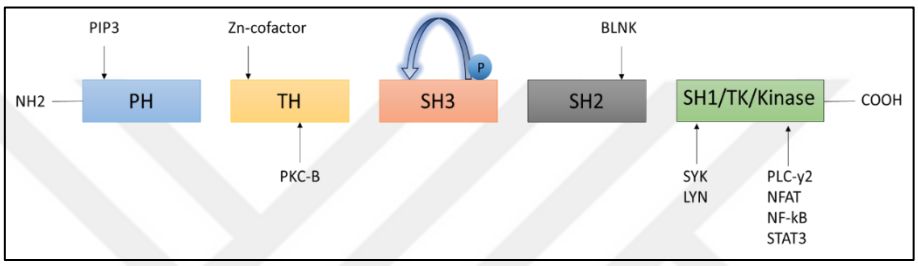

Figure 7 *BTK* protein structure.

PH Pleckstrin homology, TH Tec Homology, SH3 The SRC Homology 3 Domain, SH2 The SRC Homology 2 Domain, SH1 The SRC Homology 1 Domain.

The SRC-family kinase LYN phosphorylates *BTK* in response to BCR stimulation. As a result, activated *BTK* phosphorylates its own substrate (phospholipase C-2, a downstream molecule). The intracellular calcium level rises, and transcription factors involved in B-cell proliferation, differentiation, and survival become active. Most hematopoietic cells, such as macrophages, neutrophils, and mast cells, express *BTK*, whereas T cells and plasma cells do not. Any mutation in the *BTK* gene leads to primary humoral immunodeficiency diseases (such as XLA) (22).

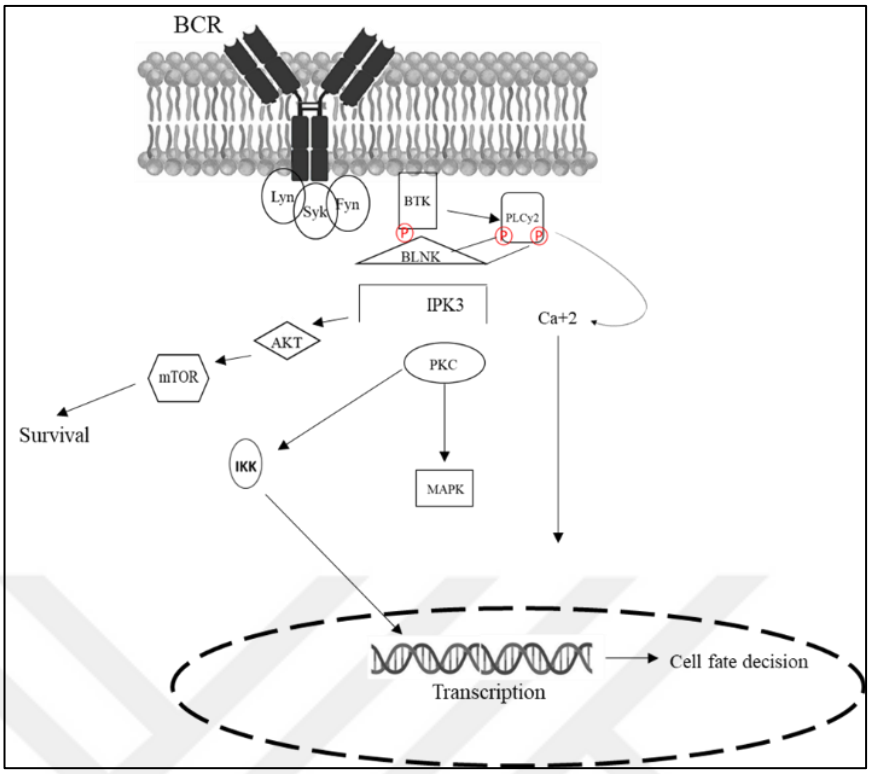

Figure 8 *BTK* pathway in cell.

When the ligand activates the B cell receptor (BCR), the Lyn/Syk/Fyn complex is formed and *BTK* is activated by phosphrescence and survives by activating the AKT/mTOR pathway from the signaling pathway. Likewise, it determines cell fate by initiating transcription via the PKC/IKK pathway (23).

Bruton's tyrosine kinase (BTK) plays a fundamental role in the survival and differentiation of B-lineage cells, so it is a suitable candidate as a drug target. *BTK* inhibitors (*BTK*is) are used extensively in the clinic and there are at least 22 available today. First-in-class Ibrutinib forms a covalent bond with a cysteine at the catalytic site of the kinase and is irreversible. The next-generation inhibitors, acalabrutinib, tirabrutinib, and zanubrutinib, have been approved in USA-Europe, USA-Europe-China, Japan, respectively. However, it is generally used in B-lymphocyte tumors, it is not widely used in immunodeficiency diseases (24).

2.5 Mutagenesis Strategy

Site-directed mutagenesis (SDM) is one of the most important techniques used in gene editing in molecular biology to investigate the cause-and-effect mechanisms of genetic variations, and their effects to the structure and function on proteins (25).

The two basic DNA modification techniques can be divided into polymerase chain reaction (PCR) and site-directed mutagenesis (SDM). A new era began with the development of PCR-based SDM in 1990. The technique used to amplify a specific region of DNA is called polymerase chain reaction (PCR) (26). Many SDM techniques involve different versions of PCR, including inverse PCR (27), proofreading DNA polymerase PCR (28), rapid mutagenesis method (29) and also some commercial SDM kits.

These techniques are generally efficient for plasmids smaller than 3.1 kb, and very low or high GC rates also affect the success rate. In addition, some types of mutations that are desired to be created may inhibit the polymerase function. Creating mutation on plasmid larger than 8 kb, a smaller subclone containing the mutagenesis site is required, but subcloning is slow and an inefficient process (30).

Common to all SDM techniques is the use of the DpnI enzyme to remove parental DNA before transformation. DpnI cleaves methylated DNA so that non-mutated DNA is not found at the later stages (31).

Finally, the most important step in SDM is the primer design. Primers need to be 18-30 bp in length to specifically bind only to the target site and for the TM temperature to remain within the 46-70^{0}C range. It must also be designed to opposite orientation because it requires amplification of the entire plasmid, not just a specific region. Primer length and GC content are the most determining factors for the TM value. Primers below 46^{0}C or even 55^{0}C in general do not bind very specifically, and if it is above 70^{0}C or even 65^{0}C, polymerase may not work efficiently, so the correct TM range should be set. For more stable and long-term operation, it is recommended

to use high fidelity polymerase instead of standard polymerase. Also, for deletions and insertions, the change site should be at the 5' end, but for point mutations, the mutation should be in the middle of the primers (31).

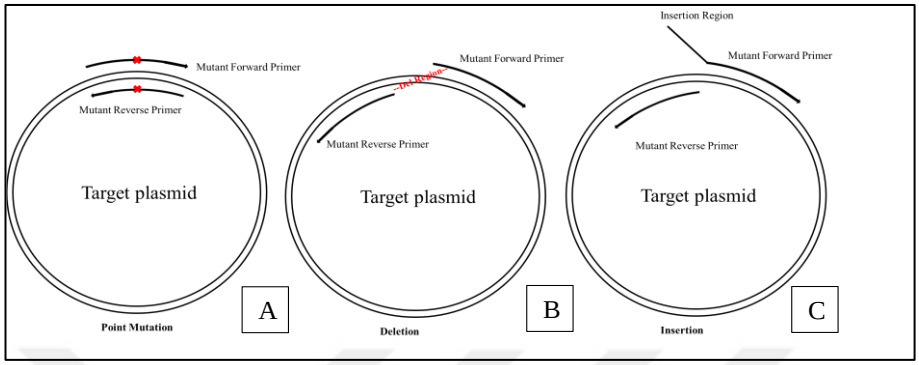

Figure 9 Types of site directed mutagenesis strategies.

Symbolize creating strategy of point mutation (A), Symbolize creating strategy of deletion (B) and insertion (C).

2.6 Gene Therapy/Gene Editing

The US Food and Drug Administration (FDA) defines gene therapy as products "that mediate their effects by transcription and/or translation of transferred genetic material and/or by integrating into the host genome and that are administered as nucleic acids, viruses, or genetically engineered microorganisms. The products may be used to modify cells in vivo or transferred to cells ex vivo prior to administration to the recipient" (32-33).

There are different types of gene therapy for regulation of gene expression.

2.6.1 Types of gene therapy

It is critical to remember that there are numerous types of gene therapy treatment alternatives, each with unique characteristics, such as somatic, germline, and preventive strategies (34).

Germline editing occurs in all cells of the body. It holds the potential to eliminate hereditary disorders from an entire community. Somatic editing is limited to specific identified body cells. The modified genes do not get inherited by future generations. Preventive editing eradicates the risk of developing disorders (34).

Gene therapy may involve many different techniques, including augmentation, inhibition, and/or cell destruction. The choice of technique to be used is determined by conditions such as the probability of occurrence and the disease being studied (35).

Manipulating a disease-causing gene by correcting or managing its expression shows how important gene therapy is. In addition, the manipulation of a gene requires three major steps. Firstly, it is necessary to correctly evaluate the gene that causes the disease. Secondly, it contains creating a duplicate DNA which matches the defected gene to solve the health problem. Finally, create a system that can deliver the new gene into a patient's human body (35).

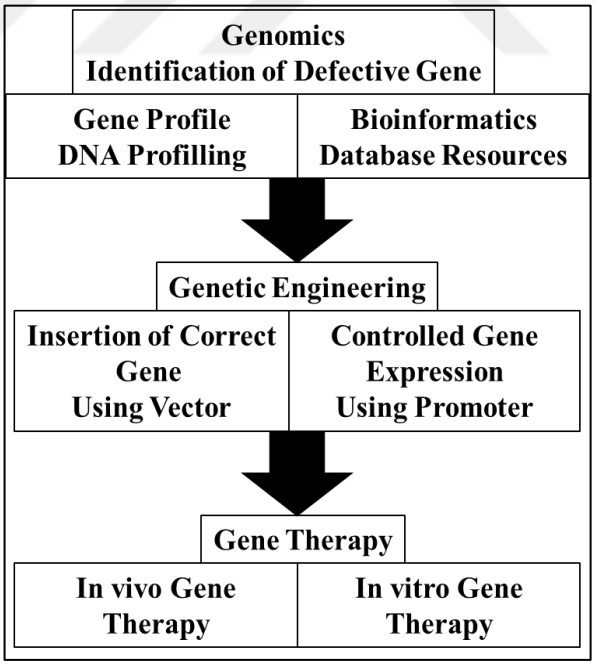

Figure 10 Main steps of gene therapy.

1) Identification of defective genes: Gene or DNA profiling and/or bioinformatics studies. 2) Genetic engineering: insertion of correct gene using vector and/or controlling gene expression with promoter. 3) Gene therapy: Completion of treatment or diagnosis by in vivo and/or in vitro studies (36).

2.6.2 Gene therapy at clinical trials

The first gene therapy attempts were not entirely effective due to nonspecific integrations or insertional mutagenesis. For instance, in a trial of gene therapy conducted in 20 patients 5 developed leukemia and one of them died. On the other hand, two patients recovered partially, and 17 patients recovered entirely. Despite these disadvantages, gene therapy outperforms allogeneic bone marrow transplantation in terms of clinical cure and mortality (37).

Many gene therapy trials are now being conducted around the world (primarily in the USA). There are 695 gene therapy clinical studies in the ClinicalTrials.gov database. Most of these studies are aimed at cancer treatment. Both non-viral and viral methods are used in these studies, but reaching the phase III stage is difficult in both methods (37). Currently, according to the FDA, there are 22 Approved Cellular and Gene Therapy Products (38).

In addition, gene therapy can be divided into two main categories: In vivo gene delivery of manipulated viral vector vehicle into the host and ex vivo genetically engineered stem cells (39).

Over the last 30 years, gene therapy has faced many challenges and negative results, but it is a method that is developing faster than modern drugs. Each new solution to the difficulties encountered plays an important role in the improvement of the method. Fundamentally, however, the success of a gene therapy largely depends on the stability of the transferred foreign genetic molecule in the host (39).

According to the Alnasser, S. study in 2021 there are close to 4000 genetically related disorders such as cancer, AIDS, cystic fibrosis cardiovascular disease and the cells to be targeted for these diseases differ (Table 1) (39).

Table 1 Cell types and related diseases.
In general, there are groups of cells that are related to the causation of certain diseases (39-40).

Cells	Diseases
Lung	Cytosis fibrosis
T cells, Muscle cells	Infectious
Bone marrow	Immunodeficiency
Muscle, Liver	Hemophilia
Hematopoietic progenitor, T cells, Muscle cells, Tumor cells	Cancer
Endothelial, Muscle	Cardiovascular
Synovial Lining cells	Arthritis
Neurons, Brain	Neurological

2.7 CRISPR/Cas9 As Genome Editing Tool

New gene editing tools make it easier and more precise to cure hereditary disorders. As a result, experiments including ex-vivo homologous recombination, TALENS, and Zinc Finger Nucleases have been carried out. None, however, outperformed the clustered, regularly interspersed short palindromic repeats (CRISPR/Cas9) system (40-41).

This technique was first discovered in bacteria and archaea as a critical defense mechanism against invading viruses. Specific areas of the human genome are targeted using CRISPR/Cas9, many of which are intended to have a therapeutic effect. CRISPR/success Cas9's and efficiency are based on sequence specificity, comparable to the RNAi technique. Host DNA repair systems can impair gene expression, with a modest insertion and/or deletion (indel) of two to six nucleotides frequently resulting in a frameshift mutation and termination. Short-guided RNA and ds-DNA templates containing Cas-like enzyme are present in the genes, allowing homology-dependent recombination to occur (41).

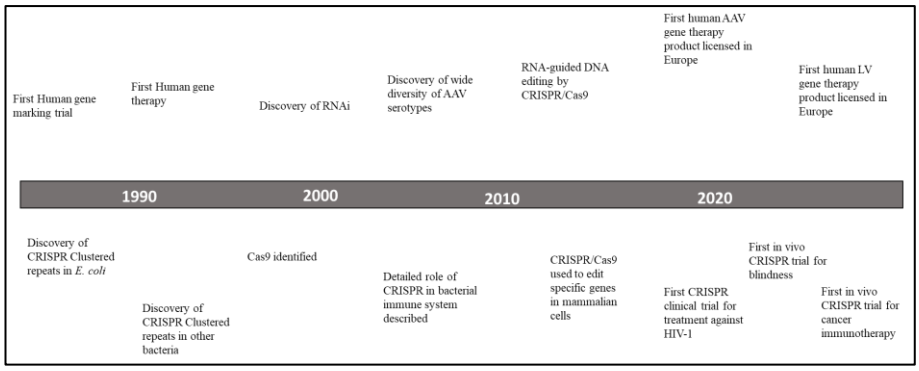

Figure 11 Brief history of gene editing approaches (41-42).

By the mid-1980s, gene transfer into mammalian cells was common practice. Retrovirus-based gene transfer did indeed offer significant advantages due to the robust integration of their genomes into the chromosomes of the host cells. However, a lack of understanding of gene control, concerns about the potential effects of exogenous DNA in the host cell and other key ethical issues meant that gene therapy remained controversial. (42-43). Some of these ethical concerns arose following a contentious, unlicensed human gene therapy trial on two patients with thalassemia in the 1980s (44-45).

Evidence of the principle of -retrovirus gene transfer to hematopoietic stem cells was demonstrated in the early 1990s, and it was used to treat severe combined immunodeficiency (SCID) (46-47). In 1991, Anderson et al. completed the first FDA-approved clinical trial. A gamma-retrovirus that promotes the production of adenosine deaminase was infected into patients' peripheral blood CD34+ cells. While the trial's immediate benefits are still being debated, it could be considered the first "successful" human gene therapy trial, at least in terms of safety concerns. X-SCID was successfully treated in 11 children in 2000 (48). This was achieved by introducing the gamma chain of the common interleukin receptor into the bone marrow using a retrovirus vector based on the mouse leukaemia virus (MLV) (48).

The first effective trial of gene therapy for melanoma treatment was undertaken in 2006. The α- and β-chains of a melanoma antigen (MART-1)-specific T cell receptor (TCR) were encoded by a retrovirus. The TCR used in the study was cloned

from a patient who had experienced regression following adaptive transfer treatment. This retrovirus was transduced and re-administered to peripheral blood cells, resulting in total regression in two of fifteen patients. This was the first-time immune cells were genetically engineered to attack cancer in a human gene therapy trial (49).

The discovery of zinc finger nucleases (ZFNs) in 2005 followed by the discovery of transcription activator-like effector nucleases (TALENs) in 2011 brought a new perspective to gene editing technology and the work gained momentum (50-51).

In 2014, ZFNs were used to treat HIV/AIDS by engineering derived T cells from patient, knocking out of the virus (52). A different field was soon developed with TALENs: Engineered T cells were used to fight B cell lymphomas (53).

A breakthrough for gene therapy researchers in 2012 was the development of CRISPR-Cas9 as a gene-editing tool. In 2016, the first trial of ex vivo CRISPR T cell therapy for non-small cell lung cancer (NSCLC) was performed (54).

In 2018, a trial of CRISPR cell therapy was performed using hematopoietic stem and progenitor cells (HSPCs) to normalize fetal hemoglobin production in a sickle cell disease (SCD) patient (55). In 2019, the first in vivo CRISPR gene therapy trials were performed, and the CEP290 gene was rearranged in a patient with Leber's congenital amaurosis (LCA) (56).

2.8 CRISPR/Cas9 Technology

Many genetic disorders are known to be caused by gene mutations. The most well-known genome editing technologies are zinc fingers nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and CRISPR-Cas9 (57).

A CRISPR-Cas9 is a natural mechanism for editing DNA sequences present in 50% of bacteria and 90% of archaea. A CRISPR locus including a parted by a non-repetitive sequence named spacer, a small palindromic direct repeat, and is identified

by bioinformatics tools. In the first stage, when spacers encounter exogenous genetic elements, they keep it as a memory, so if these elements re-enter the spacer, the system initiates the expression and intervention stages. Based on the exchange of structure and Cas operons, the CRISPR-Cas system is separated into several systems. This system is divided into two categories: multi-subunit effector complexes systems and effector complex's whole functionality is performed by a protein (58).

A protospacer adjacent motif (PAM) in the DNA sequence of the target gene is required for CRISPR/Cas-based genome editing and gene therapy. A PAM is a short DNA sequence. It consists of 2-6 base pairs. For example, the most widely used SpCas9 recognizes the NGG PAM. CRISPR/Cas-based genome editing and gene therapy are limited by the PAM requirement. Any genetic disease can be treated with CRISPR/Cas technology (59-60).

The first contains three types: Type I contains Cas3, which expresses a superfamily 2 helix based on single-stranded DNA (ssDNA) leading to uncoiling of both DNA/DNA and RNA/DNA duplexes. Type III has Cas10, which expresses a multi-domain protein. Finally, Type IV has an effector complex that includes a csf1 gene as the signature gene of the aforementioned system (61)

2.8.1 Strategies of CRISPR/Cas9-based gene therapy

In 2012 CRISPR/Cas strategy is recognized as a genome editing tool and can be used to replace, remove, or correct undesirable genes that reason of genetic diseases (62).

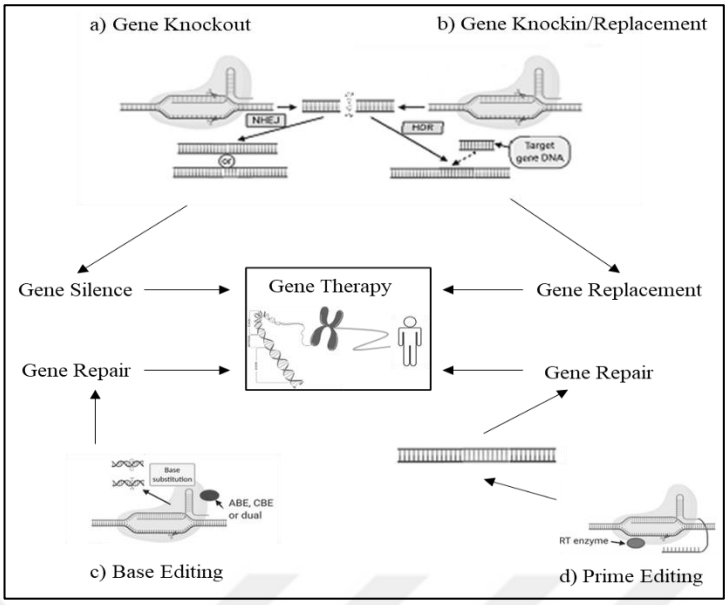

Figure 12 Strategies of gene therapy via CRISPR/Cas9.

Gene can be manipulated in three main ways. These are silencing, replacement or repair. Gene knockout, gene knockin, base editing, and prime editing are strategies for CRISPR/Cas9 (63).

When the CRISPR/Cas system is introduced into a cell, the gRNAs direct the Cas enzyme to a specific target DNA sequence with a PAM complementary to the gRNA, and the Cas nuclease cuts the double strand of the DNA, resulting in a double-strand break (DSB). Cells have evolved DNA repair mechanisms throughout the course of their extensive evolutionary history to fix damaged DNA, including DSBs. The most typical form of DNA repair, known as non-homologous DNA end joining (NHEJ), involves directly uniting two damaged DNA molecules (64). NHEJ repair often causes a frameshift change for the original DNA sequence because a single CRISPR/Cas9 cut typically deletes a few nucleotides, but in other circumstances, NHEJ repair also adds a few nucleotides and produces the same outcomes. The results of NHEJ repair include nonsense mutations or frameshift changes that silence the altered genes. As a result, the CRISPR/Cas9 system can be utilized for gene editing to silence or remove a defective gene (65).

In in vivo or in vitro studies, CRISPR/Cas9 has been shown to be simple and more effective than other traditional gene editing methods. For this reason, it is the most widely used gene editing method since its discovery. Knockin or knockout can be used

to regulate the unwanted effect of a gene. However, in the last 5 years, base or prime editing has been used to create specific differences, such as point mutations, in the gene (66-67-68).

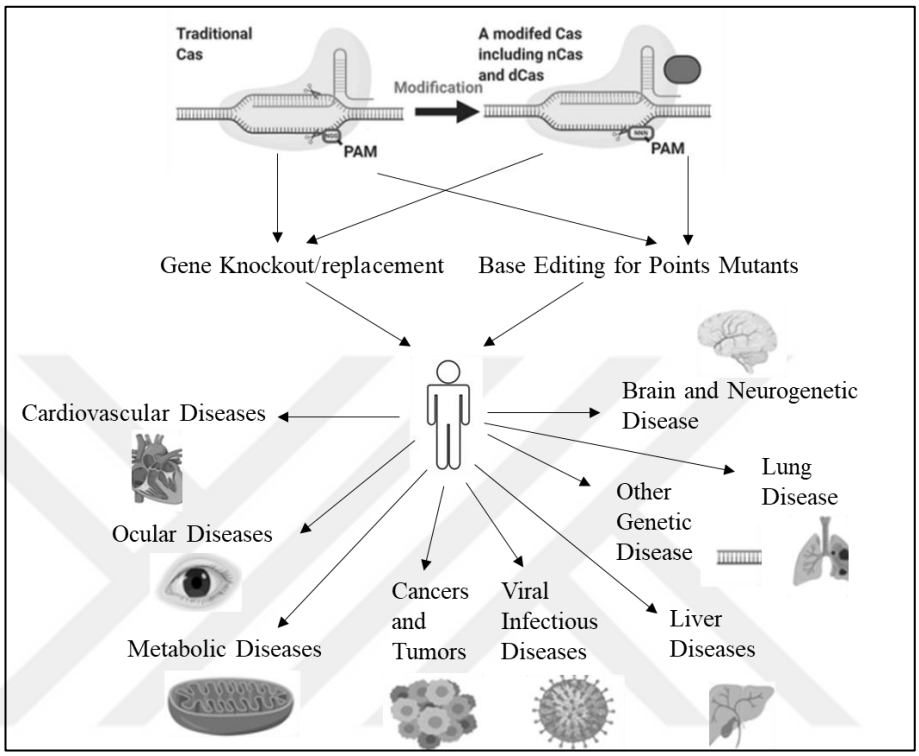

Figure 13 Potential approaches for gene therapy.
Gene modifications with traditional and/or modified approaches are options for potential treatment of various diseases (70).

CRISPR/Cas-related studies have shown its great potentials, past years, on gene editing and can be used to treat any human disease caused by a genetic mutation or alteration in a genetic component (69).

In this study, our primary aim was to fix point mutation in *BTK* gene that leads to XLA by using CRISPR/Cas9 gene editing tool. To achieve this aim, the point mutation was first introduced into 3T3 cells, followed by the assessment of therapeutic potential for XLA therapy using the engineered CRISPR/Cas9 vector system targeting the BTK gene. For this purpose, in vitro analyzes such as flow cytometry, western blot, PCR and RT-qPCR were used to determine the effects of treatment at DNA, RNA and protein level.

3 MATERIALS AND METHODS

3.1 Materials

In this section, the materials used in the experiments carried out in the thesis study are listed.

3.1.1 Chemicals

- 10% APS (Thermo Fisher, USA)
- 10% SDS (Merck, Germany)
- 30% acrylamide mix (Merck, Germany)
- 5X Lyse/Fix buffer (BD Biosciences, USA)
- Agar plate (Nutrient Agar) (Merck, Germany)
- Agarose (Merck, Germany)
- Ampicillin (Merck, Germany)
- Coomassie Blue dye (Merck, Germany)
- Dimethyl sulfoxide (DMSO) (Tekkim, Turkey)
- Dulbecco's Modified Eagle Medium, High Glucose (DMEM) (Diagnovum, Holland)
- Enhanced chemiluminescence (ECL) (Biorad, USA)
- EtBr (Sigma, Germany)
- Fetal Bovine Serum (FBS) (Diagnovum, Holland)
- Freezing medium (Diagnovum, Holland)
- FuGENE® HD Transfection Reagent (Promega, USA)
- Glycerol (Merck, Germany)
- Lysogeny Broth (LB) Medium (Merck, Germany)
- PCR Master mix (Genemark, Turkey)
- Milk Powder (Bioshop, Canada)
- Nuclease free water (Thermo Fisher, USA)
- Penicillin-Streptomycin (P/S) (Diagnovum, Holland)

- Perm II buffer (BD Biosciences, USA)
- Phosphate Buffered Saline (PBS) (Thermo Fisher, USA)
- Ponceau S dye (Merck, Germany)
- Relating DNA of study
- Relating primers of study
- RIPA Buffer
- RLT Buffer (Genemark, Turkey)
- Roswell Park Memorial Institute 1640 (RPMI-1640) (Diagnovum, Holland)
- Running Buffer
- Super Optimal Broth (SOC) media (Sigma, USA)
- TAE Buffer
- TBST Buffer
- TEMED (Sigma, USA)
- Transfer Buffer
- Tris Buffered Saline (TBS) (Thermo Fisher, USA)
- Trypsin-EDTA (Diagnovum, Holland)
- Tween 20 (Merck, Germany)
- 0.5 M Tris (pH=6.8) (Thermo Fisher, USA)
- 1.5 M Tris (pH=8.8) (Thermo Fisher, USA)

3.1.2 Consumables

- Cell petri dishes (LabMaker, USA)
- Centrifuge tube tubes (Isolab, Germany)
- Cryogenic Vials (Isolab, Germany)
- Flask (NEST, USA)
- Loop (NEST, USA)
- Microcentrifuge tube tubes (Isolab, Germany)
- Micropipette (Gilson, USA)
- Pipette (Thermo Fisher, USA)
- Pipette gun (Thermo Fisher, USA)

- Polyvinylidene Fluoride (PVDF) membrane (Biorad, USA)
- Spreader

3.1.3 Lab devices

- Automatic pipettes (Thermo Fisher, USA)
- Biorad Cell Counter (Biorad, USA)
- Electrophoresis instrument
- Flow cabin (Thermo Fisher, USA)
- Flow Cytometry- FACS Aria II (Becton Dickinson, USA)
- Fume Hood (Thermo Fisher, USA)
- Heat Block (Witeg, Germany)
- Incubators (Nuve, Turkey)
- PCR device (Biorad, USA)
- Pipette gun (Thermo Fisher, USA)
- Precision Balance
- Refrigerator and freezer (+4°C, -20°C, -80°C) (Nuve, Turkey)
- Spectrophotometer (Nanodrop) (Thermo Fisher, USA)
- Tabletop mini centrifuge (Becton Dickinson, USA)
- Vortex (Neuation, India)
- Water bath (Weightlab, Turkey)

3.1.4 Other requirements

- Alexa Fluor 647 Mouse anti-*BTK* (Santa Cruz, USA)
- Donkey anti-goat Antibody (Santa Cruz, USA)
- EFSco*BTK* (71) (a gift from Peter NG)
- *Escherichia coli* DH5-alpha cells (Component cells) (Thermo Fisher, USA)
- Goat anti-*BTK* Antibody (Santa Cruz, USA)
- Human Embryonic Kidney (HEK293) (purchased from ACU-Biobank)

- Immortalized Line of Human T Lymphocytes (Jurkat) (purchased from ACU-Biobank)
- Lymphoblast-like cells (Raji) (purchased from ACU-Biobank)
- Monoblastic Cell Line (U937) (purchased from ACU-Biobank)
- Mouse Fibroblast Cell Lines (3T3) (purchased from ACU-Biobank)
- Mutant EFSco*BTK*
- pcDNA3-EGFP (Addgene, China)
- pLenti-NG-PE2-BSD (Addgene, China)
- Prime editing guide RNA (pegRNA)

3.1.5 Kits

- GeneJET Plasmid Miniprep Kit (Thermo Fisher, USA)
- Human Blood RNA Purification Kit (Hibrigen, Turkey)
- PickMutant Site-directed Mutagenesis Kit (Canvax, Spain)
- PureLink™ HiPure Plasmid Filter Maxiprep Kit (Thermo Fisher, USA)
- Reverse Transcriptase First Strand cDNA Kit (Thermo Fisher, USA)
- Zymo Quick-DNA™ Miniprep Plus Kit (Zymo, USA)

3.1.6 Services

- Mutagenesis service from Genscript
- Sanger Sequencing service from ACU Labmed

3.1.7 Primers

Primers showed table 2 designed via NCBI primer blast and checked for oligoanalyzer, SNPCheck and USCS In silico.

Table 2 All primer list of thesis study.

Mutagenesis Primers (5' 3')	
Forward Primer	CCC GAG GGT GGG GGA GAA CCG TA
Reverse Primer	TGC AGG TCG ACT CAT CAG CTT TCC TCG TC
Mutagenesis Forward Primer	AGC AGC AAC AGC GAC ATC TGG GCC TT
Mutagenesis Reverse Primer	GTC GCT GTT GCT GAA CTT GCT GTA CAT C
BTK Expression Primers (5' 3')	
Forward Primer	ACA GAT TCC GAG AAG AGG TGA AG
Reverse Primer	GCC CTT CAT CAT ATA CAA CCT GGA A
BTK Primers for Sanger Sequencing Analysis (5' 3')	
Forward Primer	AGG GGG TGG TGA AGG TGT
Reverse Primer	GGT CTC GCT GTT GGT GAA CC
pegRNA Primers for Ligation Analysis (5' 3')	
Forward Primer	CGC CAG AAC ACA GGT GTC GT
Reverse Primer	TGC AGC CCA GCT TGC TAG C

3.2 Methods

In this section, experiments conducted in the thesis study are described.

3.2.1 Cell culture

5 different cell (3T3, Raji, HEK293, U937, Jurkat) lines were studied at cell culture. Cells were cultured with the protocols outlined below. Each cell line was frozen in RLT buffer (for RNA isolation), RIPA buffer (for Western blotting) and freezing medium (for alive cell freezing) at the end of cell culture.

To start cell culture, cryogenic tubes were taken from the -80°C freezer or liquid nitrogen tank and quickly transferred to 37°C. When the medium of the cryogenic tube were completely thawed, the cells were quickly distributed into 15mL conical-bottom centrifuge tubes and centrifuged for suspension cells 125 g for 5 minutes and adherent cells 300 g for 5 minutes. By discarding the supernatant, it was resuspended in the medium in a volume suitable for the flask and transferred to the flask and taken to the incubator.

To finished culture, adherent cells were collected by trypsinization, and suspended cells were collected without trypsinization. It was washed with culture medium and transferred to 15 mL conical bottom centrifuge tubes and centrifuged. The supernatant was discarded and the cells in the pellet were first dissolved with 1.5 mL of FBS and then added to 1.5 mL of freezing medium. Cells taken into 2 different cryogenic tubes were obtained to Mr. Frosty and put into the at -80°C. It was then transferred to liquid nitrogen.

While the cell culture was continuing, medium exchange and passage were made when necessary. Since the adherent cells adhere to the flask, the old medium was taken without trypsinization. Since the dead cells do not have adherent properties, they were in the old medium. The required amount of fresh new medium was put into the flask and the flask was put into the incubator. Since the suspended cells are in the medium, they are first taken into a 15 mL centrifuge tube. Of the cells centrifuged at 125 g for 5 minutes, the viable cells were considered to be in the pellet, and the dead cells were considered to be in the supernatant. The supernatant was discarded. On the other hand, the pellet is dissolved in the new medium and transferred to the flask and put into the incubator.

In adherent cell cultures, the old medium is discarded. The remaining medium was cleaned with 1X PBS in an amount to cover the surface of the flask. In the same way, Trypsin-EDTA is added to cover the flask surface and left in the incubator for about 5-6 minutes. The adherent properties of the cells are lost, and they start to float, medium is added to stop trypsinization. After the cells in the flask were collected into a 15 mL centrifuge tube, they were centrifuged at 300 g for 5 minutes. The supernatant is discarded. The pellet is dissolved in the new medium and the cells are counted in the automatic Biorad Cell Counter. The required amount of cells were separated into new flasks and put in an incubator. In suspended cell cultures, all cells together with the old medium were collected into 15 mL centrifuge tubes. Centrifuge at 125 g for 5 minutes and discard the supernatant. The pellet was dissolved in the new medium and the cells were counted in the automatic Biorad Cell Counter. The required number of cells were separated into new flasks with medium and put in an incubator.

3.2.2 Sample preparation and analyzing for assessment of *BTK* mRNA and protein expression levels

The *BTK* analysis was performed at the mRNA and protein level in different cell lines via flow cytometry, PCR, QPCR and western blotting.

3.2.2.1 Flow cytometry

Benjamin Bortol Protocol was used for Flow Cytometry. First, the cells were removed from their medium by centrifugation. 100 µL of cell suspension, approximately 500.000 cells, were placed in sterile FACS tubes. 1X Lyse/Fix buffer prepared; 5X Lyse/Fix buffer with MQ and pre-warm the buffer at 37^0C before use. Pre-chilled Perm II buffer on ice or 4^0C. 100 µL of 1X Lyse/Fix buffer was placed on 100 µL of cell suspension and kept on ice for 10 minutes. 2 mL of PBS was added and centrifuged at 1100 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended with 1 mL of cold Perm II buffer and kept on ice for 30 min. 2 mL of PBS was added and centrifuged at 1100 rpm for 5 min. The supernatant was discarded, and the pellet was thawed with 2 mL of PBS, then centrifuged at 1100 rpm for 5 minutes. The supernatant was discarded, and the pellet was dissolved with 25 µL of anti-*BTK* (1:10 diluted) and incubated for 30 min at room temperature in the dark. Cells were washed with 2 mL of PBS and centrifuged at 1100 rpm for 5 min. The supernatant was discarded and resuspended with 150 µL of FACS flow. Finally, it was placed in the flow device and the measurement was done.

3.2.2.2 Polymerase chain reaction (PCR)

The RNA was isolated from cell lines frozen in RLT buffer at the end of cell culture. Then, cDNA was obtained by Reverse Transcriptase PCR. *Bruton's tyrosine kinase* (*BTK)* gene expression in cell lines was examined using these cDNA samples and appropriate primers.

Human Blood RNA Purification Kit (Catalog no: TR03) were used. Samples kept in RLT buffer at -80^{0}C were taken (350 µL). 350 µL of 70% ethanol was added to the samples and vortexed. Samples were taken into the RNA spin column. It was centrifuged at 12.000 g for 1 minute. Collection tube was discarded. 500 µL of RNA wash solution I was added onto the column. It was centrifuged at 12.000 g for 1 min. The following solution was prepared separately for each sample and added on the column: 80 µL DNAse I Incubation Buffer + 2 µL DNAse I.

It was incubated for 15 min at room temperature. 500 µL of RNA wash solution I was added onto the column. It was centrifuged at 12.000 g for 1 minute. RNA wash solution II was added on the column. It was centrifuged at 12.000 g for 1 minute. The last two steps were repeated. After the collection tube was emptied, the empty column was centrifuged at 12.000 g for 3 minutes. Column was placed in 1.5 mL microcentrifuge tube and 50 µL of nuclease free water was added to it. It was incubated for 1 min at room temperature. It was centrifuged at 12.000 g for 1 minute.

Revert aid First Strand cDNA Synthesis Kit (K1622) was used. RNA sample preparated according to the 500 ng RNA in 10 uL.

- Take U937 RNA sample X uL + (10-X) uL dH2O.
- Take Raji RNA sample Y uL + (10-Y) uL dH2O.
- Take 3T3 RNA sample Z uL + (10-Z) uL dH2O.
- Take HEK293 RNA Sample A uL + (10-A) uL dH2O.

All kit components were stored at -20^{0}C. When used, it worked on ice. First, the following reactions were prepared.

Table 3 First reactions of cDNA preparation.

Reaction 1		**Reaction 2**		**Reaction 3**		**Reaction 4**	
Chemicals	Volume	Chemicals	Volume	Chemicals	Volume	Chemicals	Volume
U937 RNA	10 uL	3T3 RNA	10 uL	Raji RNA	10 uL	HEK293 RNA	10 uL
Oligo(dT) Primer	0,5 uL	Oligo(dT) Primer	0,5 uL	Oligo(dT) Primer	0,5 uL	Oligo(dT) Primer	0,5 uL
Random Primer	0,5 uL	Random Primer	0,5 uL	Random Primer	0,5 uL	Random Primer	0,5 uL
dH20	1 uL	dH20	1 uL	dH20	1 uL	dH20	1 uL

1) Since the GC rate is high, mix gently and then spin. Incubate at 65^0C for 5 minutes. Then quickly put it back on the ice.
2) Do following reactions to the tubes, respectively.

Table 4 Second reactions of cDNA preparation.

Reaction 1		Reaction 2		Reaction 3		Reaction 4	
Chemicals	Volume	Chemicals	Volume	Chemicals	Volume	Chemicals	Volume
5X Reaction Buffer	4 uL	5X Reaction Buffer	4 uL	5X Reaction Buffer	4 uL	5X Reaction Buffer	4 uL
RNAse Inhibitor	1 uL	RNAse Inhibitor	1 uL	RNAse Inhibitor	1 uL	RNAse Inhibitor	1 uL
10 mM dNTP mix	2 uL	10 mM dNTP mix	2 uL	10 mM dNTP mix	2 uL	10 mM dNTP mix	2 uL
M-MuLV RT	1 uL	M-MuLV RT	1 uL	M-MuLV RT	1 uL	M-MuLV RT	1 uL

3) Gently mix and spin.
4) Incubate at 45^0C for 1 hour.
5) Incubate at 70^0C for 5 minutes.

Direct PCR can be performed on the obtained cDNAs. It is left at -20^0C for storage less than 1 week. For longer storage, it is left at -80^0C. 3 different control PCRs were be performed.

1) First, cDNA will be obtained. Will work on ice again.
2) Do following reactions to the tubes, respectively.

Table 5 Control reactions for cDNA formation.

Reaction 4 (RT Negative Control)		Reaction 5 (RNA Template Negative Control)		Reaction 6 (Positive Control)	
Chemicals	Volume	Chemicals	Volume	Chemicals	Volume
Control GAPDH RNA Template	2 uL	Oligo(dT) Primer	0,5 uL	Control GAPDH RNA Template	2 uL
Oligo(dT) Primer	0,5 uL	Random Primer	0,5 uL	Oligo(dT) Primer	0,5 uL
Random Primer	0,5 uL	5X Reaction Buffer	4 uL	Random Primer	0,5 uL
X Reaction Buffer	4 uL	RNAse Inhibitor	1 uL	5X Reaction Buffer	4 uL
RNAse Inhibitor	1 uL	10 mM dNTP mix	2 uL	RNAse Inhibitor	1 uL
10 mM dNTP mix	2 uL	M-MuL V RT	1 uL	10 mM dNTP mix	2 uL
dH20	10 uL	dH20	11 uL	M-MuL V RT	1 uL
				dH20	9 uL

1) Mix and spin gently.
2) Then incubate at 42°C for 1 hour (This step can be done with the cDNA above).
3) Incubate at 70°C for 5 minutes.
4) Do following reactions to the tubes, respectively and add 2 uL control samples.

Table 6 PCR preparation for control samples.

Reaction 7 (Control PCR) (1X)	
Chemicals	Volume
5X PCR Master Mix	5 uL
GAPDH Forward Primer (10 uM)	1 uL
GAPDH Reverse Primer (10 uM)	1 uL
dH20	16 uL

5) The following PCR conditions were set up and briefly hold the samples at $+4^0$C.

Table 7 PCR condition of control samples.

Steps	Initial Denaturation	Denaturation	Annealing	Extension
Temperature	94°C	94°C	58°C	72°C
Time	3 min	30 sec	30 sec	45 sec
Cycles	1	35		1

PCR is required after cDNA synthesis. A 98 bp product is obtained (*BTK*). 100 ng of cDNA sample was prepared (recommended in 2 µL).

1) The following reaction 8 in table 8 is set up 23 µL of each sample. Add 2 µL of samples onto the reaction.

Table 8 PCR preparation of experiment samples.

Reaction 8 (Sample PCR) (1X)	
Chemicals	Volume
5X PCR Master Mix	5 uL
BTK Forward Primer (1 uM)	0,5 uL
BTK Reverse Primer (1 uM)	0,5 uL
dH20	17 uL

2) The following PCR conditions were set up as table 9.

Table 9 PCR condition of experiment samples.

Steps	Initial Denaturation	Denaturation	Annealing	Extension	Long Extension	Stop
Temperature	94°C	94°C	60°C	72°C	72°C	10°C
Time	3 min	45 sec	45 sec	45 sec	10 min	Hold
Cycles	1	35			1	**

The PCR reaction was prepared as shown in the following table 10. The prepared mix was placed in 0.2 µl PCR tubes for each sample. 2 µl of DNA was added for each sample and 2 µl of dH2O for the negative control.

Table 10 PCR preparation of standards.

Consumables/Chemicals	**1 rxn**	**5 rxn**
PCR Master mix (5X)	5 ul	25 ul
BTK Forward Primer (1 uM)	1 ul	5 ul
BTK Reverse Primer (1 uM)	1 ul	5 ul
dH20	16 ul	80 ul
TOTAL	23 ul	115 ul

3.2.2.3 Agarose gel electrophoresis

Agarose LE was weighed in the amount determined according to the DNA size to be run. After weighing, it was placed in a beaker and 1X TAE Buffer was added as much as the tank would take. It was dissolved in the microwave at 700 setting and 2.5 µl of EtBr was added in it. The gel poured into the tank was waited for gelation. The comb was removed, and the samples were run in the gel at the specified voltage and time.

3.2.2.4 Real-time PCR (qPCR)

The qPCR shown below was set up for 500 ng of adjusted cDNA samples (HEK293, Raji, 3T3 and U937).

Table 11 qPCR template preparation.

1	2	3	5	6	7	
3T3-*BTK*-1	3T3-*BTK*-2	3T3-*BTK*-3	HEK293-*BTK*-1	HEK293-*BTK*-2	HEK293-*BTK*-3	A
3T3-BACT-1	3T3-BACT-2	3T3-BACT-3	HEK293-BACT-1	HEK293-BACT-2	HEK293-BACT-3	B
U937-*BTK*-1	U937-*BTK*-2	U937-*BTK*-3	*BTK*-NC		BACT-NC	D
U937-BACT-1	U937-BACT-2	U937-BACT-3				E
RAJI-*BTK*-1	RAJI-*BTK*-2	RAJI-*BTK*-3				G
RAJI-BACT-1	RAJI-BACT-2	RAJI-BACT-3				H

18 µL of mix and 2 µL of cDNA of the indicated cell samples were added in each well. For the negative control, 2 µL of ddH20 was added.

Table 12 qPCR preparation protocol.

Component	Volume (1 rxn)	Total Volume (13 rxn)
2 × Taq Pro Universal SYBR qPCR Master Mix	10 uL	130 uL
BTK forward primer (10 uM) / B-actin forward primer (10 uM)	0,4 uL	5,2 uL
BTK reverse primer (10 uM) / B-actin reverse primer (10 uM)	0,4 uL	5,2 uL
ddH20	7,2 uL	93,6 uL
Total	18 uL	234 uL

qPCR conditions were adjusted as follows.

Table 13 qPCR condition.

Initial Denaturation		Cycling Reaction (X40)	Melting Curve	
95°C	95°C	58°C	95°C	60°C
30 sec	10 sec	30 sec		

3.2.3.4 Western blotting

Firstly, 1,5 mm gel glass were cleaned and placed in the gel casting module. Seperating gel were prepared as shown at table 14 and poured between of two glasses. After that, stacking gel was poured quickly on top of the running gel.

Table 14 Preparation of SDS-PAGE Gels.

Seperating Gel (10%)			Stacking Gel (4%)	
Chemical	Volume		Chemical	Volume
ddH20	3,2 mL		ddH20	3 mL
30% Acrylamide	2,67 mL		30% Acrylamide	0,67 mL
1,5 M Tris pH 8.8	2 mL		0,5 M Tris pH 6.8	1,25 mL
10% SDS	80 uL		10% SDS	50 uL
10% APS	80 uL		10% APS	50 uL
TEMED	8 uL		TEMED	5 uL

The comb was placed in the stacking gel. The comb was removed, and glass plates were placed carefully. The space between gels was filled with 1X running buffer.

After gel preparation, a BTK protein expression level was measured in 4 different cell lines. First, 5x10^6 cells were proliferated and prepared in RIPA buffer. Protein levels were then measured with the Bradford reagent and an equal amount (10 µg) of protein was prepared for western blotting.

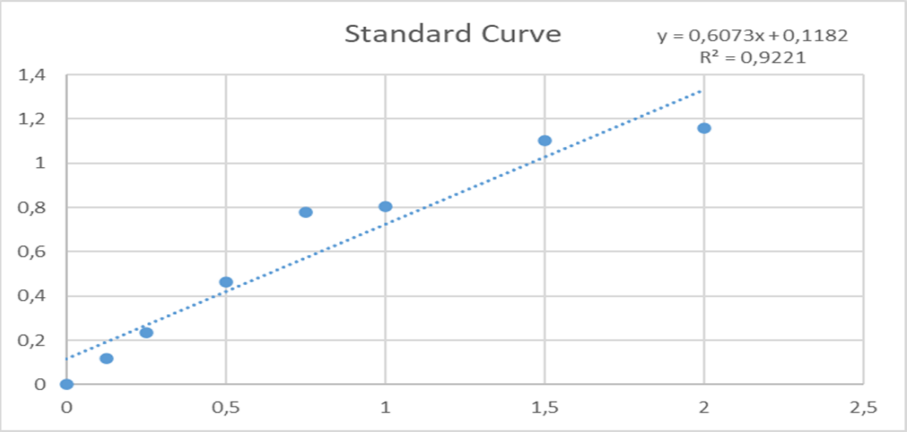

Figure 14 Standard Curve with BSA.

Required amount of the sample buffer was added to the lysates. After the sample buffer was added, samples were boiled in the shaker for 5 minutes at 95°C. After boiling, quick spin was done to collect the water vapors.

Table 15 Protein Concentration of Cell Lines

	First Read	Second Read	Mean	Blank Minus	Conc. (mg/uL)
HEK293	2,086	2,037	2,0615	1,6675	2,55113
3T3	2,079	2,137	2,108	1,714	2,6277
Raji	2,166	2,336	2,251	1,857	2,86316
U937	2,386	2,106	2,246	1,852	2,85493

Samples and the marker were loaded in the slots and run for 80 V 80 min and 100 V 60 min.

Later two sponges, four Whatman papers (9x7 cm) were placed in blot buffer. PVDF membrane (9x7 cm) was incubated for 2 minutes in a centrifuge tube with methanol for activation, after that placed in blot buffer. The blotting system was built up:

- o Sponge (wet, on the black side of the clamp)
- o 2* Whatman (wet, no air bubbles)
- o Gel (The sides of the gel was loosened from the glass and placed on the second filter paper then gel was separated from the glass.)
- o PVDF gel (inside of the gel was marked)
- o 2* Whatman (wet, the air bubbles were rolled out with a pipet.)
- o Sponge (wet, on white side of the clamp)

The clamp was closed and placed in the holder (black side to black side). The holder was placed on ice. The tank was filled with cold blot buffer and an ice block was placed in the tank. Run for 100 V 2 hours. 2,5 grams of milk powder were dissolved in 50 ml TBS-T in roller.

Blots were stained with ponceau S and incubated to see protein bands then de-stained with water. Then blots were put in 50 ml centrifuge tube tubes. 5 ml of milk solution was added to a centrifuge tube. The blots were shaken during 1 hour on roller. After 1 hour, milk solution was removed, then washed with 0.05% PBST. Blot was

stained with the first antibody diluted in milk solution (5 ml milk solution with 5µL first antibody) first and incubated at 4°C on a roller bank overnight. Next day, blot was rinsed TBS. Blot was stained with the second antibody diluted in milk solution (5 ml milk solution with 5µL secondary antibody) for 1,5 hours at RT on a roller bank. Milk solution was removed, blot was washed 3 times with 1X TBS for each 5 min, and 3 times with 1x in TBS-T for 5 min and finally 1X PBS. 2 ml ECL mix was prepared. (Solution A: solution B = 1: 1). The blot was placed in a cassette and interested bands were marked with WB pen. The blot was covered with ECL then incubated 5 min in the dark. In that time, ECL on the blot was controlled to cover. Image was taken.

3.2.3 CRISPR/Cas9 vector design

To create the CRISPR/Cas9 system, two different plasmid vectors and a 200 bp double-stranded oligonucleotide were designed.

The first plasmid vector was EFSco*BTK*.

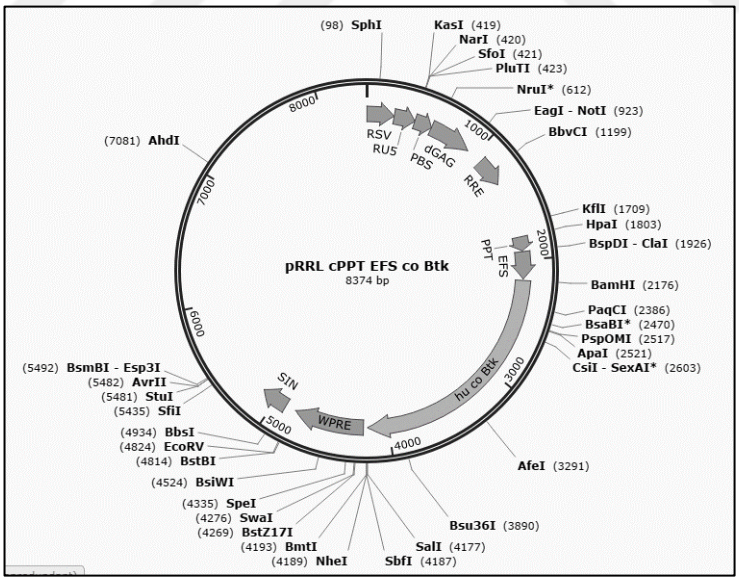

Figure 15 EFSco*BTK* plasmid.

Human *BTK* gene optimized EFS promoter. Plasmid has self-inactivating (SIN) feature. Restriction enzymes are shown at Figure. WPRE Woodchuck Hepatitis Virus (WHV) Posttranscriptional Regulatory Element (72).

The other plasmid vector was ordered via Addgene: pLenti-NG-PE2-BSD Addgene code: #176933 (Lentiviral based plasmid vector containing Sp-NG Cas9).

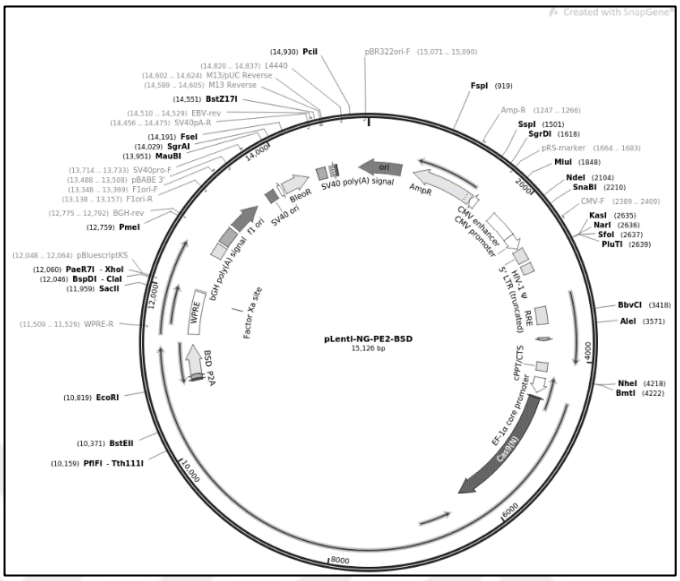

Figure 16 pLenti-NG-PE2-BSD plasmid.

There is Sp-NG-Cas9 optimized EF-1a. It is lentiviral based vector. It has prime editing 2 system sequences and blasticidin (BSD) (from Addgene).

Finally, to obtain a suitable plasmid vector for pegRNA, the designed 200 bp pegRNA sequence was ligated into the EFSco*BTK* plasmid by cutting out the *BTK*.

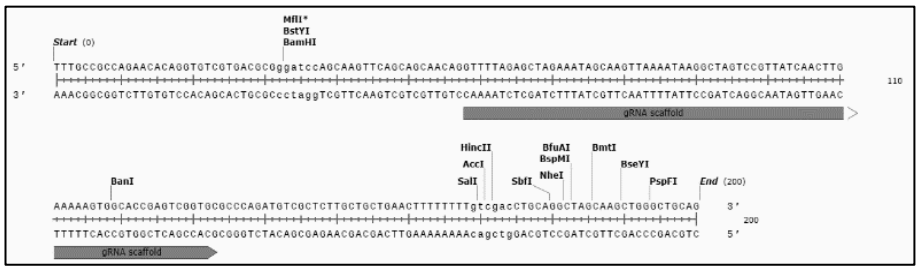

Figure 17 Design of pegRNA.

A special guide RNA, pegRNA, is required for the prime editing CRISPR/Cas9 system. pegRNA sequences created by pegFinder. BamHI and SalI cut the oligonucleotide included pegRNA and ligated to EFSco*BTK* vector (without *BTK*).

The variant (ID008) that was determined in our previous study was chosen as target to design CRISPR/Cas9 vector. The variant c.1731A>C was a missense

mutation at exon 17 on *BTK* gene. The patient showed no B-cells but a slight *BTK* protein was detected in the Western Blot analysis.

The variant c.1731A>C was a missense mutation at exon 17 on *BTK* gene.

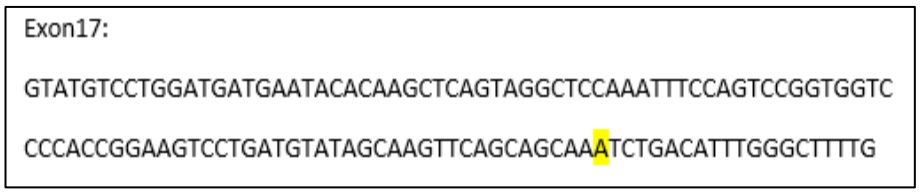

Figure 18 Wild type base on exon 17 of *BTK* (ENG00000010671) gene from Ensembl.

Plasmids that on the below were cultured in LB agar medium, subsequently LB medium overnight for future studies according to the protocol of Culturing of Bacteria.

- The first plasmid vector is EFSco*BTK*.
- The other plasmid vector was ordered via Addgene: pLenti-NG-PE2-BSD Addgene code: #176933 (Lentiviral based plasmid vector containing Sp-NG Cas9).

After that, designed 200 bp pegRNA oligonucleotide was inserted in EFSco*BTK* plasmid by RE cutting & ligation according to the protocols of Restriction Enzyme Digestion and Ligation, respectively, to obtained EFScopegRNA plasmid.

3.2.3.1 DNA extraction from gel

First, the gel piece containing the 6373 bp plasmid was cut with a sterile scalpel. Based on approximately 100 mg gel~100 µL Buffer QG, 3 volumes of Buffer QG were added. Since the measured gel piece was 100 mg, 300 µL of Buffer QG was added. It was incubated at 50^0C for 10 minutes. The gel and buffer mixture were vortexed at 2–3-minute intervals. After incubation, 100 µL of 100% isopropanol was added to the mixture. It was added to QIAquick spin column. It was centrifuged for 1 min at 12,000 g. Discard flow-through and 500 µl of Buffer QG was added into column. It was

centrifuged for 1 min at 12,000 g. After discard flow-through, 750 µl of Buffer PE was added and centrifuged at 12.000 g for 1 min. The QIAquick spin column was placed in a sterile microcentrifuge tube. 35 µl of Buffer EB was added and incubated for 1 minute at room temperature. It was centrifuged for 1 min at 12,000 g. Measurements were taken in the nanodrop and placed at -80°C.

3.2.3.2 Restriction enzymes cutting

CutSmart 10X Buffer, enzymes, plasmid and nuclease free water was placed in a reaction tube. The reaction tube was incubated at 37°C for 1 hour. Then it was kept at 65°C for 15 minutes to stop the cutting. The cutting product was loaded into 1% gel and run at 100 Volts for 1 hour.

As a special case, DpnI enzyme digestion was performed overnight at 37°C.

3.2.3.3 Ligation

T4 DNA Ligase (5 U/µL) (EL0011) was used. According to this;

Firstly, the EFSco*BTK* plasmid was digested with BamHI and SalI and run in 1% agarose gel. As a result, the EFSco backbone portion was separated by 6373 bp and the *BTK* portion by 2001 bp. The EFSco backbone was purified with the DNA extraction from gel protocol. Purified EFSco put at -80°C.

The pegRNA sequence was synthesized by commercial services and diluted with 20 µL of ddH20, and then the amount to be used for ligation was determined by measuring in the nanodrop. PegRNA from 2 µL (20 ng) was digested with BamHI and SalI to obtain a sticky end.

Calculation was made with NEB Ligation Calculator.

Accordingly, the following ligation reaction was prepared and left to incubate at 16^0C for overnight.

Table 16 Ligation preparation for ligate between of EFSco and pegRNA.

Component	Volume
EFSco backbone	0,5 uL (20 ng)
pegRNA (with stick end)	20 uL (20 ng)
10X T4 DNA Ligase Buffer	2,5 uL (1X)
T4 DNA Ligase	2 uL

3.2.4 In-vitro mutagenesis

The primers described below were designed for mutagenesis studies. *BTK* primers (1) were designed to be plasmid and site-specific to be 100% match, but Mutagenesis primers (2) were not designed to be 100% match to detect the A>C change (our plasmid G>C).

The PCR reaction was set up according to the protocol specified in the kit. Specified PCR conditions were run again shown at table 16.

Table 17 PCR condition of mutagenesis in mutagenesis kit.

Temperature	98°C	98°C	70°C	72°C	72°C	4°C
Time	3 min	10 sec	30 sec	9 min	10 sec	Hold
		35 X				

At the end of the PCR, the samples were placed at -80°C.

Transformation into *E. coli* DH5a cells was performed as specified in the kit protocol.

1) *E. coli* DH5a cells from -80°C were placed on ice.

2) 50 µL of *E. coli* DH5a was placed in 1.5 mL sterile microcentrifuge tubes.
3) Three tubes were prepared. In the first, 15 µL of the PCR sample obtained because of PCR and thought to carry the determined variant was placed. A negative PCR sample (which does not contain the EFSco*BTK* plasmid) was placed in the second. In the last one, no plasmid was added in order to be transformation negative.
4) The samples, which were kept in ice for 30 minutes, were quickly taken to 42°C and kept for 45 seconds.
5) Finally, it was quickly put on ice and left for 2 minutes.
6) Samples were plated on LB agar plate containing ampicillin.
7) Left overnight at 37°C.
8) After 16 hours, 5 selected colonies were taken into 5 mL LB broth containing ampicillin and cultured (37°C/180 rpm) for 12 hours.
9) After 12 hours, 500 µL was taken from the samples and placed in 500 µL glycerol (50%) and put at -80°C.

Plasmid isolation was performed from the remaining samples by applying the protocol specified in the title Zymo Universal Mini-Prep Kit and the samples were measured in the nanodrop.

PCR was performed on the isolated plasmids according to the protocol specified in the title 3.2.2.2 Polymerase Chain Reaction (PCR) and then sent to Labmed for Sanger sequencing.

Alternative protocols were made. Firstly, the master mix included in the kit mentioned above was used. Two separate PCR tubes were set up as follows.

Table 18 PCR preparation of alternative mutagenesis protocol.

	PCR Tube I	Final Concentration
Master mix Proofreading DNA pol. (2X)	10 uL	1 X
BTK reverse primer	1 uL	0,5 uM
BTK forward mutagenesis primer	1 uL	0,5 uM
DNA plasmid (EFSco*BTK* plasmid)	1 uL	300 ng
dH20	7 uL	-
	PCR Tube II	Final Concentration
Master mix Proofreading DNA pol. (2X)	10 uL	1 X
BTK forward primer	1 uL	0,5 uM
BTK reverse mutagenesis primer	1 uL	0,5 uM
DNA plasmid (EFSco*BTK* plasmid)	1 uL	300 ng
dH20	7 uL	-

PCR conditions are shown below, respectively; PCR Tube I and PCR Tube II.

Table 19 PCR conditions of alternative mutagenesis protocol.

Temperature	98°C	98°C	68°C	72°C	72°C	4°C
Time	3 min	10 sec	30 sec	2 min	10 sec	Hold
		35 X				
Temperature	98°C	98°C	68°C	72°C	72°C	4°C
Time	3 min	10 sec	30 sec	30 sec	10 sec	Hold
		35 X				

After the PCR, the following manual step was applied.

1) Two PCR tubes were mixed.
2) PCR conditions were applied.
3) Cutting was done with DpnI enzyme.
4) The transformation used in the kit protocol was performed.

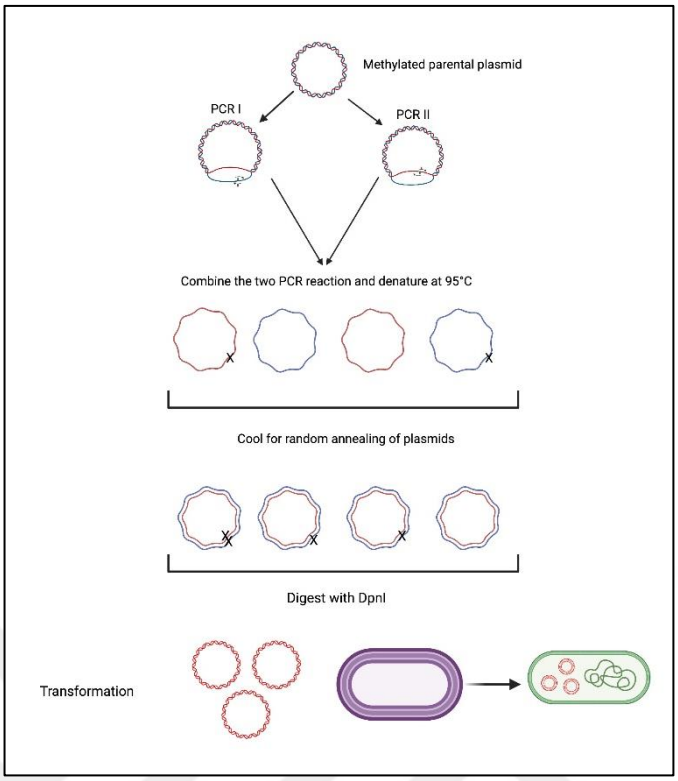

Figure 19 Schema of mutagenesis alternative protocol (73).
(This Figure is drawn with BioRender).

Mutagenesis was taken as a service. By the techniques described above the mutagenesis cannot be fully achieved. Hence the mutagenesis could not be created. Therefore, mutagenesis service was obtained from Genscript. In this service, 2000 ng of plasmid dried on whatmann paper and 2000 ng of plasmid at room temperature were sent to Singapore laboratory of Genscript.

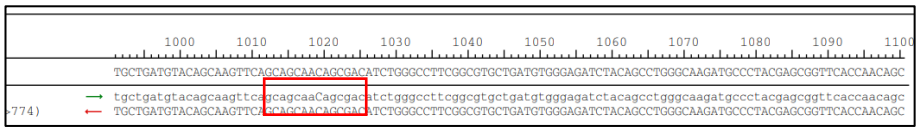

Figure 20 Mutagenesis sanger sequencing from Genscript.
Variant is shown in red square (G>C in plasmid).

3.2.4.1 Transformation

The competent cell at -80°C was placed on the ice block. Plasmid standing at -20°C was taken. 50 µl of cells are taken into 2 microcentrifuge tube tubes. 15 µl of

plasmid was placed in one of the tubes and accepted as positive control, and 15 µl of dH2O was placed in the other and considered negative control. Sample tubes were placed in an ice block and incubated at +4°C for 30 minutes. Tubes were placed on the heat block previously set to 42°C and incubated for 45 seconds. Samples were taken back on the ice block and incubated for 5 minutes. 600 µl of SOC medium was added to two 15 mL centrifuge tubes. The samples were placed in SOC medium and incubated for 1 hour at an angle of 45° in a 200-rpm incubator at 37°C. 300 µl samples were dropped on the agar plate dropwise and spread with a spreader. Agar plates were incubated overnight at 37°C.

3.2.4.2 Culturing of bacteria

First, the bacterial stock to be used was selected and placed on ice. Different protocols were followed according to the status of the bacterial main stock.

A sterile loop was immersed in the main stock and the loop was mixed in the liquid. Then, it was streaked onto the previously prepared LB Agar medium (containing ampicillin). The main stock was placed at -80^{0}C and cultured overnight at 37^{0}C in plate containing LB agar medium. A medium-sized, colony was selected from the colonies formed after overnight incubation. Again, with the help of a sterile loop, this colony was taken and placed in 5 mL of LB medium (containing ampicillin) in the 50 mL centrifuge tube prepared before and mixed. It was incubated for 12-16 hours at 37^{0}C at 180-200 rpm. The LB Agar was stored at +4^{0}C for 1 month. The LB Medium was stored by making glycerol stock.

500 µL of the main stock was taken and placed in 4.5 mL of LB medium (containing ampicillin) in a 50 mL centrifuge tube previously prepared. It was incubated for 12-16 hours at 37^{0}C at 180-200 rpm. The LB Medium was stored by making glycerol stock.

The same protocol was followed for larger scale production; 1/10 odds; culture/weather and 1/5 odds; main stock/medium.

3.2.4.3 Plasmid isolation

PureLink™ HiPure Plasmid Filter Maxiprep Kit, Zymo Quick-DNA™ Miniprep Plus Kit and GeneJET Plasmid Miniprep Kit were used, respectively.

First, the Equilibration Buffer (EQ1) included in the kit was put into the filtration cartridge and it was expected to flow thanks to gravity. Meanwhile, *E. coli DH5a* culture, which was left overnight in 500 mL LB medium before isolation, was taken into blue capped bottles and 4000 g for 10 minutes centrifuged, and the supernatant was discarded. 10 mL of Resuspension Buffer (R3) with RNase A was added to the bottles and the pellet was resuspended. 10 mL Lysis Buffer (L7) was added when there was a homogeneous image. Pipette done, never vortex. After homogenization, it was kept at room temperature for 5 min. 10 mL of Precipitation Buffer (N3) was added to it and inverted rapidly until homogenized, no vortexing. The resulting homogenized solution was added to the column and allowed to flow by gravity. The column was then washed in the same way with 10 mL of Wash Buffer (W8). The inner filtration cartridge in the column system was removed and washed again with 50 mL Wash Buffer (W8). The flow-through was discarded and a 15 mL Elution Buffer (E4) column was added to elute the plasmids through the filter. All solution was allowed to flow through the filter into a sterile centrifuge tube by gravity. The resulting plasmid was measured with a nanodrop device. The samples were placed at -80^{0}C.

E. coli DH5a culture, which was left overnight in 5 mL (500 µL of sample glycerol stock was made) before isolation, was centrifuged at 500 g for 10 minutes, and the supernatant was discarded. The pellet was resuspended with 200 µL of DNA elution Buffer. 200 µL of Bio Fluid & Cell Buffer (Red) and 20 µL of Proteinase K were placed on the sample. The sample, which was homogenized with the help of vortex, was incubated at 55^{0}C for 10 minutes. 420 µL of Genomic Binding Buffer was added to the sample and homogenized with the help of vortex. The homogenized sample was placed on the Zymo-Spin IIC-XLR column in the Collection tube and centrifuged at 12.000 g for 1 minute. The collection tube was discarded, and the column was placed in a different collection tube. 400 µL of DNA Pre-Wash Buffer was added and

centrifuged at 12.000 g for 1 min. Flow-through was discarded. 700 µL of g-DNA Wash Buffer was added and centrifuged again at 12.000 g for 1 min. Flow-through discarded. Finally, 200 µL of g-DNA Was Buffer was added and centrifuged at 12.000 g for 1 minute, the collection tube was discarded. DNA Elution Buffer was preheated at 65^0C and 50 µL of buffer was placed on the column, which was placed in a sterile 1.5 mL ependorf. It was incubated for 5 min at room temperature. It was centrifuged at 12,000 g for 1 min and measured in a nanodrop. The samples were placed at -80^0C.

The bacterial culture was transferred to a sterile microcentrifuge tube to a maximum volume of 5 mL. It was centrifuged for 1 min at 12,000g. The supernatant was discarded and the pellet dissolved with 250µL resuspension solution. 250 µL Lysis Solution was added to the homogenised solution and mixed by inverting 4-6 times. 350 µL neutralisation solution was added and mixed by inversion 4-6 times. The solution was centrifuged for 5 minutes at 12,000 g. The supernatant was transferred to the GeneJET spin column. The supernatant was transferred to the GeneJET spin column and centrifuged at 12,000g for 1 minute. 500 µL of the wash solution was added to the GeneJET spin column and the supernatant was centrifuged at 12,000 g for 1 minute. The flow-through was discarded and the same procedure was repeated. The empty tube was centrifuged for a final time at 12,000g for 1 minute. The GeneJET spin column was loaded with a new and sterile 1.5 mL Ependorf, 50 µL of elution buffer was added to the column and it was held at room temperature for 2 minutes. It was centrifuged at 12,000g for 1 minute, measured in a nanodrop and stored at -20^0C.

3.2.5 Transfection of mutant plasmid and CRISPR/Cas9 system

$2x10^5$ 3T3 cells were seeded in 6 well plates before one day from transfection and cultured according to the protocol except the medium (DMEM without FBS and P/S).

Plasmid stocks were prepared according to the table 20.

Table 20 Preparation of plasmids for transfection.

Plasmid (Concentration)	ng/well	uL/well	Total wells	Total ng needed	Total uL needed
EFScoBTK (1340 ng/uL)	2000 ng	1,49 uL	18 well	36.000 ng	26,82 uL
Mutant EFScoBTK (1229,6 ng/uL)	2000 ng	1,62 uL	18 well	36.000 ng	29,16 uL
EFScopegRNA plasmid (585,8 ng/uL)	500 ng	0,85 uL	6 well	3.000 ng	5,1 uL
Cas9 plasmid (1224,5 ng/uL)	500 ng	0,40 uL	6 well	3.000 ng	2,4 uL

After plasmid preparation, samples were prepared according to the table 21.

Table 21 Plate design for transfection study.

Flow	RNA	Western	Plasmid	Fugene	Total	per well mix	per well medium
48H							
Unt	Unt	Unt	0,00	0,00			
mBTK	mBTK	mBTK	9,72	29,16	38,88	6,48	93,52
wtBTK	wtBTK	wtBTK	8,94	26,82	35,76	5,96	94,04
mBTK_EFS	mBTK_EFS	mBTK_EFS	18,66	55,62	74,28	12,38	87,62
mBTK_Peg_Cas	mBTK_Peg_Cas	mBTK_Peg_Cas	26,16	78,48	104,64	17,44	82,56
			3	3			

During the transfection, DMEM (w/o FBS and P/S) was added first, then Fugene was added and it was incubated at room temperature for 15 minutes. Finally, plasmids were added it and the mix applied on the cells after 15 minutes at room temperature. After 48 hours cells were collected and stocked in RLT buffer for qPCR and RIPA buffer for western blotting. Last cells were collected in PBS and applied flow cytometry protocol for checking intracellular *BTK*.

- 1,62 uL plasmid m*BTK* + 4,86 uL Fugene + 93,52 uL DMEM
- 1,49 uL plasmid EFSco*BTK* + 4,47 uL Fugene + 94,04 uL DMEM
- 3,11 uL plasmid m*BTK* ve EFSco*BTK* + 9,33 uL Fugene + 87,56 uL DMEM
- 1,62 uL plasmid m*BTK* + 1,49 uL EFSco*BTK* + 0,85 uL pegRNA + 0,40 uL cas9 + 13,08 uL Fugene + 82,56 uL

4 RESULTS

4.1 Assessment of *BTK* mRNA and Protein Expression Levels

4.1.1 Analysis of intracellular *BTK* in different cell lines

First, *BTK* expression in various cell lines were determined by flow cytometry and determined the model cell line to optimize our proof of principle. According to these results as expected Jurkat, a T-cell line, presented a low *BTK* expression (3.63%) and Raji and U937 cells showed similar *BTK* levels as respectively (50.89% and 53.98%). 3T3 cell lines showed lowest *BTK* levels as 1.73% (Figure 21).

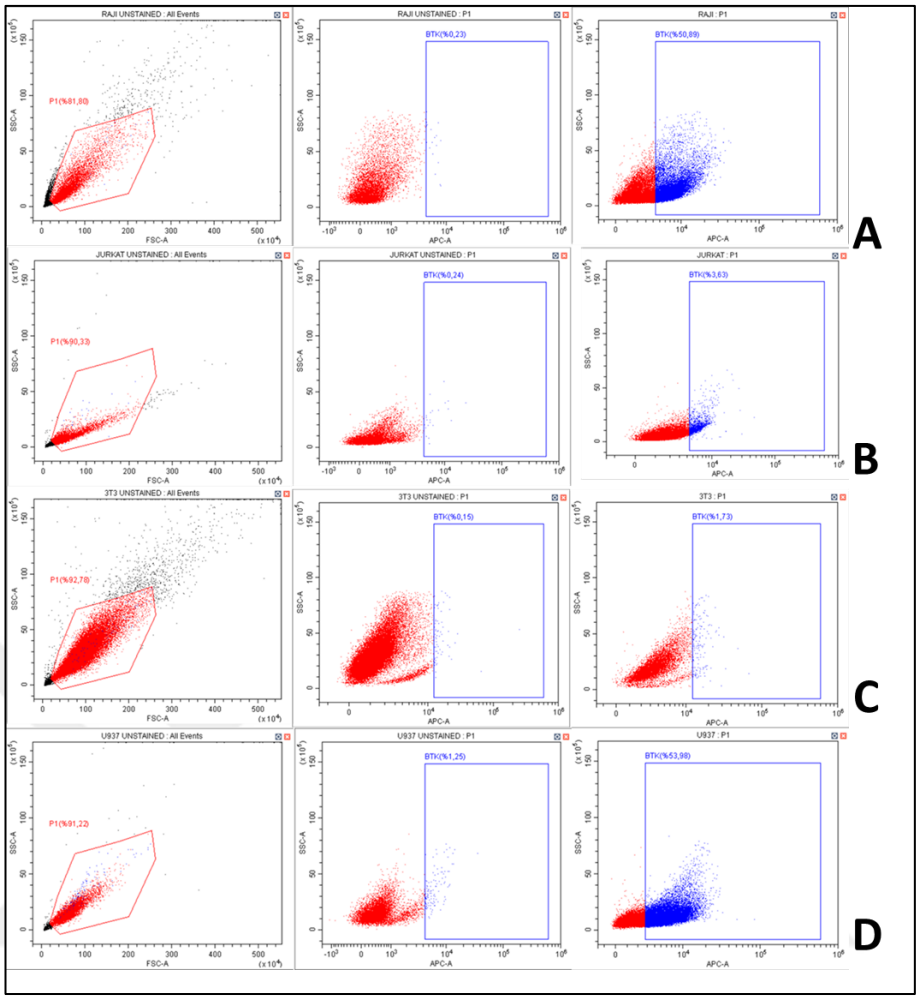

Figure 21 Intracellular *BTK* level of different cell lines by flow cytometry.

Column 1 shows the viable cell population. Column 2 shows *BTK* before staining and Column 3 shows positive *BTK* after staining with Alexa Fluor 647 anti-*BTK*. A. *BTK* level is 50.89% in Raji cells. B. *BTK* level is 3.63% in Jurkat. C. *BTK* level is 1.73% in 3T3. D. *BTK* level is 53.98% in U937.

4.1.2 Relative expression of *BTK* gene in different cell lines

The *BTK* mRNA levels in different cell lines were determined by both RT-PCR and qPCR (Figure 22 and Figure 23).

To determine the *BTK* expression on mRNA level, total RNA was isolated from HEK293, 3T3, Raji and U937 according to the mentioned protocol. Samples were measured in the nanodrop and stored at -80°C.

After RNA isolation, cDNA isolation was performed and is shown in Figure 24. The *BTK* mRNA expression could not be determined in 3T3 cells by RT-PCR, but in Raji and U937 cells.

GAPDH positive control has 496 bp products and negative controls has no bands.

As shown in Figure 22, cDNA was made from HEK293 cells to compare with 3T3 cells and it was observed again that *BTK* gene was expressed in U937 and Raji cells, but little or no expression in 3T3 and HEK293 cells. However, there was a faint band in HEK293 compared to 3T3.

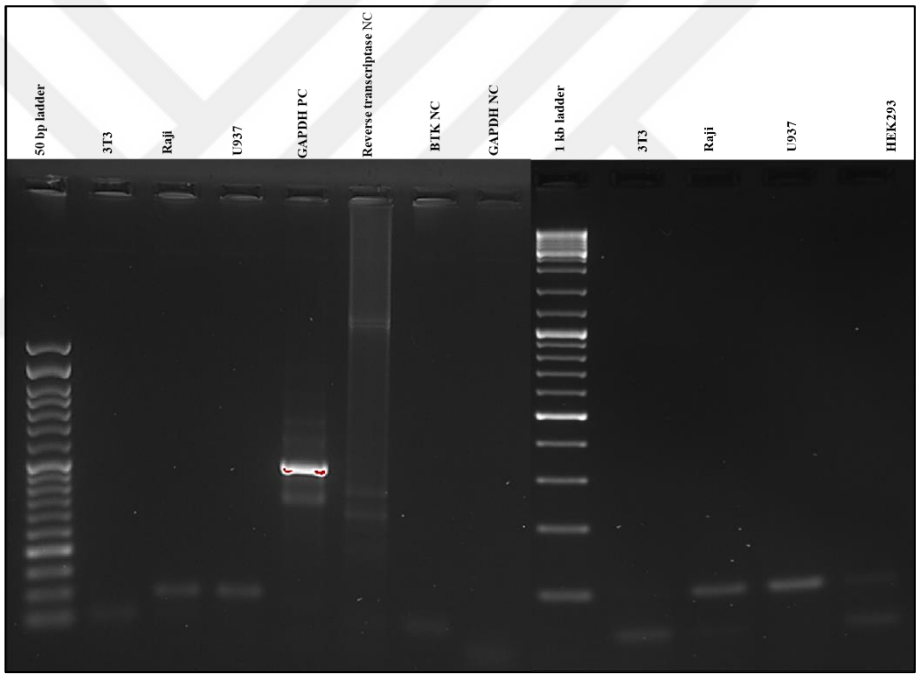

Figure 22 RT-PCR Results of Cell Lines and Controls

In qPCR study, the lowest mRNA *BTK* expression was determined in 3T3 and the highest was in U937 as shown in Figure 23.

Three replicates were studied by qPCR for verification. No significant difference between replicates of cell lines samples was observed ($p<0.05$) (Figure 23).

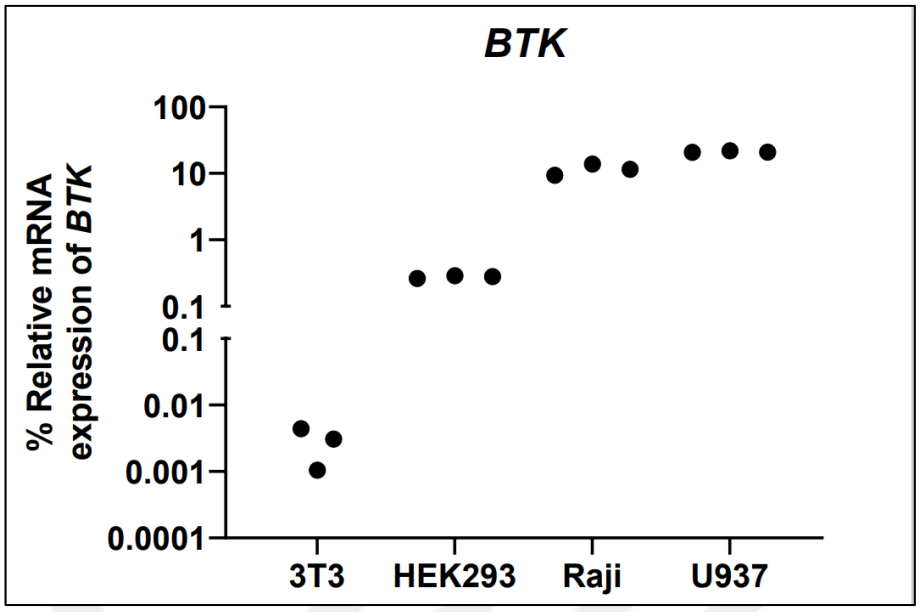

Figure 23 Three replicates' values of qPCR experiment.

4.1.3 Analysis of BTK protein

A western blotting study was performed to determine the BTK protein expression level in different cell lines. As showed in Figure 24, although the expression level is very low in 3T3 and HEK293 cell lines, it is quite high in Raji and U937 cell lines.

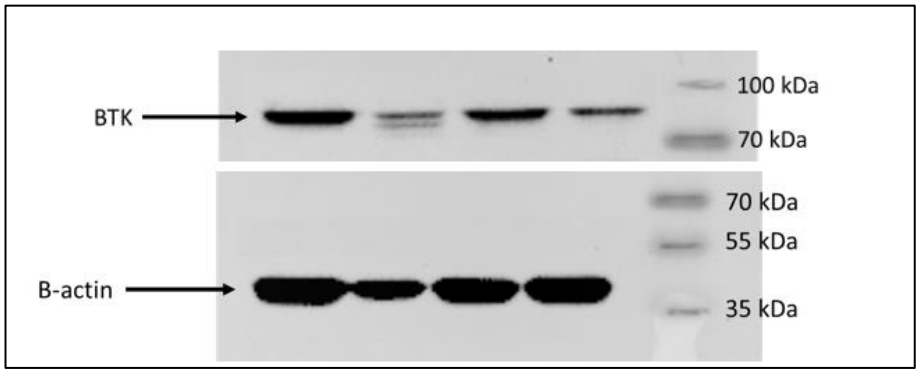

Figure 24 BTK protein of Cell Lines; U937-HEK293-Raji-3T3.
B-actin was used as internal control.

The western blot intensities were also measured by Biorad Image Lab and evaluated and 3T3 cell line showed the lowest protein amount among all other cell lines (Figure 25).

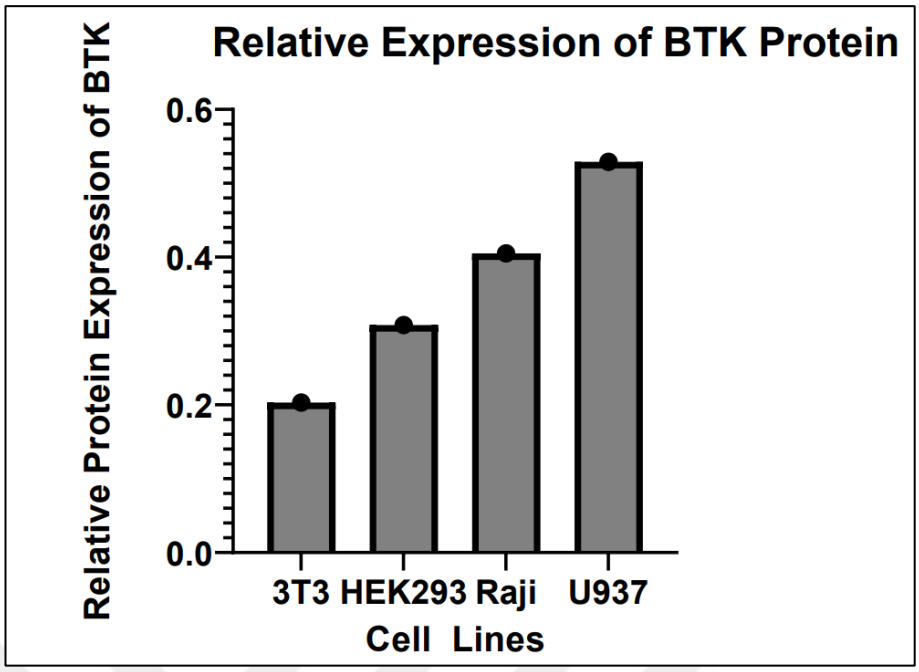

Figure 25 Relative protein expression of BTK protein by using Biorad Image Lab.

4.2 Confirmation of Plasmids

EFSco*BTK* plasmids were transformed into *E. coli DH5a* cells according to the protocol. Then, maxi purification was performed to increase the amount of plasmid and used for future studies. The plasmids were digested with BamHI and EcoRI restriction enzymes to validate that the obtained plasmid was EFSco*BTK* (Figure 26). EFSco*BTK* was digested into three pieces and this result confirmed that the plasmid was EFSco*BTK*.

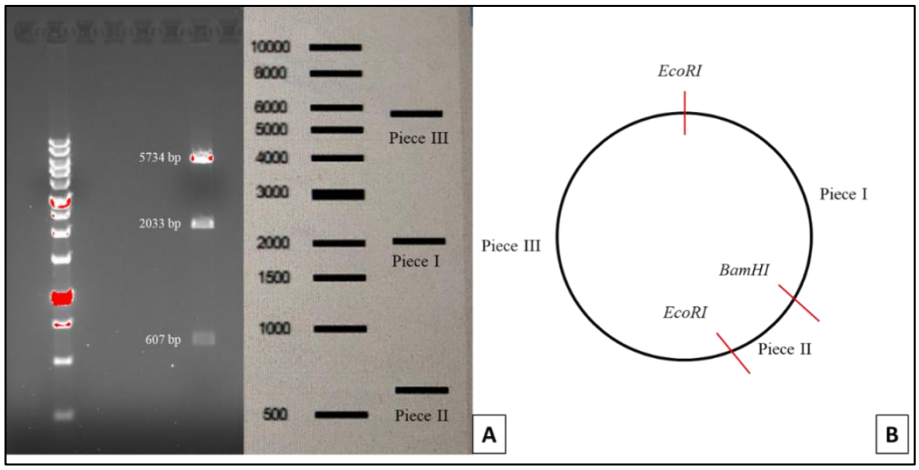

Figure 26 A. EcoRI and BamHI cutting of EFSco*BTK*.
Bands are respectively 5734 bp, 2033 bp and 607 bp. B. EcoRI and BamHI cutting of EFSco*BTK* of vector schema.

The pLenti-NG-PE2-BSD plasmid was purchased via Addgene. It was cultured and isolated from *E. coli DH5a*. Later, it was digested with EcoRI and NheI restriction enzyme and confirmed (Figure 27).

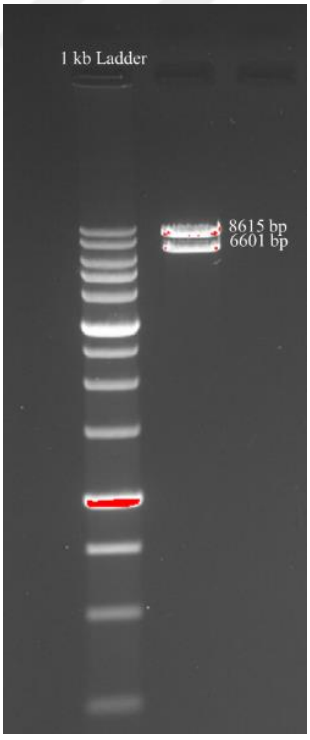

Figure 27 pLenti-NG-PE2-BSD plasmid with cutting EcoRI and NheI.
Band sizes are 8615 bp and 6601 bp, respectively.

As previously mentioned, EFSco*BTK* was digested with BamHI and SalI enzymes and BamHI and NheI enzymes to generate the EFScopegRNA plasmid. As seen in Figure 28, although there was no significant cut-off difference, BamHI and SalI were chosen for the continuity of the next experiments and the cutting efficiency.

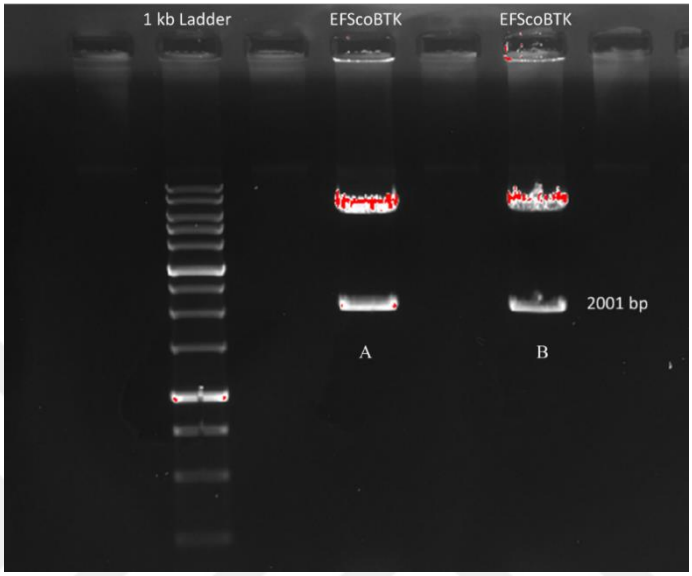

Figure 28 EFSco*BTK* cutting.
(A) EFSco*BTK* with BamHI and NheI (B) EFSco*BTK* with BamHI and SalI.

2000 ng of EFSco*BTK* plasmid was digested with BamHI and SalI for pegRNA ligation. After loaded the entire amount into the gel, it was cut with a sterile scalpel as shown in Figure 29 and the EFSco backbone, *BTK*-free plasmid, was isolated as described in the methods.

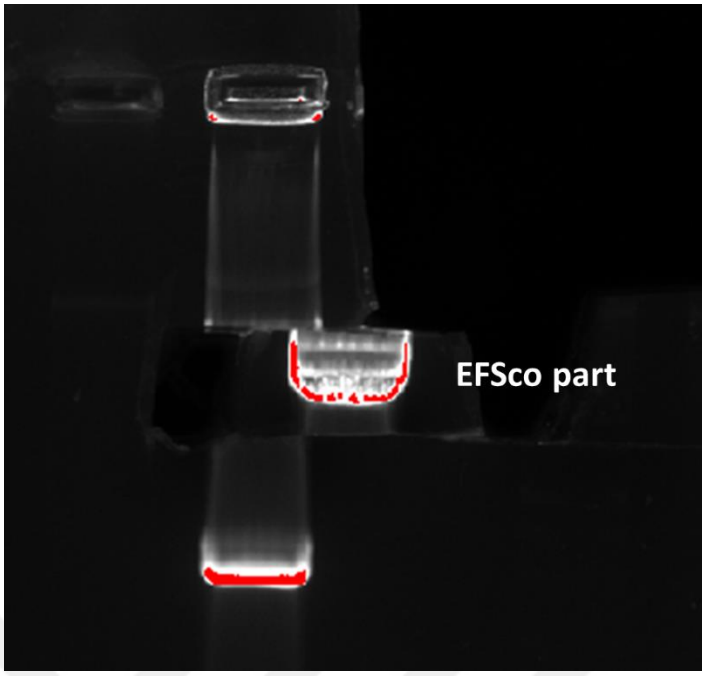

Figure 29 EFSco*BTK* with BamHI and SalI for obtain empty vector backbone.
The cut gel is shifted to the left to better understand the EFSco part of the plasmid.

The new plasmid generated by inserting the pegRNA into EFSco vector was called EFScopegRNA. Since there was more than 6 kb difference between insert and vector, 1:1 ligation was performed. As a result, a successful ligation occurred, but a non-ligate vector that did not enter the ligation process was also seen due to 1:1 ratio (Figure 30).

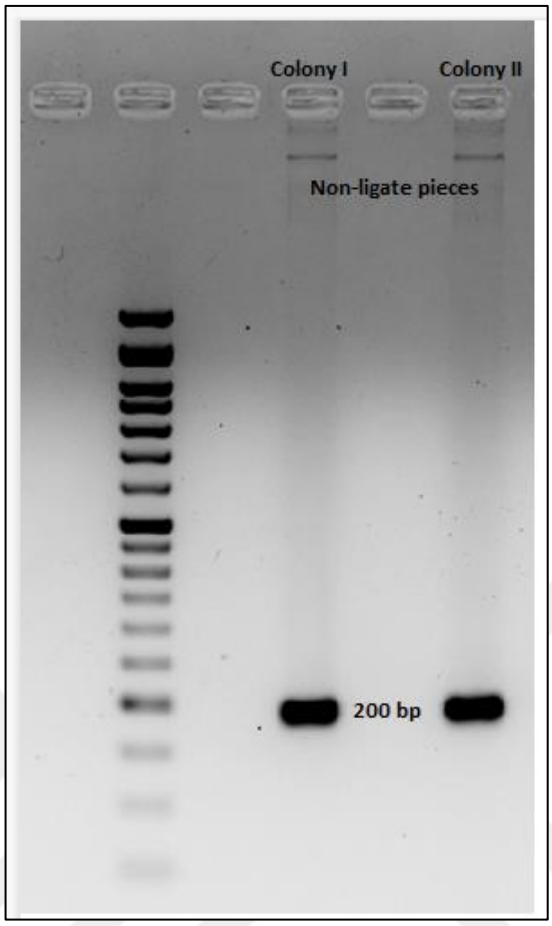

Figure 30 EFScopegRNA ligation PCR control.

4.3 Creation of c.1731A>C Missense Variant

Pick-mutant site directed mutagenesis kit were used. There were no colonies on negative control plate. On the other hand, based on standard procedure, there were many colonies on transformation plate and were selected in groups of 5 and cultured as mentioned before (Figure 31).

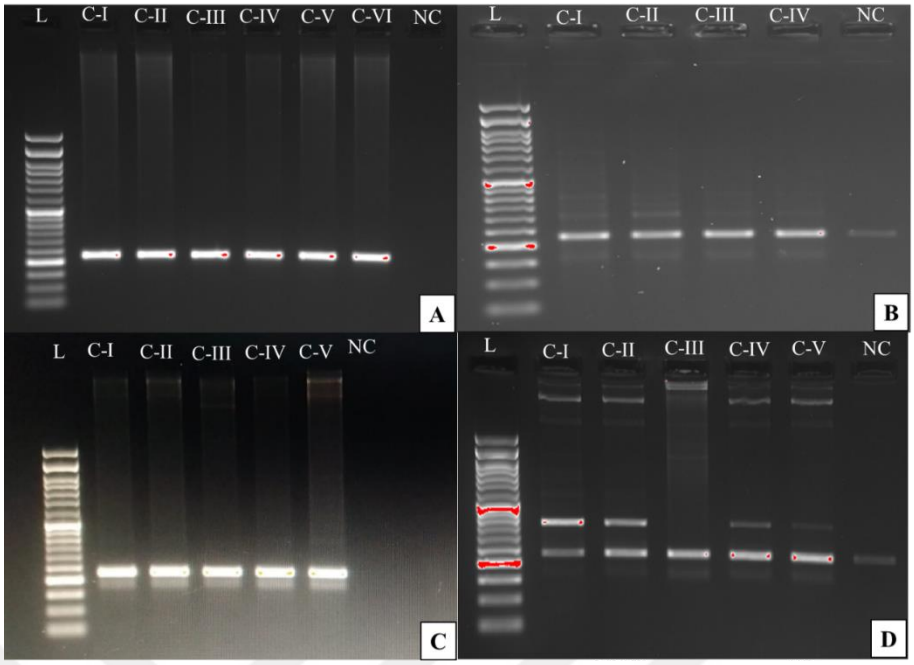

Figure 31 Example Images of Agarose Gel Electrophoresis of PCR Products of Missense Variant G>C.
(A-B) Mutation by Pick-mutant mutagenesis kit. (C-D) Mutation by Alternative Protocol.

After plasmid isolation from colonies, plasmids were measured at nanodrop, as shown in Table 22.

Table 22 Plasmid concentration of transformant colonies**.**

Sample	Concentration (ng/uL)	A260/280	A260/230
Colony I	145.0	1.90	0.33
Colony II	266.0	1.90	2.14
Colony III	140.3	1.88	2.01
Colony IV	142.8	1.87	1.31
Colony V	262.1	1.91	2.21

When the results were examined, no mutation was observed in two colonies and S reading was observed in three colonies. This reading says Guanidine or Cytosine. For this reason, a different and manual method was tried by searching the literature. Unfortunately, the method failed to create mutation (Figure 32).

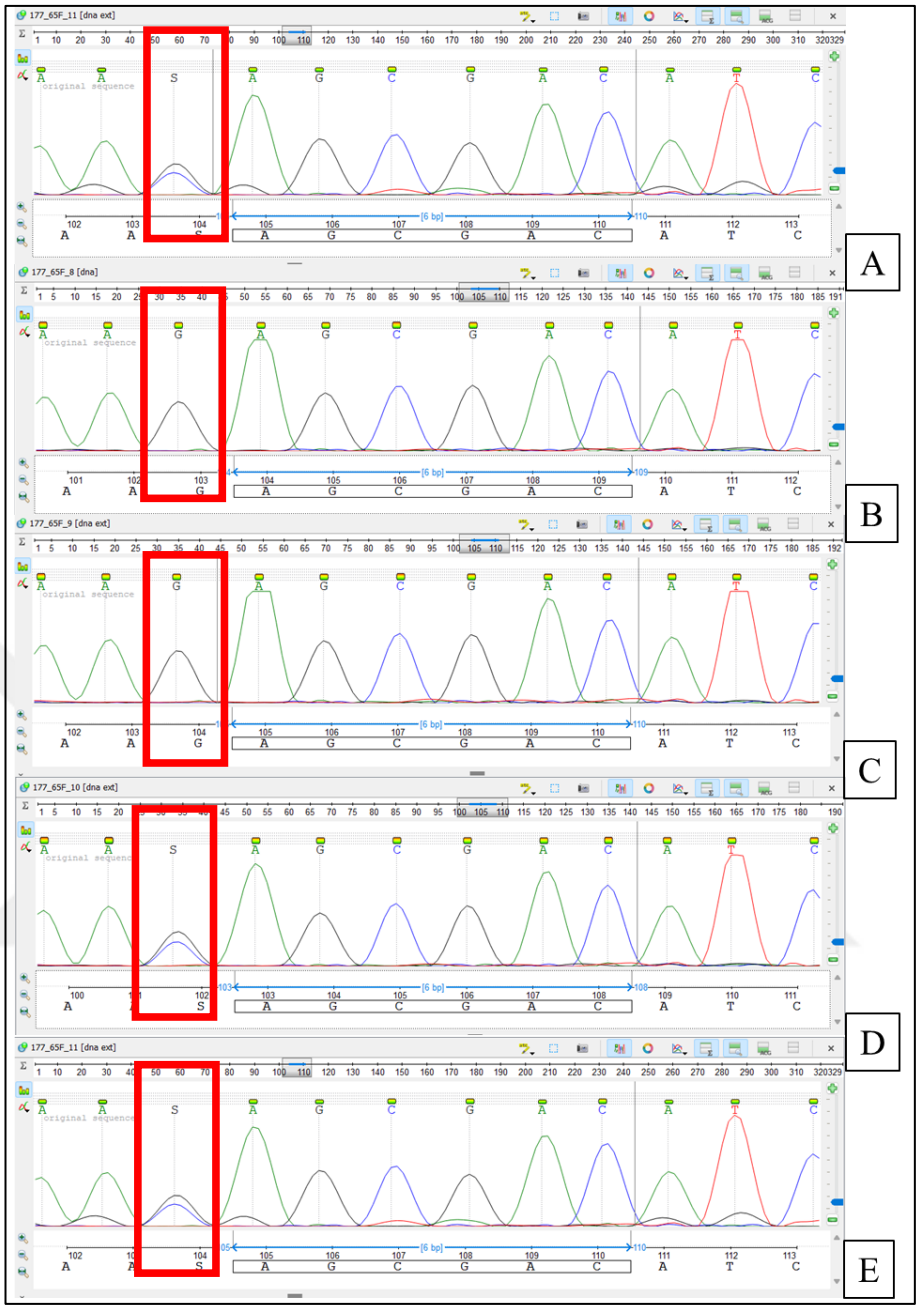

Figure 32 Sanger analysis of transformant colonies.
The diagram displays the bases shown in the red square were the bases where mutations were expected to occur. Specifically, a heterozygous variant (A-D-E) was generated, while no mutation was observed in (B-C). In this context, the letter "S" denotes either Guanine or Cytosine, whereas Adenine is represented by A, Cytosine by C, Guanine by G, and Thymine by T.

Since the in vitro mutagenesis applications were failed was ordered as plan B in vitro mutagenesis as commercial service from Genscript (Figure 33).

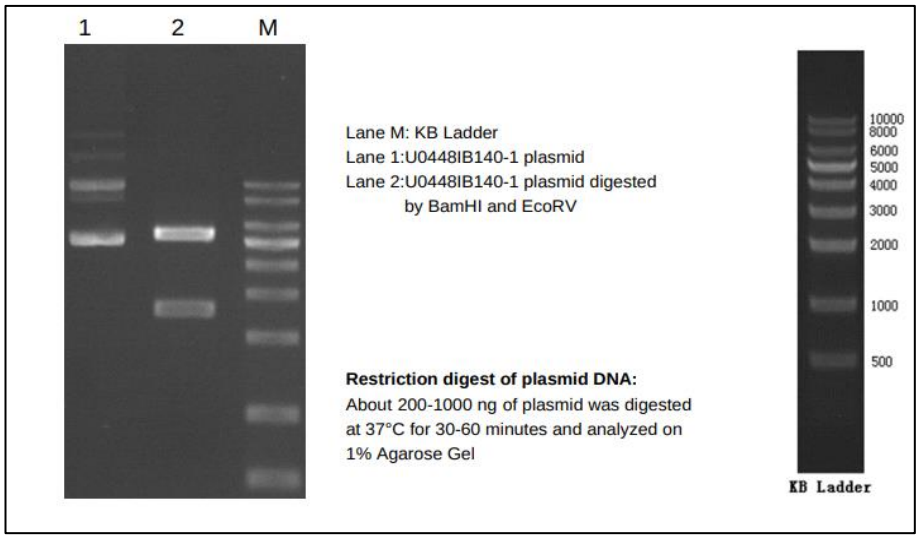

Figure 33 Mutant EFSco*BTK* plasmid confirmation from Genscript

4.4 Verification of CRISPR/Cas9 Repair Potential on 3T3 Cells

Firstly, plasmids transfected to 3T3 cells were measured in flow cytometry to determine the intracellular BTK levels. As seen in Figure 34, plasmids were transferred to 3T3 cells. According to flow cytometry results, intracellular BTK level in 3T3 cells was 0.88%. When mutant EFSco*BTK*, wtEFSco*BTK*, and both plasmids were transferred simultaneously to 3T3 cells, intracellular BTK levels were 0.03%, 0.11%, and 0.22%, respectively. Finally, when pLenti-NG-PE2-BSD and EFScopegRNA were transferred, it was 0.14%.

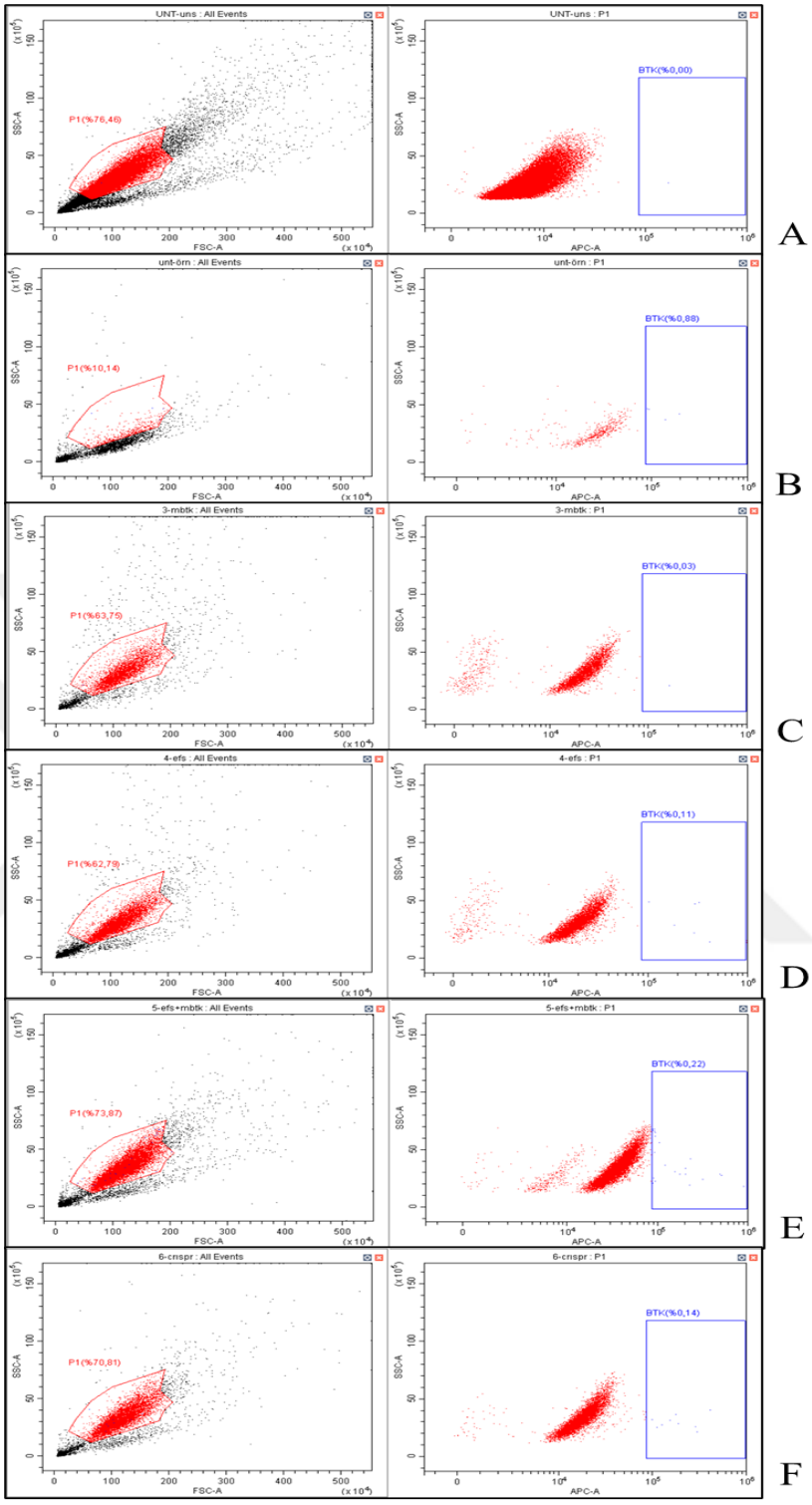

Figure 34 Intracellular BTK level of transfected 3T3 cell lines by flow cytometry. Column 1 shows the viable cell population. Column 2 shows positive *BTK* after staining with Alexa Fluor 647 anti-*BTK*. A. Unstained wild type 3T3. B. Stained wild type 3T3. C. Mutant EFSco*BTK* tranfected 3T3. D. EFScoBTK tranfected 3T3. E. Mutant EFSco*BTK* + EFSco*BTK* transfected 3T3. F. Mutant EFSco*BTK* + EFScopegRNA + pLenti-NG-PE2-BSD transfected 3T3.

4.5 Verification of CRISPR/Cas9 Repair Potential on HEK293 Cells

Firstly, plasmids were transfected to HEK293 cells as described before and measured in flow cytometry to determine intracellular BTK levels. As shown in Figure 35, plasmids transfected successfully to HEK293 cells. According to flow cytometry results, intracellular BTK level in HEK293 cells was 5.45%. When mutant EFSco*BTK*, wtEFSco*BTK*, and both plasmids were transferred simultaneously to HEK293 cells, intracellular BTK levels were 1.00%, 30.00%, and 1.06%, respectively. Finally, when pLenti-NG-PE2-BSD and EFScopegRNA were transferred, it was 2.12%.

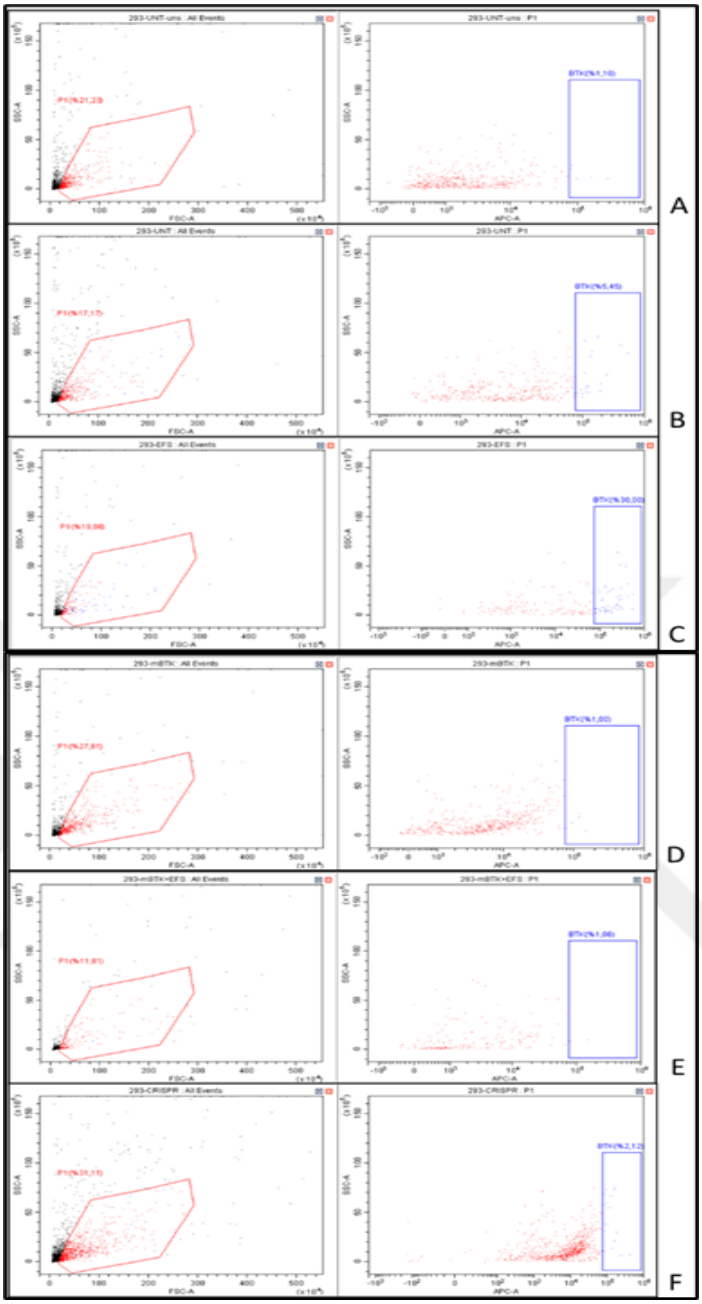

Figure 35 Intracellular BTK level of transfected HEK293 cell lines by flow cytometry. Column 1 shows the viable cell population. Column 2 shows positive BTK after staining with Alexa Fluor 647 anti-*BTK*. A. Unstained wild type HEK293. B. Stained wild type HEK293. C. EFSco*BTK* tranfected HEK293. D. Mutant EFScoBEK transfected HEK293 E. Mutant EFSco*BTK* + EFSco*BTK* transfected HEK293. F. Mutant EFSco*BTK* + EFScopegRNA + pLenti-NG-PE2-BSD transfected HEK293.

5 DISCUSSION

X-Linked Agammaglobulinemia (XLA) is a rare genetic disease that primarily affects males, characterized by low number or lack of B cells, recurrent infections, and agammaglobulinemia. The disease is caused by the mutations in the *Bruton's tyrosine kinase* (*BTK*) gene, which is involved in primary humoral immune defense. The current treatments for XLA, such as immunoglobulin replacement, are expensive and ineffective in restoring B cell function (74).

CRISPR/Cas9 is a natural system for editing DNA sequences found in many bacteria and archaea. It has become an important tool in gene therapy studies in recent years due to its ability to target specific DNA sequences and introduce precise changes. In this study the prime editing (PE) CRISPR/Cas9 system was used, which is a newer and more efficient version of the CRISPR/Cas9 system (75).

Here we propose a gene editing approach using CRISPR/Cas9 technology to potentially treat XLA. To test this approach, a missense variant from a group of XLA patients was selected and aimed to restore *BTK* gene by a third generation CRIPR7Cas9 technology.

According to the literature *BTK*, is expressed outside the bone marrow, thymus, retina, smooth muscle, placenta and with related tissues and cells (76). Hence, first we aimed to determine the most suitable cell line to optimize in our treatment model. We analyzed HEK293, Raji, U937, Jurkat and 3T3 cell lines. Our study results showed that the 3T3 cell lined showed the lowest *BTK* mRNA expression. 3T3 cell line is a mouse fibroblast cell model and consistent with the literature, *BTK* was not expressed in mouse fibroblast cells and human embryonic kidney cells (HEK293) (77). As expected *BTK* was shown to be highly expressed in lymphoblast-like cells (RAJI) and pro-monocytic model cells (U937), both at mRNA and protein levels and this was in line with the literature (78). Jurkat is T-cell model and we used Jurkat as a negative control. According to the literature, *BTK* should not be observed in Jurkat cells because T cells do not express *BTK* (79). Our intracellular BTK results, also confirmed at

mRNA and protein level, that Jurkat does not express BTK (80). As shown previously, we also confirmed that 3T3 cells has almost no intracellular *BTK* (81) expression. The mRNA levels were very low but there was still a slight protein expression in 3T3 cells. Although level was much lower than in all the other cell lines, there was still a detectable band. It is a well-known fact that the mRNA and protein expression levels are not always in the same level and although there is a minimum amount of mRNA, there still can be a detectable amount of protein expression (82).

XLA is mostly caused by the mutations in the *BTK* gene. The current standard treatment for XLA is subcutaneous or intravenous injection of antibodies from healthy donors. Although there are some gene therapy attempts against XLA, there is still no clinically available treatment.

The primary aim of this thesis was to optimize a third generation CRISPR/Cas9 technology to restore a *BTK* variant that was detected in an XLA patient. From the gene editing point of view, there is only one study that targets XLA by CRISPR/Cas9. Gray et al. focused on establishing targeted integration into the *BTK* gene's intron 1 with CRISPR/Cas9 (83). The researchers used K562 (with *BTK* expression) and Jurkat, (without *BTK* expression) cell lines for modelling. On the other hand, with the support of our findings, 3T3 cells were chosen for further analysis due to their high level of proliferation and low level of *BTK* expression. It was planned that the control of low level *BTK* expression would be easier for future studies and the use of mouse fibroblast cell line was assumed appropriate for future animal experiments which will be based on this study.

To optimize a gene editing strategy, we chose the c.1731A>C missense variant that was detected in a XLA patient from our cohort. We tried to mimic the variation in the 3T3 cell line and fix the variant by CRISPR/Cas9 method.

Firstly, the site-directed mutagenesis approach was used to create the missense variation. Despite commercialized or manual methods, the mutation cannot be created. Hence, we obtained the mutated plasmid from a service provider and confirmed by

quality control studies. The reason of failure of the site-directed mutagenesis method can be listed as (84); 1) High plasmid size (9 kb plasmid), 2) High TM value (68-70^{0}C), 3) Type of PCR Polymerase (Phu turbo polymerase) 4) Incorrect temperature calibrations of laboratory devices.

A pick-mutant site-directed mutagenesis kit was used, but according to the kit's protocol, separate arrangement of AB and CD segment was not suitable for our study because EFSco*BTK* is almost 8kb which is quite high. Instead, a more common and standard protocol such as QuikChange could be tried. However, successful results could not be obtained when proceeding through the manual PCR protocols as well. The main reason for this was again that the plasmid size was too high (8 kb). Although the Sanger sequences results showed a heterozygous change was obtained, but it was ambiguous (stated as "S" which could be a G or a C) it was not used for the precision of the study. On the other hand, DNA polymerases extension temperature is 72^{0}C. However, the recommended extension temperature for many high-fidelity polymerases is 68^{0}C. According to calculations, mutagenesis primers have 70^{0}C, so the polymerase may not have worked properly, and the mutation could not be created. Moreover, high fidelity polymerase is not always sufficient, different polymerases such as Phu turbo polymerases could have been used (85) but due to resource and time limitations not every kind of polymerase could be obtained. Hence, we provided the mutated plasmid by commercial service providers and confirmed the mutation by Sanger sequencing.

During in vitro mutagenesis studies we also designed the prime editing CRISPR/Cas9 vectors by bioinformatics tools to target the *BTK* gene. Apart from the literature, this study has three different focus points. First, prime editing, which is accepted as the third generation of the CRISPR/Cas9 system, was used in this study. The most important advantage of prime editing is the elimination of the requirement of incorporating a DNA donor to carry out gene editing (86). Another important point about the study is that we tired a personalized approach by imitating a novel variant. Since all plasmid systems are lentiviral based, it provides the advantage of being able

to return to the lentiviral system for advanced studies and allows for more permanent gene editing.

When the CRISPR/Cas9 vector system is analyzed, the main components are the need for a vector containing cas9 and a vector containing pegRNA with PE. Since Cas9 is a standard protein, a ready vector was purchased, but there is no variant-specific guide RNA, so it was designed with pegFinder and synthesized by editing in accordance with the prime editing system. However, instead of a plasmid synthesis from scratch, the EFSco*BTK* plasmid, which can be used as a ready-made backbone, was used and EFScopegRNA was created.

As the last part of the study, we aimed to treat the mimicked 3T3 cells by variant specific CRISPR/Cas9 vectors. We expected that *BTK* expression would increase when EFSco*BTK* plasmid is transferred to 3T3 cells and when the CRISPR/Cas9 vector system is transferred, the mutation would be repaired and *BTK* expression restored. After confirming the vector system, 3T3 cells transfected with different plasmids. First, 3T3 cells treated with both EFSco*BTK* and mutant EFSco*BTK* plasmid the *BTK* expression level was highly increased. On the other hand, 3T3 transfected with mutant EFSco*BTK* there was also a slight increase. This is an expected results because we already know that the patient, had no B-cells but low level of *BTK* protein expression. This expression could also be detected from the monocytes of the patient. This result may show that the c.1731A>C variant affects the function of the *BTK* protein, but not the production and further analysis are needed to confirm this hypothesis. Unfortunately, the 3T3 cells that were also treated with specific CRISPR/Cas9 vectors showed no increase in *BTK* expression. This was probably due to low transfection efficiency, which was around 1%. The transfection efficiency in 3T3 cells could not be increased in repetitive experiments. Also, literature search confirmed us that 3T3 cells result with low level of transfections. The studies showed that 3T3 cells needed high number of passages after thawing but due to time limit we decided to continue the study with HEK293 cells, which also showed very low levels of *BTK* (87).

In HEK293 cells the untreated cells showed 5.45% *BTK* and when we transformed the cells with EFScoBTK the expression level went to up 30.00%. The mutant EFScoBTK transfected cells showed 1.00% *BTK* and CRISPR/Cas9 transfected cells were 2.12%. However, for HEK293, less cells are seen than expected because an analysis error was made in terms of cell size in flow cytometry settings.

Here we aimed to develop a CRISPR/Cas9 based gene editing application to c.1731A>C variant in XLA. Although the transfection efficiency was still low the obtained results are promising for further analysis. This study is one of the first attempts to generate a treatment system against XLA by gene editing technology.

6 CONCLUSION

Although not very successful, the preliminary result of this study shows us that the system is working, and gene editing technology needs an optimized settings regardless of the system chosen. It also shows that variant specific CRISPR/Cas9 designs can be used as a key solution not only for rare diseases such as XLA, but also for more common diseases such as cancer. However, further studies should evaluate whether this approach is suitable for a potential treatment for XLA.

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