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GENE THERAPY IN TREATING SICKLE CELL ANEMIA DISEASE

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***Abstract*—Sickle cell anemia disease or in short known as SCD is an inherited and serious condition affecting the body's blood and various organs. This disease can give effects to the red blood cells that can cause sickle-like episodes that cause pain and other symptoms. People with SCD are generally well in between episodes of sickling which may cause a long term of complications. Certain situations may trigger sickling, like cold, low oxygen levels, infection or lack of fluid in the body (dehydration). This complication of disease can be prevented from the root with a good treatment. Therefore, early diagnosis and specialized treatment for SCD are recommended from doctors. Based on the previous research, the method used in treating this disease is stem cell transplantation that can be found in bone marrow. Actually, every transplant of bone marrow has risks and bad effects on the patient. It can give an infection to the immune system like attack the new stem cells that will cause failure to the transplant. Moreover, once the stem cell of the donor doesn't match with the recipients, the new immune system cell of donors would attack a few organs of recipients. So, these complications are very dangerous to the donors and recipients. Based on the medical biotechnology that has a lot of benefits, this research will focus on treating disease by gene therapy with CRISPR/Cas9 as a method and an adeno-associated virus (AAV) as a vector. In this research which is based on application of biotechnology, it will be focusing on gene therapy. Gene therapy is also one of the best treatments for this disease. The main reason for patients with sickle cell is causing the disorder of the adult hemoglobin gene, not the fetal hemoglobin gene during the gene mutation. So, this gene therapy in the sickle cell operates by growing the production of the selected gene by restoring the transition performance again to* **fetal hemoglobin, which does not sickle, and subsequently decreasing sickle hemoglobin and thereby increasing fetal hemoglobin.**

***Keywords*— Sickle cell anemia disease, treatment, gene mutation, fetal hemoglobin**

1.0 INTRODUCTION OF BIOTECHNOLOGY

Biotechnology is the contribution of all living beings and biological plants to the creation of innovative and usable goods. It may also be defined as the usage of biological products for the manufacture of a certain substance for other purposes. The biotechnology industry has recently made a major contribution to society,

not just in science but also in the economy. It has been claimed that technical advancement potential would improve the competitive capability of an organization. Technology creativity capability may also be improved by intra-organizational learning practices. In fact, empirical work should have a significant effect on technical success that would increase the pace of innovation [1], [2],[3].

There are two approaches that have been proposed for the creation of scientific-capabilities. Firstly, engaging in fundamental science would create an impetus for experimental discoveries. Second, empirical information from different contexts can be learned and added to the production phase. The biotechnology sector is the most desirable sector that can be researched because it consists of a great deal of creativity and is highly known for its academic work [4].

1.2 Types of Application in Biotechnology

Biotechnology has been applied in many types of areas such as medical, industrial and also agriculture. Generally, medical biotechnology involves the usage of living cells and other cell products to enhance human well-being. Medicine is intended to discover treatments as well as to get rid of and avoid diseases. The medical biotechnology concerned involves the usage of a few methods for scientific purposes in order to discover new or more efficient forms of recognizing diseases, sustaining human wellbeing and human cell biology.

For agricultural biotechnology, generally it focuses on the production of modifying plants' genetics for the function of raising crop yields or adding those plants on their characteristics which offer them the benefits of growing in regions which impose the plant for some kind of stress factor, namely the weather and pests. For certain situations, the process entails scientists discovering a phenotype, locating the gene that triggers it, and then inserting the gene into another plant so that it receives the beneficial phenotype, making it more yield or more resilient than it has previously been.

In applications of industrial biotechnology, it usually comes from the development of cellular constructs to the creation of biological components for various uses. For example, it involves the production of innovative technologies in the manufacturing of beer and food, the building sector, personal care goods and the washing of detergents [5].

Based on the topic chosen which is gene therapy in treating sickle cell anaemia disease, this research will focus on medical biotechnology.

1.3 Medical Biotechnology

This chapter is focusing on medical biotechnology. Medicine will focus on treating the serious disease such as *E. coli* and dangerous viruses like influenza, also the production of insulin, technology used in scientific and medicine.

Historically, Herb Boyer and Stanley Cohen started the modern era of medical biotechnology in 1973, when they were able to establish a method to incorporate DNA into the *E. coli.* The coli bacterium created a transgenic bacterium. Later, this recombinant DNA technique was used to successfully insert human insulin genes into *E. coli*. The *E. coli* genetically modified and has been known to synthesize human insulin [6].

Other than that, medical biotechnology also referred to red biotechnology which is a part of biological technologies to health and medicine drug research and growth. Breakthroughs in these and similar medical areas have revolutionized the medicine as practice for a more precise evaluation of the illness. It is also about proteomic testing for disease prevention and more reliable techniques for the production of medications which are aimed at the molecular stage and thus conceivably more powerful yet low toxic with the prospect of gene therapy to treat previously incurable diseases [7].

Besides, medical biotechnology is characterized as the use of biotechnological techniques to develop products of medicine that can be used for the detection, treatment and prevention of diseases. Established items which are the best of medicinal biotechnology are antibiotics that are used to cure infections of bacteria related with the chemicals that are found in crops are considered like bio-pesticides. Human insulin is the most impressive advancement of scientific biotechnology, which can be developed beyond the human body. The drug illustrates the development of medicinal science in previous years. Insulin is often considered a blessing to modern biotechnology, equivalent to growth hormones. This medicinal product has been manufactured utilizing recombinant DNA technology, which allows development in large amounts [8].

Next, in medical biotechnology includes the study and advancement of technologies used in

the scientific, medicinal sectors and agriculture. Medical biotechnology is the use of cell materials and living cells to manufacture and study medical and medicinal products in helping to avoid and cure human diseases. Many scientific biotechnologists operate in industrial environments and are clinical. Most scientific biotechnologists are working in industrial or university environments. In scientific labs, these specialists perform trials as part of medical science studies such as commercial biotechnologists work on producing vaccines and medicines. Scientific biotechnology has helped introduce natural chemicals, environmental clean-up technologies to the sector and insect-resistant crops [9].

In the area of medical biotechnology, one studies how diseases influence the human body at the cellular stage. Its goal is to cure and avoid illnesses, thus raising the lifetime of the individual. For example, to increase mobility for people with disabilities.

Medical biotechnology can be explained in a few big sectors and roles nowadays. First, is pharmacology. Pharmacology in conjunction with biotechnology is one of the latest and increasing areas. It includes the concepts of biotechnology for the production of medicines. Bio preparations such as antibodies, vaccinations and nucleic acid medicines are a large range of medical medications on the market.

Secondly is gene therapy. It includes the usage of DNA as a therapeutic agent for the diagnosis of a specific disease. It primarily entails replacing a mutant gene with a corrective gene. Gene therapy has seen considerable strides in the last two decades. Within a brief amount of time, it has moved from a theoretical stage to a technical level, as well as clinical trials against a number of deadly diseases. The most important developments included gene therapy for multiple hereditary conditions such as extreme mixed immunodeficiency, persistent granulomatous condition, aids, hemophilia, Parkinson's disease, HIV, measles, and even other acquired diseases.

Thirdly is stem cells. A stem cell is a cell that has the ability to grow into some form of cell in the human body. Normally, the stem cells are inserted into the injured regions of the body and, in the correct circumstances, the injured region is repaired. Most commonly, these stem cells are grown in the clinic, first to maintain good care, and eventually injected into the sick individual. The key field where the stem cells have demonstrated their usefulness is bone marrow transplantation, the replacement of damaged cardiac tissue following a heart attack, and the replacement of damaged nerve tissue for spinal cord injuries. Stem cells are actually being used to cure several illnesses. The largest source of stem cells is the fetus itself.

Next is tissue engineering. It requires developing human tissues outside the body for subsequent usage. So far, tissue engineers have developed bone marrow, cartilage and artificial skin. People are currently working on the development of artificial kidney, liver and pancreas.

Lastly is bioprocessing. This is one of the latest fields of study in the industrial development of human products, drugs and others. It is produced through genetically engineered bacteria and viruses. This contributes to the development of the target substance in vast amounts in a limited period of time with comparatively less expense. One of the main uses of bioprocessing is insulin, a human protein responsible for raising blood sugar rates following ingestion of carbohydrates. It achieved so by inserting the human insulin gene in bacteria, cultivated, and enabling the production of insulin that is refined, extracted and marketed to diabetes worldwide.

Medical biotechnology is one of the rarest fields, there is a shortage of qualified sectors. Over the coming years, however, there would be an immediate need for well-trained and qualified medical and biotech fresheners. If the pace of development persists, the day will arrive when medical biotechnology will become a cornerstone in the area of health research [10].

1.4 Gene Therapy in Treating Sickle Cell Anaemia Disease

This research focuses on gene therapy. Gene therapy is one of the roles for medical biotechnology nowadays. Gene therapy involves the injection of genes into human tissues and cells to cure a disorder, often genetic disorders in which a faulty mutated allele involves substituting with a healthy version [11]. Gene therapy involves the introduction of new genes to some cells to repair malfunctioning or defective genes. Researchers usually accomplish by utilizing a virus to pass genetic loads into cells, as the viruses have developed to do with their own genetic content. Gene therapy is expected to cure a broad variety of disorders, such as cystic fibrosis tumors, asthma, lung failure, AIDS and hemophilia. As stated, gene therapy can treat and cure the sickle cell anemia disease with further process.

Some researchers agreed that sickle cell disorder develops when the infant inherits defective genes for the development of hemoglobin from their parents. This condition shows that it is linked with recurrent early life illnesses and sometimes induces mortality faster than the general population. So, gene therapy replaces these defective genes with regular genes. Historically, sickle cell anemia disease is a category of genetic disorders that arise from the development of defective hemoglobin genes. This situation gives an effect in red blood cells the formation of irregular hemoglobin [8]. Hemoglobin is responsible for carrying oxygen throughout the body in red blood cells. Individuals with the disease contain abnormal hemoglobin red blood cells and change from normal round disk shape to narrow sickle shapes as shown in Figure 1.

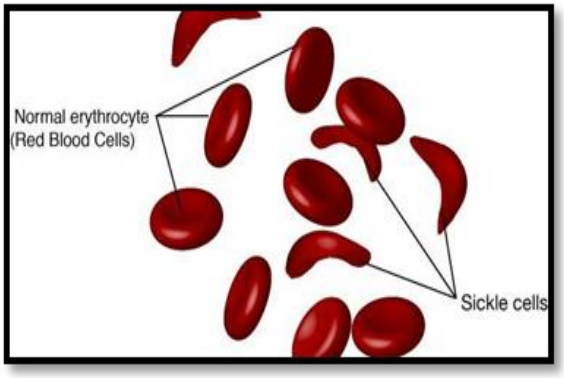

Figure 1: The shape of normal red blood cell and sickle cell [8]

The type of the sickle is the end product of a sequence of biophysical processes and dynamic biochemicals inside the red cell following the de-oxygenation of hemoglobin sickle (HbS). Sickle-shaped cells do not move easily into narrow blood vessels as disk-shaped red blood cells do. Therefore, the creation of a gene therapy method has been pursued for several years. Gene therapy provides a strong hope for the potential of medical research. This includes the insertion of genetic material into a cell to cure a disease. Previously, momentum was created when it was seen that adeno-associated virus (AAV) vectors comprising globin genes from normal human and regulatory regions from the beta-globin locus control area [12].

Generally, gene therapy is the insertion of genes into human tissues and cells to cure a disorder. Many of the diseases handled in this manner are inherited abnormalities triggered by malfunctions of gene mutation. Adeno-associated viruses (AAV) are the most commonly employed vectors for gene therapy. Any of the various forms of viruses used as vectors in gene therapy include lentiviruses, retroviruses, parvovirus, adenoviruses, hepatitis virus, herpes simplex virus and vaccine virus [12].

2.0 INTRODUCTION

Sickle cell anemia is a disease that occurs when mutations occur in the HBB genes that are responsible in providing production instruction for β- globin protein. Haemoglobin are made of four protein subunits that consist of 2 α-like globin peptides and 2 β-like globin peptides [12]. Specifically, α-like globin cluster gene is (ζ, α_1 and α_2) are located on chromosome 16 and the β-like globin gene cluster (ε, G_γ, A_γ, δ andβ) are located on chromosome 11[1]. Adults and fetuses have different sets of globin protein, adult haemoglobin (HbA) have ($\alpha_2\beta_2$) while fetal haemoglobin (HbF) protein subunits are ($\alpha_2\gamma_2$)[1]. The γ protein in the HbF gradually changed into β protein upon labour. This is the point where usually the symptom for sickle cell disease and β- thalassemia will manifest the clinical symptom. The mutation that causes the disease is homozygous point mutation (A-T) in the sixth codon of the β-globin/HBB gene. The mutated gene will produce sickle haemoglobin (HbS, $\alpha_2\beta^S{}_2$) [13].

The method implemented in treating sickle cell disease was found unexpectedly in 1984 during the treatment of leukaemia in children with SCD and it cures both leukaemia and sickle cell disease. This event then was the first step that became an example for hematopoietic stem cell transplant (HSCT) [12]. Currently, allogeneic HSCT is the primary cure for the disease with disease free survival greater than 80% with human leukocyte antigen (HLA) matched sibling donor transplant. However, most of the time finding a suitable sibling donor is hard. While, using an unrelated donor increases morbidity and mortality that are associated with the transplant and not to mention the immune side effects that come with the allogeneic HSCT [14]. Hence, with the knowledge of γ protein in the HbF and the new discovery in gene editing tool by using the programmable nucleases (ZFNs, TALENs and CRISPR Cas9). The technology can be used to correct the mutated gene. Now, the focus is on the step and mechanism of the gene therapy by using CRISPR Cas9 and stem cells. Gene

therapy generally involve three main procedure [14]:

(i) Harvesting the stem cell

(ii) Modifying and introducing the genetic material

(iii) Chemotherapy conditioning before transplanting them in patients.

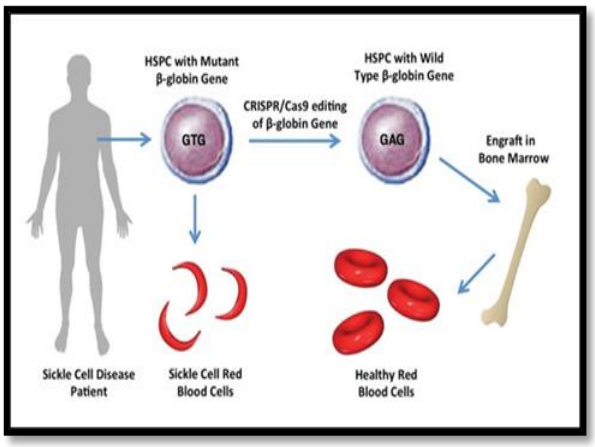

Figure 2: The overview of the step need to be accomplish for a successful gene therapy for Sickle Cell Disease [15]

2.1 Harvesting autologous hematopoietic stem and progenitor cell

HSCT requires harvesting of allogeneic or autologous Hematopoietic stem and progenitor cells (HSPCs) that usually come from peripheral blood (PB) under particular situations, bone marrow (BM) during the entire life and cord blood (CB) upon birth. There are different types of HSPC harvesting and it is important to make sure the procedure used not only increases the number of HSPCs harvested but also ensures the safety of the patient while doing the procedure.

2.1.1 Bone marrow (BM) harvesting

BM harvesting was mainly developed to perform allogeneic HSPC transplantation and later autologous transplantation. But recently BM is mostly used for allogeneic HSPC transplantation [12]. The procedure needs to be done by one or two hematologists using a needle with multiple side holes. The aspiration point should be at posterior iliac crest (Figure 3) and the aspiration volume range should be restricted to 15-20 ml only [16]. Before starting the procedure, the needle used needs to be washed with heparin/saline solution. While harvesting the HSPC, the collecting bag that contains anticoagulants needs to be agitated continuously to avoid coagulation. Half way through the process total nucleated cell (TNC) count is performed to estimate the optimum BM volume harvested within the allowed range [17]. The dose for cell harvesting suitable for allogeneic transplantation is 3-5x10^8 TNCs per kilogram of recipient body weight. Generally a higher number of TNC is better for both harvesting and transplanting HSPC, especially in acute myeloid leukemia (AML). The last step after harvesting the BM is sending them to the cell therapy unit for ABO (blood group) compatibility examination [18].

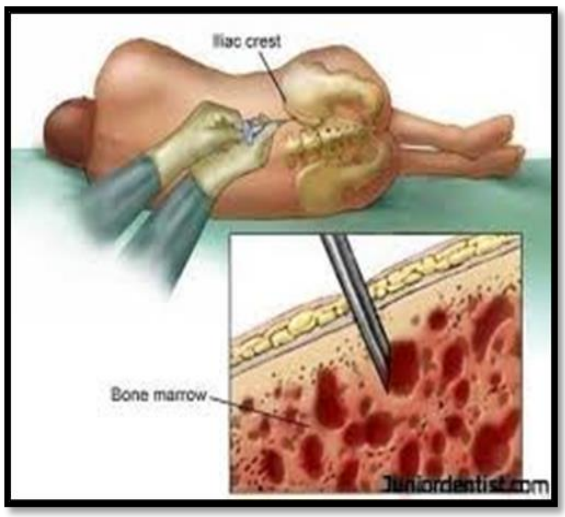

Figure 3: The aspiration process from the posterior iliac crest [19].

2.1.2 Cord Blood (CB) Harvesting

The protocol to harvest the CB is still very much similar with the original method [20]. Basically the harvesting starts right after the birth of the baby where the umbilical cord is double clamp and then the cord will be punctured under sterile condition [18]. The blood from the umbilical cord then will flow into an anti-coagulated sterile closed harvesting system. It is important to train the birth unit staff in harvesting CB procedure in order to reduce the rejection rate due to the bacterial contamination and clotting [18]. The window for CBU harvesting and processing is strictly limited to 24-36 hour to ensure cell viability. The HSPCs harvest from the CB is generally

108+28 ml, 12.5+5.3x106 TNC and approximately CD34+ cells [20].

2.1.3 Peripheral Blood (PB) Harvesting

HSPCs presence in blood was unknown until a discovery in 1971. However, in normal conditions there are no HSPCs present inside the PB. Hence, the HSPC needs to be drawn or mobilized from BM [21]. The mobilization technique for autologous and allogeneic transplant patients is different [12]. Mobilization of HSPC from the donor starts with the donor receiving G-CSF (filgrastim or lenograstim) 10 µg/kg/day 4-5 prior to the apheresis [22]. Apheresis is a process to separate the blood into different components according to its specific gravity. Key factor that associated with high efficiency of the harvesting is male sex, higher BMI, higher premobilization WBC and the use of lenogratism [23].

On the other hand, in patients, instead of treating them with G-CSF only. There are other methods usually implemented to immobilize the HSPC from the BM to PB. The first method is combining chemotherapies with growth factor i.e. G-CSF to treat underlying disease inside the patient [5]. Some examples of the disease are Hodgkin's disease (HD) or solid tumour and inducing the CD34+ to immobilize into PB [6]. The second method is cyclophosphamide associated with hematopoietic growth factor that can be used in treatment of multiple myeloma (MM). According to C. Marmier et al., the use of chemotherapy regimen only might cause failure but with the presence of GM-CSF or G-CSF, it induces increasing numbers of CD34+ harvested [10].

2.2 Modifying the Sickle Cell Disease genetic material

2.2.1 Choosing the suitable vector to carry the gene

There are few aspects that were considered before choosing a viral vector to carry the donor gene. Criteria that the researcher usually after is the ability to attach and enter the targeted cell, able to enter the nucleus, can maintain the ability to express itself over a period of time, lack of toxicity and is non-pathogenic to the human body. Over the years there are a lot of vectors that were discovered by scientists. For example Retrovirus (RV), lentivirus (LV), adeno-associated virus (AAV) and integration-deficient lentiviral (IDLV). In the next part it will be focusing on AAV as the vector [14]

The size of the AAV packaging is 4.7 kb at the site of the donor. Inverted terminal repeat (ITR) and the homology arm ore part that are dispensable in the vector [25]. Commonly the size of the ITR is 270 bp while the homology arm should not be more than 400 bp. With that being said, the approximate size of the gene that can be inserted is around 3.6 kb [25]. Since the HR process is to manipulate the DNA exactly at the double strand break. The standard HR donor design are two homology arm that are in between the transgene expression cassette or the mutation that will be introduced [14].

The arm should be splitting at the CRISPR cut site and if it happens that the modification area is separated from the CRISPR site. The homology arm should be laying in between the cut site and modification site [26] . There are two considerations that need to be taken in this case. First, if the target site is not disrupted by the introduction modification, Cas9 might need an INDEL introduction after HR takes place and the second one if there homology sequence between the target DNA and the donor region between the cut and joining site changes. There are high possibilities termination of HR occurs due to the nascent DNA leaving the donor and annealing back to the chromosomal region. One step that can be taken to minimize the effect is to reduce or minimize the use of homology in the both regions (cut site and desired cut).

Modification of the chromosome utilizes the homologous recombination (HR) between the genomic DNA and targeting vector. The targeting vector will be bioengineered so that it will take the donor DNA and align it in the genome so that it will be in the same locus as the code for the protein needed. However, precise integration of the insert DNA uses homology-directed repair (HDR) of spontaneous double strand break (DSB) just like the concept of using sister chromatid as the template. After transfection of the donor DNA with sequence homologous to the targeted locus to be modified the HR will be utilized to repair spontaneous DSB.

2.2.2 Gene targeting by using programmable nucleases to correct the defective gene

After choosing the suitable vector to carry the gene, methods to modify the gene are rapidly developed for the last decade. The main purpose of the editing tools are to make a targeted double-stranded DNA breaks (DSB) by using different method such as site directed nucleases (ZFNs), transcription activator like effector nucleases (TALENs), and clustered

regularly interspaced short palindromic repeat and associated Cas9 (CRISPR/Cas9). The programmable nucleases in the focus are CRISPR/Cas9. Owing to the ease of use properties of the CRISPR/Cas9 method, it quickly became famous among the researchers. Comparing Jinek and colleagues research on the method ZFNs, TALENs and CRISPR/Cas9 (Figure 4). The findings show that CRISPR/Cas9 have better precision to target HBB locus near the β^S mutation.

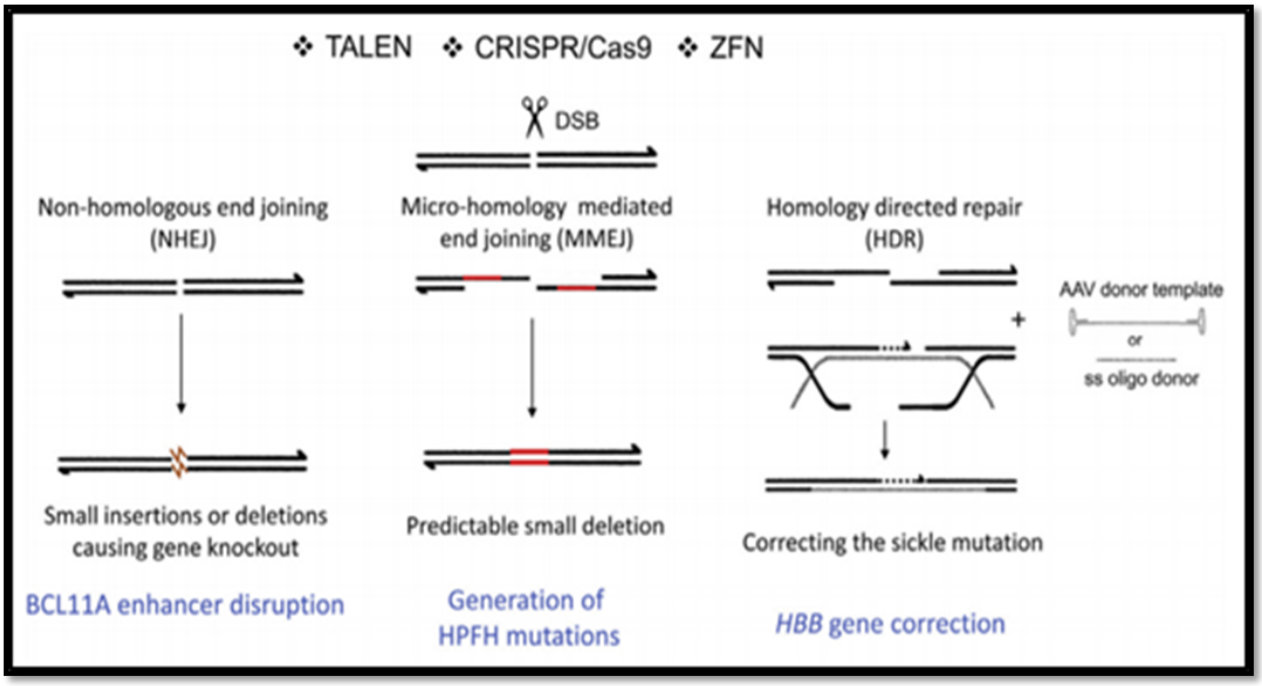

Figure 4: The figure depicts how ZFNs, TALENs and CRISPR/Cas9 nucleases correction mechanism [4]

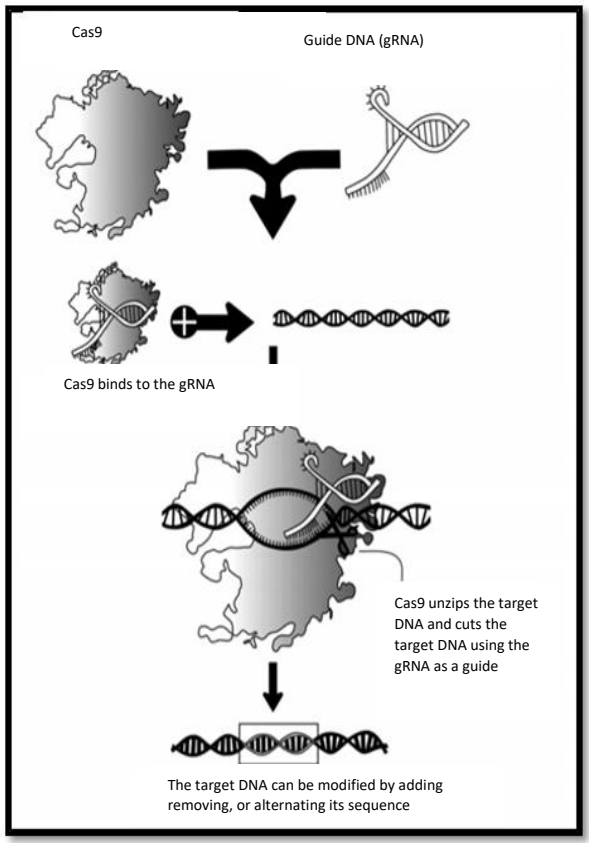

Figure 5: Mechanism of CRISPR/Cas9 [16]

CRISPR/Cas9 key mechanism (Figure 5) in this genome editing method is the least complex of producing the gRNA. The importance of gRNA is to guide the tracrRNA-crRNA chimera complex to the specific DNA strand and create a targeted double-stranded DNA breaks (DSB). The gRNA will serve as the indicator of where should the complex go and the Cas9 is the endonuclease that will break the DNA strand. The cell then will try to fix itself by a mechanism called Homology Directed repair (HDR). Specifically for treating the sickle cell anaemia there are two way a gene could be manipulated:

(i) CRISPR/Cas9 repairing the haemoglobin S into normal HbA and:

(ii) increasing the production of Haemoglobin F (HbF).

First approach to mediate the HbS gene is by repairing them into the normal β-globin protein [1]. The process needs the presence of the Cas 9 protein, gRNA, and donor DNA at the same place and at the same time. When the Cas9/gRNA is delivered into the cell as the ribonucleoprotein complex along with the donor DNA (DNA homology template) it will increase the DSB rate and increase the gene targeting frequency [28]. The whole then will identify the nearest locus of β-globin protein, the complex will anneal the DNA and the pairing of gRNA with the DNA is through Watson-Crick base pairing [28]. According to Handel and colleagues it shows that the high editing performance of the plasmid-based CRISPR/Cas9 system in cell lines does not have high correction rate in human primary T cells and HSPCs. In order to overcome the problem chemically modified nucleotides are added to the both terminals of the gRNA to increase their stability in the human primary T cell and HSPCs [28][27].

When the endonuclease/Cas9 creates a locus specific double strand break (DSB). Natural body mechanisms will take place to correct and two highly conserved competing mechanisms are the Non homologous end joining (NHEJ) and Homologous Recombinant [28]. NHEJ is the default method throughout the cell cycle to repair break by ligation of the DNA without end processing. Usually it will have insertion and deletion (INDEL) at the break site [28]. On the other hand HR occurs at the S or G2 phase of the cell cycle and this window offers an undamaged sister chromatid to serve as the repair template [14]. By using this principle we can harness HR to create precise DNA changes by providing an exogenous DNA donor template. The condition is, the donor template needs to have homology arms that are identical to the DSB [29]. HR also can avoid single-nucleotide polymorphism (SNPs) disease and allow the entire genome reading frame can be inserted at the specific genomic site[15].Once the gene were edited, haematopoiesis take place and it is the process that generate all of the blood and immune cell from HSCs with self-renewing capacity [30]. Despite the method developed to identify and evaluate the self-renewing HSCs by immunophenotyping, CD34+ cell surface marker has been readily used to identify a heterogeneous population of HSPCs. Generally it has 0.1-1% HSCs with long term repopulation capacity [28],[29].

The second approach used to tackle the problem is increasing the production of the HbF. As mentioned in the introduction, upon delivery the γ-globin gene was silenced and replaced by β-globin gene to produce HbA[12]. So manipulate these events by reducing the expression of the factor that silencing the γ-globin gene will produce more γ-globin genes [31]. BCL11A is one of the factors that maintain the production of the HbF in postnatal cells and unlike KLF1, MYB and LRF it does not give side effects to the erythropoiesis [30].

2.3 Chemotherapy Conditioning Before Transplantation

Before executing hematopoietic stem cell transplant (HSCT), conditioning therapy is needed to remove existing disease. To create more space for engraftment in the bone marrow and immunosuppression to decrease the risk of rejection of the donor cell by the host cell [21]. The conditioning therapy involves combination of chemotherapy and/or immunotherapy using different regiments and concoction of drugs. The main objective of the treatment is to avoid risk of relapse and rejection rate [32]. This can be achieved by 'reduced intensity' or non myeloablation treatment that is relatively less toxic than the myeloablation treatment where the concoction is pumped in larger dosage to create space or ablate the bone marrow for engraftment to take place [32].

3.0 ADVANTAGES OF GENE THERAPY

As stated, the previous method used in treated sickle cell anemia disease is stem cell transplantation. But, with the high technology nowadays, another method that can replace the method is application of gene therapy with CRISPR/Cas9 as a tool and a vector.

Injection of new functioning genes into an individual's tissue or cell to alter the genetic condition and remove the faulty gene called gene therapy. It is a modern field of study, and several scientific initiatives are either at the trial stage or at the early phases. Nevertheless, hopes are strong, so hopefully be able to get rid of genetic disorders faster. Gene therapy may have two forms which are germline gene therapy and somatic gene therapy. It is particularly important in the treatment of genetic sickle cell anemia disease. Throughout this, gene therapy provides a variety of benefits in treating and curing this disease.

Firstly, gene therapy is one of the sources of hope. Genetic deficiency persists often after extensive tests, and many individuals are affected or impaired by these disorders and find their lives. In fact, in 2009 in the United States, 3% of congenital defects have been reported in all births, the pleasure of raising a child is unexpectedly missed and substituted with the pain and difficulty to sustain such a challenging existence. Specific patients and relatives feel discouraged, because attempts to improve the problem by moving to various stages of healthcare are often frustrated as there is no solution for these diseases. Gene therapy designed to remedy birth abnormalities that are responsible for more than half of child deaths is laudable. This can improve that, so all of the unborn babies will be grown to their prime and delivered safely. Besides, gene therapy guarantees a treatment and cure for this sickle cell anaemia disease that is currently incurable that causes agonizing pain in many [33].

Secondly, the effects of therapy are timeless and long-lasting. When a dysfunctional gene is substituting with a healthy condition of a gene like sickle cell anemia disease, there are low possibilities of recovery, so that is typically a one-off step that can keep symptoms-free. In fact, gene therapy is not only a remedy for the patient suffering from a specific disease, but also a remedy for the whole family. When deleting and terminating a gene that predisposes one to sickle cell anemia disease, the faulty genes may not be passed to their descendants, but to a new working chromosome.

Next, gene therapy can eradicate this disease and improve the quality of life. Using treatment involving gene therapy, previously incurable conditions can be cured and removed. Gene modifications, especially reproductive genes utilizing the germline process, may assist to avoid the transfer of faulty genes and thus no further occurrence of disease. Gene therapy does not only rely on diseases. There are a few conditions that can render life endless, such as infertility, are still known, and it is expected that soon enough gene therapy will trigger reproductive genes and enable people to have babies with a good and normal condition [34].

So, gene therapy is the best solution in treating and curing the patient with the sickle cell anemia disease. With a good treatment, this disease can be avoided early from the root.

3.1 Complications of Stem Cell Transplantation

Stem cell transplantation (SCT) refers to transplantation of the hematopoietic stem cells (HSCs) from a donor into an individual. Dynamic biological entities that interact intimately with/and are influenced by/the physiology of the recipient by transplanted human bone marrow or stem cells are. This procedure is very risky. Cultured human stem cells are maintained under conditions that promote either the self-renewing expansion of undifferentiated progenitors or the acquisition of differentiated properties indicative of the phenotype the cells will assume before they are transplanted. Additional fine-tuning occurs as a consequence of instructions received from the cells' physiologic microenvironments within the

recipient after incompletely differentiated human stem cells are transplanted[35],[35],[36].

In treating sickle cell anaemia disease, there are two major barriers to stem cell transplants. First is the lack of appropriate donors and secondly is the risk of serious illness associated with donor-to-patient stem cell transplant. There are only about 300 of these transplants performed to date based on these reasons. Material which contains donor T-cells also for donor-to-patient stem cell transplants use. It can cause graft-versus-host disease (GVHD) which is a significant cause of illness and even death in stem cell transplants and causes these donor T-cells to react to the patient's body as foreign [37],[38],[39].

Lack of donors is another problem with stem cell transplant for SCD. Usually, a close genetic match to the patient is the preferred donor, especially a patient's brother or sister. The closer the donor cells are to the patient's cells, the less severe the immune reaction is likely to be principal in using closely matched donors to reduce the risk of GVHD [40].

Next, the transplant procedure is very risky and may be psychologically devastating and traumatizing for the patient, has serious physical and psychological side effects or even death will be faced by the patient.

In addition, especially for patients who do not receive a bone marrow transplant from a well-matched brother or sister donor, bone marrow transplant carries its own dangers and limitations. In fact, about 10% of patients that make bone marrow transplants die from the treatment is about 10% [41].

Bleeding, pneumonia, and severe infection are other very serious complications. Long-term consequences may include a higher risk for cancer and infertility even in those who are cured [42].

3.2 Advantages of Adeno-associated Virus (AAV)

This gene therapy had used Adeno-associated virus (AAV) as a vector in treating sickle cell anemia disease. Generally, this AAV has several unique benefits and advantages for this treatment. Transducing non-dividing cells can be performed by the AAV vectors. It is able to sustain a self-inactivating design without losing its potency. It is important and plays a big role in designing efficacious beta-globin vectors [35].

Recombinant adeno-associated virus (AAV) is usually preferred in the procedure and step of delivery of the gene into the targeting cells due to its mild immune response, high titer, overall safety and ability to infect a wide range of cells. Primates and humans may be infected by native AAV, however, for humans, it has not been documented to present minimal risk and cause disease. By comparing with the others viral vectors like adenovirus, AAV is response in animal models which induces mild immune, rendering AAV is the best and suitable virus for distributing in vivo genes and the researchers also agreed that this method is suitable especially for who are concerned about their health and also in treating sickle cell anaemia disease. There is a risk when using a certain virus, like lenti-virus or retrovirus, which can interrupt gene activity by sporadic interaction events. There is a low chance in inserting mutagenesis since AAV may not penetrate into the main or host cell genome. Once the AAV acts as concatemers in non-dividing cells in the long term, the main function in replicating cells would be lost.

In inducing a successful infection of AAV, native AAV needs co-infection with the aid virus so that the replication will be defective. The Aid Free Expression Mechanism of AAV supplies the helpful feature essential for infection, also removing the requirement for a helper virus and supplies aid genes to different plasmids. There are 293 cells co-transfecting in the recombinant of AAV that supply and generate the necessary AAV expression plasmid with adenoviral E1 genes, pHelper plasmid and recap plasmid. In determining the serotype, the plasmid of Rep-Cap includes the genes of rep and cap. The capacity of AAV that wants to be cloned can be improved by the supply and help of duplicate and cap genes on a different plasmid, which is constrained due to its low genome scale. Adenoviral genes contain VA, E4 and E2A in the pHelper plasmid, which are essential for an effective infection and are normally contained in the helper virus. After 48 to 70 hours transfection of cells has been harvested, it will be exposed to a sequence of freeze-thaw processes to retrieve through centrifugation and extract the virus from the cells. The coarse lysate can be distilled, processed or titrated.

Although the infection's efficacy varies depending on the serotype determined by the sequence of the capsid protein, AAV is capable of infecting many different types of cells. There are a few of native AAV serotypes that have been recognized that are commonly used for recombinant AAVs with serotypes. They are 1-9 AAVs being the most used. The most commonly researched and reported serotype is AAV-2. By shuffling DNA of multiple AAV serotypes, these serotypes were produced to

create AAVs with hybrid capsids that have improved transduction efficiency in vivo (AAV-DJ/8) and in vitro (AAV-DJ) in a variety of tissues and cells.

The AAV genome must undergo second strand synthesis to generate the double stranded DNA necessary for gene expression as it is single stranded. The rate that limits the stage of AAV infection and may result in poor transduction capacity during the second strand synthesis. The AAV genome may be packed as a double-stranded DNA rather than a single-stranded AAV (scAAV) to address this restriction. The area of the ITR gene is removed and the genome stops from being partitioned into the monomers that are usually packed with scAAV. By passing the second strand of the synthesis stage, the double stranded scAAV genome is capable. Thus, the capacity to clone is limited to satisfy the added string but the vectors result in higher yields.

In using in vivo studies or if the raw viral lysate could be toxic to the target cells, virus purification is more essential. With a purification kit for AAV-2 and AAV-DJ, it can recover 60 percent or ultracentrifugation may be used to purify any AAV serotype or more of the virus present in the supernatant.

ViraDuctinTM AAV Transduction Reagent as additive can be applied to improve transduction efficiency with any AAV serotype or when infecting non-permissive cells. It takes 2-7 days to analyze gene expression of post infection, depending on the amount of virus used for transduction and the gene [37].

Besides, AAV has a low immunogenic profile and has wild type that is not associated with any pathogenicity unlike most naturally occurring viruses used in clinical trials. In contaminants of adenovirus preparations, AAV was first discovered. It is a non-enveloped virus that carries a small, approximately 4.7 kb single stranded genome and is icosahedral. These AAV vectors are providing a few advantages such as they lack all viral genes, further minimizing potential activation of innate immunity also including those that are responsible for integration into host chromosomes.

Besides being able to treat sickle cell anemia disease, AAV vector also can treat brain cells, muscle and liver and it is capable of infecting a wide range of cells. In repairing DNA via homology directed repair (HDR), AAV vectors also are able to enhance and facilitate it. With all of these characteristics, it shows that AAV vectors are able to correct disease-associated mutations especially in treating the sickle cell anemia disease [38].

3.3 Advantages of CRISPR/Cas9 as Method

By using the CRISPR/Cas9 of gene therapy in treating sickle cell anemia disease, everything can be achieved. Based on the reports of genome editing in species such as sweet orange, it highlights the clear advantages of CRISPR/Cas9 in terms of accessibility, cost, simplicity and versatility and also include the appearance of such a large number of publications based on the CRISPR/Cas9 technology in such a short time.

In making CRISPR much more straightforward to test multiple gRNAs for each target gene, it does not require any protein engineering steps unlike its predecessors. Furthermore, the amount of gRNA sequence needed to be changed to confer a different target specificity was only 20 nt in completing the process. By *in vitro* transcription using two complementary annealed oligonucleotides, any number of gRNAs can be produced. This CRISPR/Cas9 method can be used for high-throughput functional genomics applications also bringing genome editing within the budget of any molecular biology laboratory with inexpensive assembly of large gRNA libraries. Next, the CRISPR/Cas9 method can allow genomic modifications that are beyond the reach of the other nucleases by cleaving methylated DNA in human cells. It is reasonable to assume that the ability to cleave methylated DNA is intrinsic to the CRISPR/Cas9 method and not dependent on the target genome although this aspect has not been specifically explored in plants. The CRISPR/Cas9 technology is generally particularly suitable for monocots with high genomic GC content such as rice and also suitable for more versatile of genome editing in plants.

In comparing the CRISPR/Cas9 method to ZFNs and TALENs, the main practical advantage is the ease of multiplexing. It is able to edit several genes at the same time and can be particularly useful to knock out redundant genes or parallel pathways with the simultaneous introduction of DSBs at multiple sites. By targeting two widely spaced cleavage sites on the same chromosome, the same strategy can also be used to engineer large genomes by deletions or inversions. Any number of different sequence-specific gRNAs and multiplex editing with the CRISPR/Cas9 method simply requires the monomeric Cas9 protein. In contrast, ZFNs or TALENs require separate dimeric proteins specific for each target site with multiplex editing.

Furthermore, CRISPR research community has promoted the widespread uptake and use of this technology in contrast with open access

policy, for example, ZFN platform to the proprietary nature. Web tools for selecting gRNA sequences and predicting specificity and hosts active discussion groups which the community provides access to plasmids. These facilities have contributed to the rapid progress in our understanding of the system and its practical applications and encouraged newcomers to adopt the technology.

In addition, the relatively high frequency of off-target mutations reported in some of the earlier studies is one of the few criticisms of the CRISPR/Cas9 technology. A missing base (gRNA bulge) at various locations along the corresponding gRNA sequence or DNA sequences that contain an extra base (DNA bulge) have also been shown to induce off-target cleavage. As an intrinsic property that reduces the likelihood of immune system evasion by viruses with point mutations, the relaxed specificity of the CRISPR/Cas9 complex at non-seed positions in the crRNA spacer appears.

Considering the design of the gRNA is the most important strategy that has been developed to reduce off-target genome editing. CRISPR/Cas9 method recognizes target sites by Watson–Crick base pairing allowing off-target sites to be predicted more reliably by sequence analysis in contrast to ZFNs and TALENs, whose target specificity is determined by protein–DNA interactions that are often context-dependent and unpredictable. The gRNAs can be tested for off-target effects rapidly and inexpensively as the CRISPR/Cas9 method is also easy to reprogram. There were over 700 sgRNA variants in parallel to gain insight into the issue of specificity. The results show that the CRISPR method is the best option with higher success [40].

Moreover, there were also advantages of using defective gene and CRISPR. Clustered Regularly Interspaced short palindromic repeats (CRISPR)-CRISPR associated protein 9 (CRISPR/Cas9), Transcription Activator-Like Effector Nucleases (TALENs) and Zinc Finger Nucleases (ZFNs), are widely used genome editing methods that can produce the target mutation in a broad range of cell types.

The downside of ZFNs is the comparatively limited size of the ZFN monomer coding chain. The broad size of TALEN and Cas9 DNA coding may be an issue for certain viral distribution methods due to packaging limitations. In comparison to ZFNs, TALENs may be built to reach any area in the genome due to their strong modularity. In addition, recent advances in cloning strategies for the assembly of TALENs repeats in the required sequence have rendered it easier to generate site-specific TALENs [36].

4.0 JUSTIFY THE EFFECT OF THE TECHNOLOGY TOWARDS THE SOCIETY

Discovery in gene therapy is quickly becoming one of the most controversial topics in modern medicine and research as the technology blooms at a rapid rate. Everything has its own risk and side effects towards the living things. Gene therapy includes the insertion or modification of the genetic material and in humans or plants, it is becoming more common. Gene therapy has undiscovered and huge potential to be explored in treating degenerative disease such as remobilizing the paralyzed limb due to accident or stroke and it also might treat cancer [39].

In current growth of the technology, most gene therapy is limited to the disease that does not have a treatment option. Most of the time, the technology can be seen treating genetic diseases like thalassemia and sickle cell anemia. Even though there are so many amazing breakthroughs, the public are still afraid of the technology. Mainly because they have been fed by propaganda and misinformed by the media. The fear of the technology to be far more superior than what it was intended also became one of the public fear and rejection towards the emerging technology.

4.1 Somatic and somatic germline gene therapy

It is of the utmost importance to inform the society about the difference between somatic and germline gene therapy. The current work in the lab is mostly revolving around manipulating the somatic cell and there are a lot of amazing discoveries made by the researcher and there is new light in curing diseases like cancer, hemophilia and some types of blindness. On the other hand, germline study is not as extensive as somatic gene therapy but the main fundamental of the germline gene therapy is to manipulate the donor gene into the reproductive cell like sperm, ovum or blastocyst. The main objective of the therapy is to avoid development and manifestation of the sickness. Against the popular stigma of producing “designer babies' ' gene therapy function by removing or fixing the defective gene so that the babies that are born later on will be free from inheriting the dangerous birth defect. Going back to CRISPR technique, due to the high precision in editing specific parts of the genome it has promising potential to cure disease like HIV and reduce risk of harming the embryo. Contrary to popular belief the scientist is crazy to work on germline gene therapy, they

are advised to be careful of their work so that the current progress on gene therapy will not be jeopardized [39].

4.2 Gene therapy vs religious issue

One of the issues that rise among the society is the religious one. The thing that they are concerned with manipulating the gene is about “playing god.” They believed that one should not touch the fundamentals that made us human or the genome. It is hard to be discussed since it is something that is sensitive. But let us take a look at the benefits it offers and the cons that come along with it. Gene therapy treatment might be a little bit harsh but it is extremely helpful in treating disease. In some people's point of view it is what matters. But to some it is more than that, what if the change that was made at the cellular level changes more than what is expected. Furthermore, how far enough gene therapy would go. Let us take a look, would gene therapy be used only for treating chronic disease or it will use less serious disease as well. Evaluating from the current breakthrough and development in the technology it is possible for all of the options above. But this is where the Food and Drug Administration (FDA) and National Institute of Health (NIH) play their role to govern the protocol.

4.3 Availability of the treatment for all society level

Another issue that arises from gene therapy is the right of the people to receive the gene therapy treatment. Will it only be available for the rich only or should it be available to only for those who need it. According to Dr. Leopold, gene therapy has the potential to save insurance companies a lot of money, but for uninsured therapy cost may seem impossibly high and expensive and this fact is so unfortunate since it kind of dictates whether a person lives or dies. However, one advantage of gene therapy is it does not depend on the other available organ to be transplanted like organ transplantation. There are a lot of gene sources that can be harvested to be worked on and there are still enough left [39].

Hence, the number of patients that can be treated as long as the proper source of medication for the therapy is enough. For the time being using this method of treatment as the mainstream treatment like chemotherapy is still far away in the future due to the expensive cost. But, somewhere in the future and with the improved technique. Gene therapy will change the direction of the medicine's development and growth. As discussed earlier, many people will not opt for the therapy alone will sway public opinion even though it really benefits the society by improving their quality of life [39].

Scientists believe that the controversy arises due to the misconception and more education to the public will do the trick. However, if we take a closer look into the problem. The lack of trust of the public to the researcher makes people or the society itself believe that scientists are doing unethical practice in their work [39]. Ultimately, society itself will determine the success of gene therapy since they are the ones who make the decision about their own health while choosing their treatment plan. If the society does not believe the scientist, the study might be reduced and progress slower due to lack of funding.

4.4 Safety issue of the gene therapy

One of the major issues always in the spotlight is the safety of the treatment. Like mentioned beforehand, the under-educated factor on the technology and horror story about the illness or the clinical trial may be contributing to the concern on gene therapy. In 1999 the death of an 18- year old girl during the trial instilled great fear and hesitation among the society. After that incident the Subcommittee on Public Health of the committee on Health, Education, Labor and Pensions concluded that the society does not have enough knowledge on clinical trials. Clinical trials are where a lot of new treatments are being tested and it gives hope to the patient. But before being totally involved in any of the clinical trials, it is crucial for them to consider their own safety and wellbeing. It needs to outweigh their decision before jumping into any clinical trial to avoid any potential harm to them. The patient and their guardian also need to keep in their mind that the risk and side effects of the clinical trial are vary and sometimes unknown. Hence, it is also difficult to know all of them, especially the dangerous one like cancer. The symptom might develop years later. The case study that is famous for this incident is the effect of dioxin in Agent Orange when it was first introduced. During the fiasco, they did not know the extent of the toxicity of the agent until some patient that was currently in gene therapy to treat SCID developed cancer.

Next, the society or specifically patients are concerned about the use of the viral vector to introduce the donor DNA into their body. It is true that most gene treatments use viruses to deliver the gene. But one concept that they seem to be having hard time to understand is the pathogenicity of the virus is removed and their function in the treatment is only as the carrier for the correct gene to be transported.

There are cases that reported that the patient gets a negative response to the treatment when the virus is injected into their body. But that is one of many risks of the treatment. Viruses that are injected in the human body are strains and not copy. Hence, it cannot be biologically active and infect the host. For example, a herpes virus is used as the vector in the treatment would not get herpes from the treatment. Most of the time this is hard to explain and the word 'Herpes' has the stigma and makes the patient afraid of the treatment.

CONCLUSION

Sickle cell anemia is a genetic disease that was caused by the mutation that occurs at the HBB gene. The mutation causes the individual to possess HbS instead of HbA and the hemoglobin produced was sickle in shape and unable to carry oxygen efficiently throughout the body. The clinical symptom that will manifest by sickle cell disease patients is anemia due to lack of hemoglobin, episodes of pain and fatigue. The emergence of new technology. Development of gene therapy has been rapidly evolving for the last 50 years. The fundamental of the treatment is to find the defective gene and correct the gene. From extensive study done there are 3 main steps to visualize the gene therapy pathway. The first step is to harvest the stem cell from the donor. Second, the gene modification was done and the last one is chemotherapy conditioning before transplant.

The real challenge in those steps is at the second step where genes are modified. The targeted gene needs to be located and after that programmable nucleases (ZFNs, TALENs and CRISPR/Cas9) are bioengineered to create a double strand break (DSB) that will provide space for the complementary arm to correct the gene. This is where the vector plays their role as the carrier. While the body tries to correct or heal themselves, HR will take the sequence from the vector and copy them to be inserted into the break. Now, the patient can have the corrected gene and produce the normal hemoglobin or fetal hemoglobin by changing the expression factor. The advantage of the treatment is it offers better quality of life for both patient and their guardian. The treatment will tremendously help the patient to have a normal life without extended periods of bed ridden and constant visits to the hospital. Second advantage is the effect of the treatment is long lasting and timeless, the treatment targets the gene and the usage of the stem cell ensures the long period of relief. The treatment also can be used to completely eradicate the disease and maybe in the future when the germline gene therapy is less controversial. The disease could be prevented from before birth.

Different vectors were discovered but AAV is chosen because of the efficiency and it has no side effect on the repair of DSB instead it helps in promoting homologous recombinant and point to the initial step of vector optimization [41]. In addition, the use of CRISPR/Cas9 as the editing tool. Increase the precision of the treatment. This is because it is relatively easy to create a specific crRNA and tracrRNA compared to the whole sequence of proteins. The CRISPR/Cas9 complex portrayed a clear cut of the DNA while being annealed and ligand. The justification on the effect of gene therapy towards society is relatively still controversial. There are a lot of aspects that still need to be clearly and properly governed by the law enforcer. But it does not mean that it is not good. There are a lot of success stories that can be referred to. The case study for further study in America is case HGB-206 and for case study in France is HGB-205. Hence, it is important for the scientist with the press to educate the society about the knowledge and avoid more misunderstanding. Gene therapy has very promising results in medicinal and other biotechnology areas. It will be such a waste if we do not harness the knowledge and utilize it for the greater good.

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