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RESEARCH ARTICLE

CONCEPT OF GENE THERAPY: TYPES, THERAPEUTIC VECTORS (VIRAL AND NON-VIRAL), STRATEGIES FOR TREATING CANCER CELLS

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ABSTRACT

Introducing a new gene by replacing the mutant gene, is the prime goal of any gene therapy. In the present section, we will understand the mechanism of gene therapy and its types. "A process to recover the normal function of a gene by transferring a gene is known as gene therapy." Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in adults. In the past few decades, many efforts have been made to improve the prognosis of GBM, however, with limited success. Many gene therapy strategies for GBM have been developed and a few have progressed to clinical trials. Retroviral vectors have superior features for gene therapy in brain cancers, including tumor specificity, immunogenicity, and longer half-life. Early gene therapy trials in GBM patients based on transplantation of retrovirus-producing cells into the brain failed to prove efficacious. Adenoviral vectors, which can be prepared as high-titer virus solutions and undergo efficient transduction in tumor cells, failed in clinical trials, likely due to immunogenicity and instability of gene expression. Alternative therapeutics such as oncolytic viruses that specifically target and destroy cancer cells are currently under investigation. In addition to novel vectors, retroviral vectors are still attractive candidates for use in gene therapy against brain tumors. Since yields of properly-packaged viral particles from virus-producing cells have been very limited so far, gene therapy by direct injection of high- titer retroviral vectors into the patients' brains was not possible. To overcome these disadvantages, a packaging cell line that yields high-titer retroviral solutions was established by our group, enabling the direct injection of massive retroviral vector stocks directly into the brain. Mouse glioma models were effectively cured with a combination of a suicide gene/ prodrug system and a highly-concentrated retrovirus solution. Preclinical assessments, including that of replication-competent retroviruses and tumorigenicity of the combination method, have confirmed the safety of the highly-concentrated retrovirus solution. Addimited so far, gene therapy by direct injection of high- titer retroviral vectors into the patients' brains was not possible. To overcome these disadvantages, a packaging cell line that yields high-titer retroviral solutions was established by our group, enabling the direct injection of massive retroviral vector stocks directly into the brain. Mouse glioma models were effectively cured with a combination of a suicide gene/ prodrug system and a highly-concentrated retrovirus solution. Preclinical assessments, including that of replication-competent retroviruses and tumorigenicity of the combination method, have confirmed the safety of the highly-concentrated retrovirus solution. Additional studies are needed to address the clinical utility of such combination gene therapies. Taken together, these data suggest that retroviral vectors are still good candidates for development in gene therapy applications.

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INTRODUCTION

The concept of gene therapy was originated in the 60s by Rogers and Pfuderer. In 1961, K Lorraine had successfully introduced a functional gene in the mammalian cells. After several years of it, in 1971, Carl R. Merril experimented on Human Fibroblast cells and concluded that DNA could be inserted in the human genome for fixing the mutant gene Later on, in 1972. Theodore Friedman and coworkers successfully corrected the Lesch-Nyhan single gene disorder using gene therapy trials at the National Institute of Health. The idea of gene transfer in a human was given by Martine Cline, he was one of the pioneer scientists in gene therapy research. He had also attempted DNA modification techniques in 1980.In the year 1984, the first retroviral vector system was introduced by Richard Mulligan for delivering the foreign gene.

In 1990, a clinical trial of the world's first approved gene therapy performed under the close monitoring of NIH. In the early '90s, gene therapy gossip becomes stronger as it entered in the cancer treatment. In 1992, Trojan and coworkers introduced a concept of using gene therapy in cancer treatment. They had postulated that the introduction of the transgene could trigger off the oncogenic activity. In 2002, scientists had successfully treated adenosine deaminase deficiency through gene therapy. In the same year, sickle cell anemia mice were treated by introducing the artificial therapeutics gene. The era for using a non-viral vector in gene therapy was begun when polyethylene glycol was first time used as a vector for the delivery gene into brain cells in 2003.Introducing a new gene by replacing the mutant gene, is the prime goal of any gene therapy. In the present section, we will understand the mechanism of gene therapy and its types. *"A process to recover the normal function of a gene by transferring a gene is known as gene therapy."*

Current therapy for glioblastoma

Glioblastoma multiforme (GBM) is the most common and most aggressive malignant primary brain tumor in adults. Despite aggressive surgical resection coupled with optimized radiation and chemotherapy, GBM patients carry one of the worst prognoses and the median survival is 12 to 15 months (DeAngelis, 2002; Mahaley, 1989; Deen *et al*., 1993). The resistance to treatment and highly invasive property are representative bio- logical features of GBM that prohibit improvements in the prognosis after conventional therapies. The current standard of care for GBM patients includes maximum surgical resection combined with radiation and concomitant and adjuvant temozolomide (TMZ) therapy (Stupp *et al*., 2007). Even with this multimodal therapy, extension of the median survival is only two to three months. Due to the poor prognosis despite the use of multimodal therapy, there is an urgent need for the development of effective novel therapies. Gene therapy is becoming a promising therapeutic alternative among of the many strategies explored for GBM.

Gene therapy strategy for malignant tumors: Gene therapy is a technique for correcting defective genes responsible for disease development or for removing malignant cells by killing these cells. Viral vectors are used to transfer the therapeutic genes into the target cells or tissues in patients. In gene therapy for congenital disorders, most of the gene sequence is used to supplement or alter the defective function. The first clinical trial of gene therapy for a genetic disorder was performed on patients with adenosine deaminase severe combined immunodeficiency (ADA-SCID) (Blaese *et al*., 1995). The absent or mutated adenosine deaminase in this disease was delivered by retroviral vector ex vivo to complement this enzyme. Contrary to the case of congenital disorders, it is not easy to obtain the therapeutic efficacy by transfer of correct genes into malignant tumors. A small number of non-transduced cells contribute to tumor recurrence by escaping the therapy. One of the most challenging problems in gene therapy is gene delivery. Low transduction rate of therapeutic genes leads an ineffective treatment, especially in gene therapy for malignant tumor (6). Gene therapy by transfer of correct genes into tumors requires a higher rate of transduction to kill most tumor cells and current transduction methods are inadequate for eradication of metastatic or highly invasive tumors. The low transduction rate can be partially resolved by development of "suicide" gene therapy using a combination of toxic genes and prodrugs (Duarte, 2012). Suicide gene therapy capitalizes on the so-called "Bystander effect", a phenomenon in which toxic metabolites derived from the prodrug are transferred from the transduced cells expressing the toxic gene to neighboring non-transduced cells via gap junctions; this effect is recognized as a critical compo- nent that helps obtain therapeutic efficacy with suicide gene therapy (Bi *et al*., 1993). In fact, we and others reported that suicide gene therapy had a transduction rate as high as 10% - 70% and induced effective tumor regression in experimental models of brain cancer (Culver *et al*., 1992; Yamada *et al*., 1992; Tamura *et al*., 2001). The basic principle of many gene therapies for malignant tumors is based on this mechanism and the use of viral vectors for gene delivery.

Types of gene therapy: Four types of gene therapy are most popular:

- Somatic cell gene therapy
- Germline gene therapy
- *In vivo* gene therapy
- *Ex vivo* gene therapy

Somatic cell gene therapy: The somatic cell and germline gene therapy are categorized based on the cell type involved in it while the *In vivo* and *ex vivo* gene therapies are categorized based on the method of application. The cells other than germline cells are somatic cells. Bones, blood, skin, internal organs, and other vital tissues develop from somatic cells that follow mitosis cell division. 46 numbers of chromosome viz 23 pairs are present in somatic cells. "In the somatic cell gene therapy, a mutant gene within somatic cells is replaced by a transgene." The change that we made through the gene therapy remains lifetime only, it will not inherent to the next generation. And this is the limitation of the present therapy. The somatic cells are not involved in the reproduction, hence it cannot be inherited. Various ethical issues are involved in the somatic cell gene therapy, though, the success rate is noticeable.

Virus-mediated gene transfer and liposome-mediated gene transfer are widely used in SCGT (somatic cell gene therapy).Although it can be applicable for all the somatic cell types, the common target is the bone marrow. Cells present in the bone marrow are the only cells that can be divided throughout life and produce different types of blood cells. This is the reason, SCGT success rate is more for blood born disorders such as thalassemia, sickle cell anemia, and hemophilia. In the process, first, virus-like AAV (Adeno-associated virus) commonly used as a vector in somatic gene therapy, infects the isolated bone marrow cells. Once the gene inserts at target location, transformed cells are grown in a lab. The cells injected back into the bone marrow. The broad overview of the entire mechanism of somatic cell gene therapy is shown in the Figure below, Fig 1.

Somatic cell gene therapy has several limitations:

A virus can combine with the host genome and infect the cell (Viral escape).

- Also, if a gene does not insert at the specific location, it may disrupt the function of the normal gene too.
- The chances of activation of oncogene and proto-oncogene are very high.

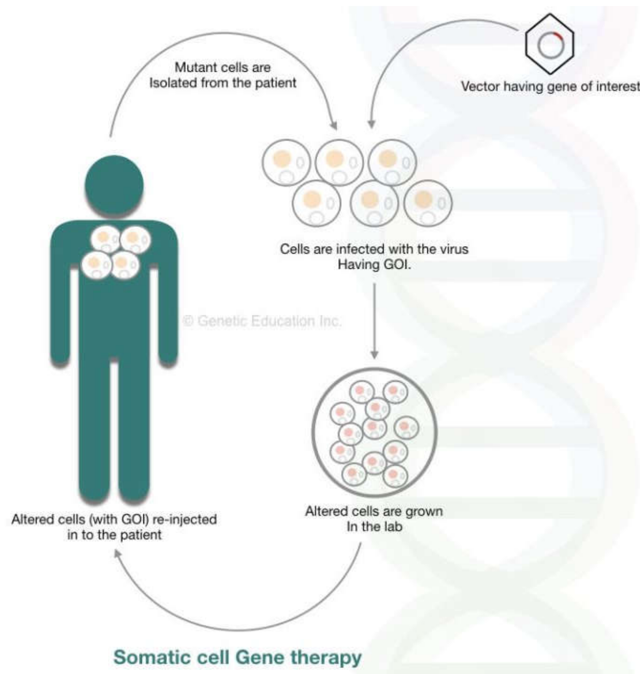

Fig .1. Somatic Cell Gene therapy

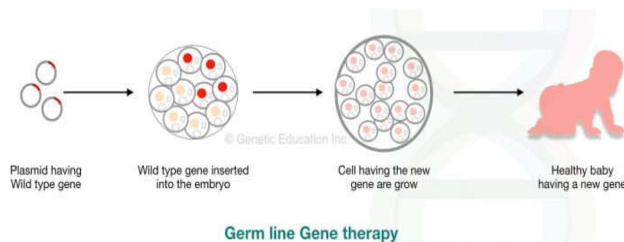

Fig.2. Germ line Gene therapy

Germline gene therapy: "Before splitting the embryo, the GOI is introduced in germ cells (sperm or egg cell), fertilized egg cell or embryo, this method of transferring gene is referred to as a germline gene therapy." Consequently, the change made in the DNA of these cells can pass it to the next generations. As we are playing live embryo here, some scientists believe that it is unethical. Germline gene therapy is prohibited worldwide. However, the comprehensive overview of the method is like this: Using viral vectors, cells from the reproductive tissues or from the un-split embryo are infected. The modified cells grow in the sterile lab environment, care must be taken while doing this. The modified cell must not be infected with other unmodified viral cells. The modified germ cells are used for *in vitro* fertilization and other artificial reproductive techniques. However, direct injection of a transgene into the embryo is preferred more. The mechanism of germline gene therapy is shown in the Figure below Fig.2.

***In vivo* gene therapy:** In the *in vivo* gene therapy, the exogenous gene is directly inserted into the target cell. The gene of interest introduced into the body through aerosols or injection. The effect of *in vivo* gene therapy is restricted to some areas. It introduced GOI only to the affected area, not all the bodily tissues. The ideal examples of *in vivo* gene therapy are cystic fibrosis and Duchenne muscular dystrophy. In cystic fibrosis, the exogenous gene (or transgene) is introduced through nasal spray (aerosol) whereas in DMD the GOI, a dystrophin gene inserted into the muscle cell through the injection. Nonetheless, some surrounding cells are infected without any reason and cause some adverse effects.

Conclusively, the *in vivo* gene therapy (Fig. 3) is not a good option for treating inherited diseases for now.

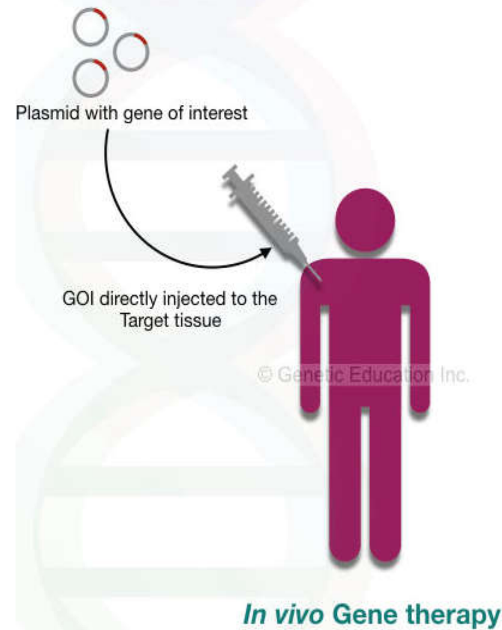

Fig .3. In Vivo Gene therapy

Ex vivo gene therapy: In the *ex vivo* gene therapy, affected cells are collected, modified in a lab (outside body system), and inserted back in our body. Here none of the gene therapy processes/steps is performed inside the body hence it is called *ex vivo*. A researcher can control the whole process, thus the present method is safer and gives more control to scientists. Although the technique is relevant for those cells which have the potency to divide. Hence only several types of tissues or cells can be altered using *ex vivo*. Bone marrow or blood cells are such types of cells often used for the *ex vivo* gene therapy because the bone marrow cells can divide throughout life. Why cell must be actively dividing?.

The reason behind choosing actively dividing cells that the transgene can spread faster in other cells. Therefore blood cells and bone marrow cells are the only choices for it. Because of this reason the *ex vivo* gene therapy is restricted to some of the blood-related disorders. Thalassemia, sickle cell anemia, thrombosis, and hemophilia can be treated using this technique. The steps of the *ex vivo* gene therapy are listed below, The defective or mutant cells are isolated from the patient. Exogenous gene is inserted in the defective cell lines using viral vectors. Under the strict sterile condition, altered cells are grown. The transformed cells are selected and grow in such conditions where it is not infected with other viruses. The modified cells are now injected back to the patient's body. The frequently used vectors for *ex vivo* gene therapy are retrovirus and AAV.

To use retrovirus, the ψ sequences of it are first removed, so that the virus cannot insert their own DNA into the host genome (We had discussed the whole mechanism in the next section of this article). AAV is an efficient vehicle for *ex vivo* gene transfer because it can efficiently infect dividing cells. Cystic fibrosis and Duchenne muscular dystrophy cannot be treated with it. The success rate of the *ex vivo* gene therapy depends on the rate of incorporation and expression of a transgene. We will discuss the hemophilia gene therapy in another article of this series in the Fig. 4.

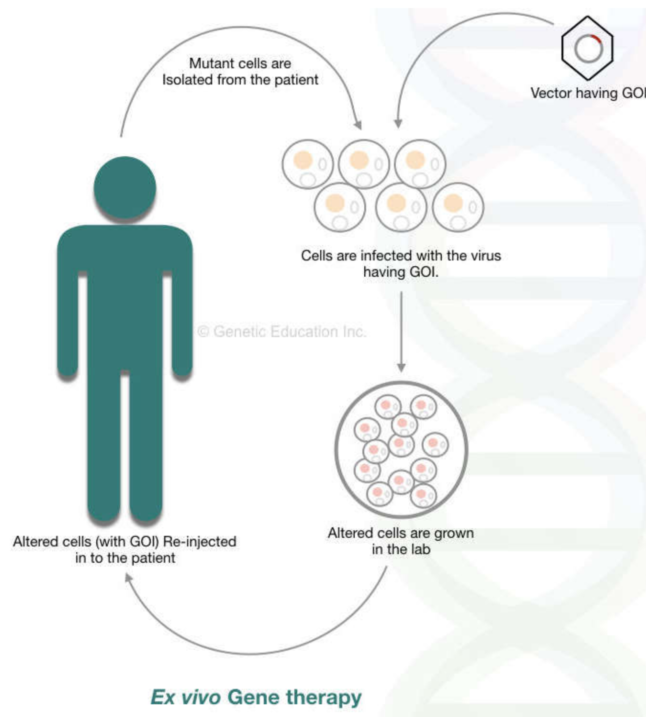

Fig .4. Ex Vivo Gene therapy

Different technique of gene therapy

Loss of function mutations and abnormal gene expression are two key reasons to cause genetic disorders. Based on the technique used, three other types of gene therapy are:

- Gene augmentation therapy
- Gene inhibition therapy
- Suicidal gene therapy

Gene augmentation therapy: The natural function of a gene is lost due to some of the polymorphism called a loss of function mutation. Due to the loss of function mutation a normal function protein does not translate. In the gene augmentation therapy, the mutant (LOF gene) is replaced by the fully functional wild type gene that translates a wild type protein again in the body. The present technique is majorly used for monogenic disorders. Cystic fibrosis-like disorders can be treated using gene augmentation therapy.

Gene inhibition therapy: Over expression of a gene causes some life-threatening disease conditions like cancer. Change in the methylation pattern or epigenetic profile of a particular gene results in the over expression/under expression of that particular gene.

In the gene inhibition therapy, the overexpressed gene is inhibited by using,

- another gene or DNA sequence
- or by interfering with the activity of the product of that gene.

The present gene therapy is the best choice for an inherited disease, infectious disease, and cancer. The over expression of the oncogene can be lessened by using this method.

Suicidal gene therapy: In some types of diseases, it is very necessary to kill the cell, especially in cancer.

For those types of cells, some of the genes called suicidal genes are introduced to kill the cells. The Gene produces a toxic product that induces a strong immune response against that cell leads to cell death. Suicidal gene therapy is specially designed for cancer therapy. The mechanism of Suicidal gene therapy is shown in the Figure below Fig.5.

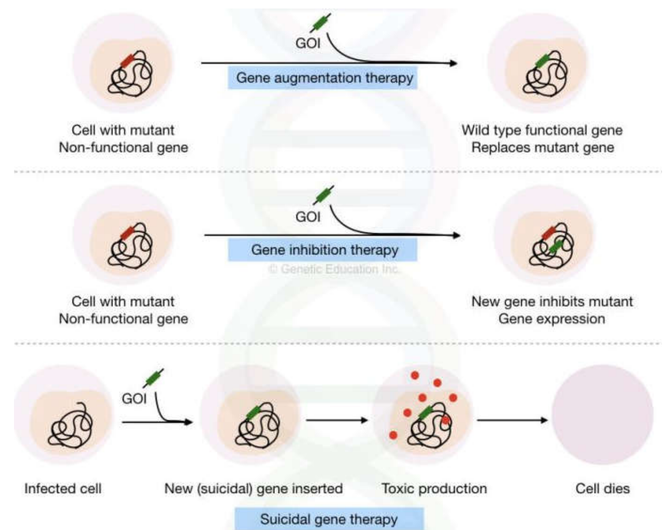

Fig 5. Suicidal Gene therapy

Vectors for gene therapy (Viral and non-viral)

Viruses, liposomes, and naked DNA are some of the vehicles used to introduce transgene into the host genome. The vehicles used to introduce the transgene is known as vectors, the utility of the vector depends on the factor enlisted below,

- The size of the exogenous gene (transgene)
- The efficiency of the delivery
- It will induce the host immune response or not
- The stability and longevity of the transgene
- Level of expression of a transgene

"If the selected vector can carry larger transgene that cannot induce immune response with good efficiency to infect the cell and higher expression rate, are selected for the gene therapy experiment." The classification the gene therapy vectors are given into the Figure below, the classification of gene therapy is shown in the Figure below Fig.6.

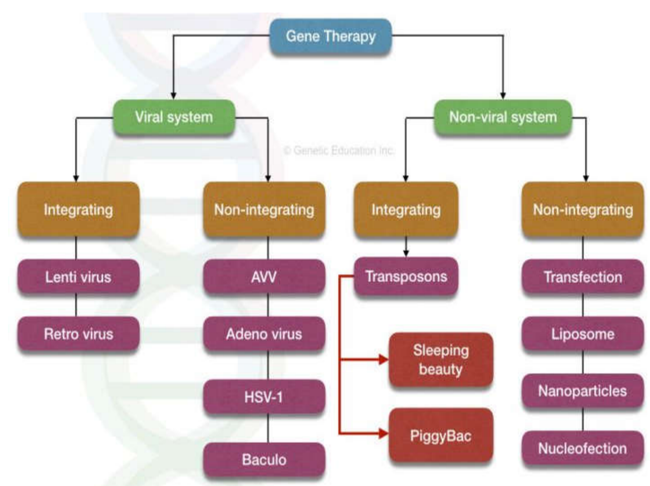

Fig .6. Classification of Gene therapy

The vectors are divided into two broader categories:

- Viral vectors
- Non-viral vectors

Viral vectors: Some of the common types of virus used in the gene therapy are listed here,

- Retrovirus
- Adenovirus
- Adeno associated virus
- Lentivirus

Retrovirus: The retrovirus is an RNA virus, the genome of it is made up of the RNA (not DNA). It has two RNA molecules in their genome. Therefore it is also known as retrovirus-mediated gene therapy. By the mechanism of reverse transcription, the RNA forms DNA (more specifically called cDNA), and the DNA is integrated into the genome of an organism. Structurally, the retrovirus contains *gag*, *pol*, *env* genes, long terminal repeats (LTRs) on both the side and Ψ sequences. The mechanism of Retrovirus is shown in the Figure below Fig.7.

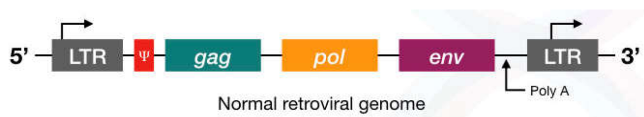

Fig .7.Normal Retroviral geneome

The *Pol* gene encodes for the reverse transcriptase enzyme. The *gag* gene encodes for the viral core protein. The *env* gene encodes for the envelope protein present on the surface of the virus helps in recognition of the receptor present on the target cell. The Ψ sequence required for the packaging of viral particles, therefore these sequences are very important for the virus to infect the host cell, although these sequences are non-coding. The LTR sequences present on both the ends help the reverse transcribed DNA to integrate into the host genome.

Adenovirus: The adenovirus is one of the best choices since long for gene therapy. The genome of it made up of double-stranded DNA. The size of the genome is 35kb, which is surrounded by the icosahedral capsid made up of 12 different proteins. It is beneficial over the retrovirus because it can naturally infect the non-dividing cells, especially, cells of respiratory and gastrointestinal tracks. Because of this reason, it is one of the best opportunities for cystic fibrosis gene therapy. One other benefit the adenovirus provides among all other vectors is that it can evade the host immune response. It is also highly-target specific and has controlled integration. Which means it can only infect or integrate the DNA into their host cells, not other surrounding cells. Among 50 different serotypes, serotypes 3 and 5 have a higher degree of tropism for the respiratory tract cells. However, due to its harmful effects, it is very necessary to disable the viral replicating mechanism. The viral gene expression can be divided into early and late phase, for understanding this we have to understand the structure of it. The virus has the 100bp terminal inverted repeats between which the 35 kb DNA is present. The Structure of adenovirus is shown in the Figure below Fig.8. The expression of the adenovirus divided into two phases the early infection phase and the late phase. The early gene expression is too low and derived from the E1, E2, E3, and E4 regions of the genome.

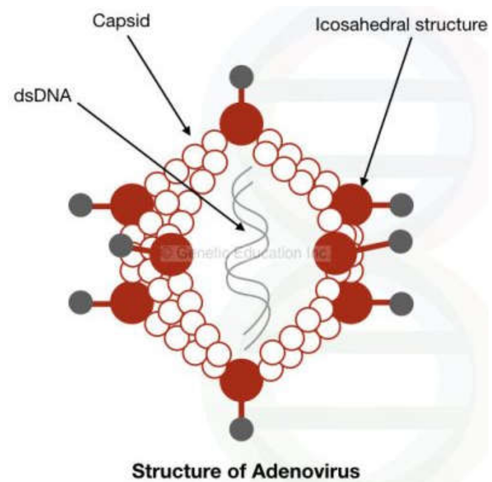

Fig .8. Structure of Adinovirus

The rest of the region involved in the late expression of the virus. Clinical trial on the adenovirus revealed that even in the inactive stage adenovirus prompts a strong immune response.

Adeno-associated virus: The genome of the Adeno-associated virus is made up of the single-stranded DNA contains only two genes "*rep*" and "*cap*". Also, the terminal repeats are present on both ends of it. The *rep* gene encodes for the protein that helps the AAV to integrate into the host genome, especially on chromosome 9. The cap gene encodes for the protein that constructs the capsid of it. Interestingly, the AAV virus is naturally non-replicative, it required one helper virus to do so. The adenovirus is used to do this function along with the AAV. Since the AAV can infect both dividing and non-dividing cells, it is the best alternative of the adenovirus. Majorly, it infects the cells of upper respiratory airways with a long-lasting expression of more than 6 months. For gene therapy, the *rap* and *cap* gene of the AAV is replaced by the transgene. By removing the *rep* gene, the virus loses its specific integration power on chromosome 9. The genomic structure of AAV is shown in the Figure below, genome of the Adeno-associated virus is shown in the Figure below Fig.9.

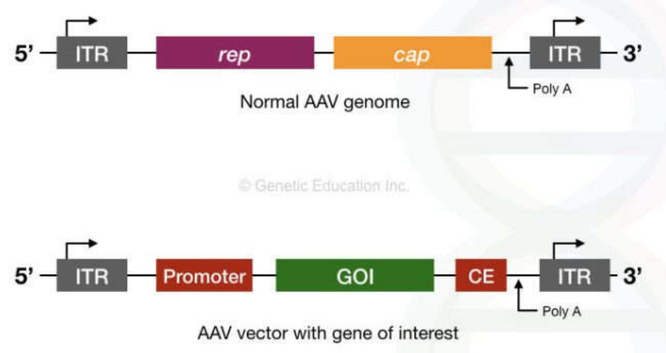

Fig .9. AAV Vector with Gene of Interest

Due to the long-lasting expression of AAV, it is now allowed in human trials for the hemophilia A gene therapy.

Lentivirus: The lentivirus is another form of the retrovirus that can even infect the non-dividing cells. HIV is one of the best examples of Lenti-retrovirus.RNA is the genetic composition of a lentivirus carries *env*, *gag*, and *pol* genes. However, HIV like lentivirus only infects the T cells. Practically, using HIV for gene therapy is not a good choice.

The summary of the viral vectors:

vector	DNA carrying capacity	Positives	Negatives
Adenovirus	8kb to 35kb	-Infect both dividings as well as non-dividing cells. -Chance of infection is less	-Provoke strong immune response. -Transient expression
AAV	Up to 4.5kb	-Longer transgene expression. -Non-pathogenic -Broad tropism	-Smaller DNA carrying capacity. -Required a helper virus
Retrovirus	Less than 8kb	-Stable integration -Infect replication cells -Suitable for *ex vivo* treatment	-Oncogenic activity -Uncontrolled integration -Can not infect non-dividing cells -Adverse effect -Provoke immune response
Lentivirus	8kb	-Infect proliferating, non-proliferating and bone marrow cells. -Self inactive	-The DNA carrying capacity is less. -The chance of infection is high. -Provoke immune response.
HSV	30kb	-Safer -Higher DNA carrying capacity -Suitable for *in vivo* gene therapy	-Difficult to produce

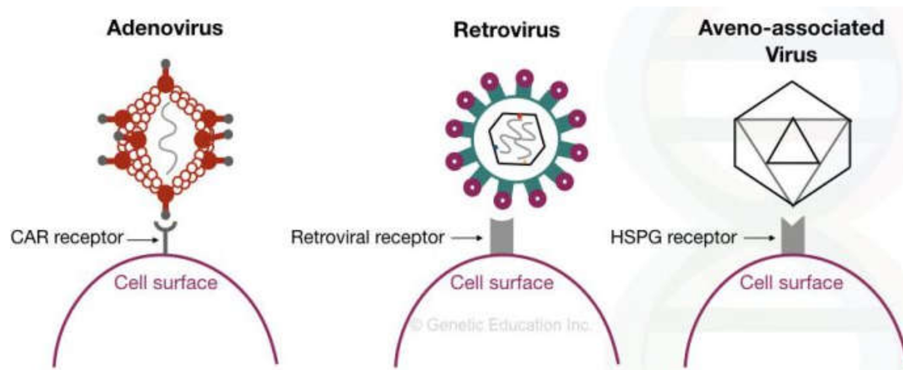

Fig .10. Adenovirus, Retrovirus, Aveno-associated Virus

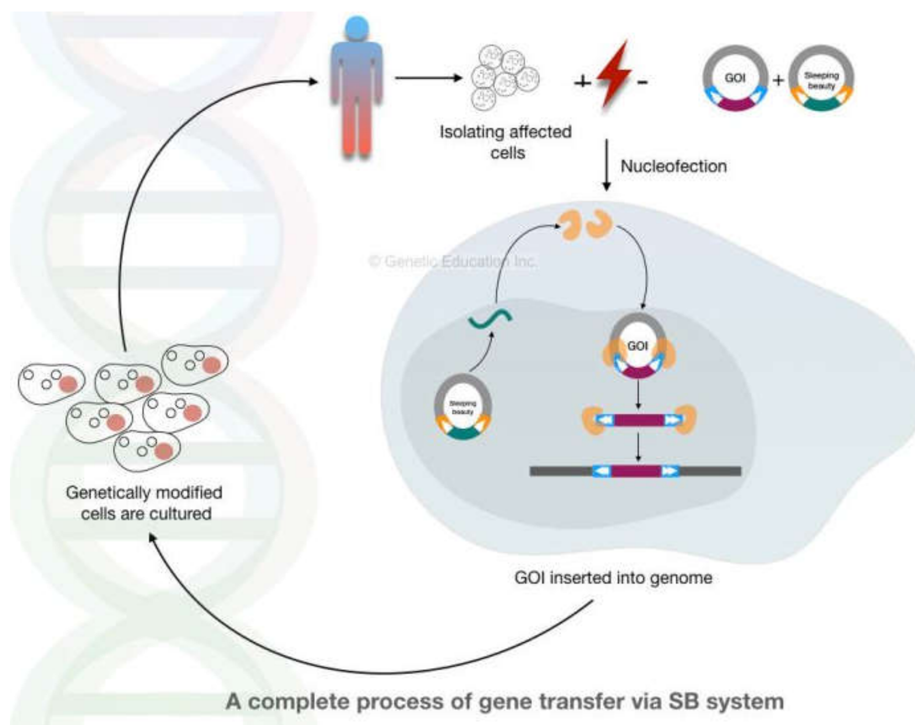

Fig 11. A Complete Process of Gene transfer via SB Systemy

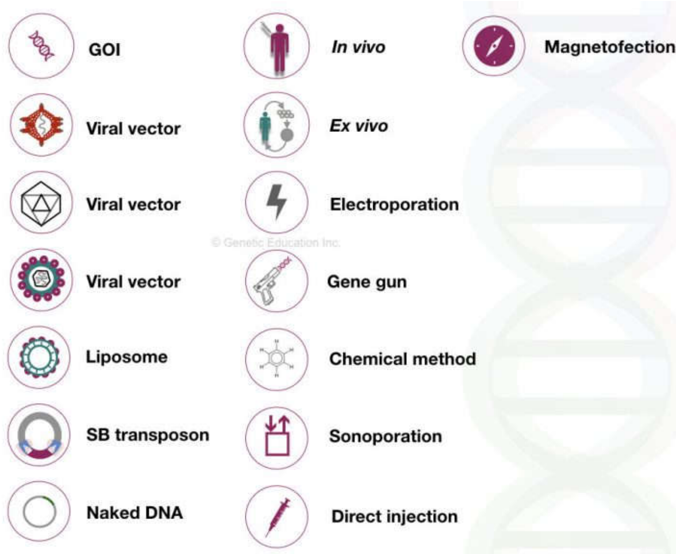

Fig .12. Magnetofectiony

The image given below shows how the viral vectors (Fig.10) insert the gene of interest into the host genome by interacting with the receptors present of the surface of the cell.

Non-viral vector: Liposome, naked DNA, nucleofection, and transposons are some of the non-viral vector-mediated methods used for the gene therapy. Why the non-viral vector will be one of the best opportunities for the gene transfer? The non-viral vectors are non-toxic, non-immunogenic, and tissue-specific. Also, it is easy to design and apply them. Some of the non-viral vectors are discussed below,

Liposome: The liposome also called lipoplex-mediated gene therapy is an artificial technique non-infected to the host cell. The liposomes are artificially synthesized molecule typically 0.025 to 2 µm in size, made up of the lipids. The DNA cannot directly be inserted in the cell because the cell surface, as well as the DNA itself, has a negative charge. Hence naturally both repel each other. The liposome is used to solve this problem. The lipid molecule is hydrophilic as well as hydrophobic in nature which protects the interior aqueous phase. The head of it is hydrophilic while the tail is hydrophobic. The liquid solution of DNA is encapsulated with the liposome lipid bilayer which helps in penetrating it. Nonetheless, due to the smaller size of it, the liposome is not a good choice for the larger size of gene transfer. Also, the liposome is non-attractive for the DNA as well as the cell surface.

This problem is encountered by introducing a positive charge on the hydrophilic domain of the lipid. A new type of upgraded version of the liposome is designed and named it as lipoplex. The lipoplex attracts both the DNA as well as the cell surface, hence, a more stable complex of lipid-DNA is created. This tube-like structure efficiently transfers DNA into the target cell. Plus, lipoplex is non-immunogenic. Due to this reason, it is the best choice over the liposome and virus-based vector. Furthermore, it is easy to prepare and can transfer a large amount of DNA. The major disadvantage of the lipoplex is that it is not as efficient as the virus-based gene therapy.

Transposon: Transposon-Mediated gene therapy is one of the emerging therapy after the CRISPR-CAS9. Why transposon? The transposons are mobile genetic elements that can move from one location to another into the genome. It also contains coding genes and terminal repeats as like the viruses. However, almost all transposons are inactive for long. The scientist has discovered active transposons from the fish fossil and named it as sleeping beauty transposon. The SB system is capable of inserting DNA into the host genome without any side effects. It escapes the host immune system too and delivers a gene of interest efficiently at the target site. Although the method still has many loopholes and limitations that need to be perfected before any pre-clinical trial. The general mechanism of the SB system is shown in the below Figure 11. Nevertheless, systems like SB transposon and CRISPR-CAS9 will become more effective in the future. We had explained sleeping beauty transposon in our previous article, the article contains all the information on the SB system, starting from their discovery to its mechanism of action.

Naked DNA: During the experiments on mice, the scientist had observed that the naked DNA directly injected into the mice muscle cells through the lesions present on the cell surface. Although theoretically, it should not possible as both the DNA and the cell surface contain negatively charged molecules that repel each other. The scientist believes that naked DNA will be useful for the production of therapeutic proteins. The transgene can be inserted into the muscle cell for the production of proteins such as insulin and thrombotic factors. Nevertheless, enough research data are not available at present in favor of naked DNA use in gene therapy. Some of the other non-viral vectors mediated gene therapy methods are discussed here.

The non-viral vectors are further divided into three broader categories.

- Physical methods
- Chemical methods
- Synthetic molecules

Physical methods: Several physical forces or procedures such as needle, gene gun, electroporation, ultra-sonication, and laser photo-poration can be used in the gene transfer.

Electroporation: The first attempt of *in vitro* electroporation was done in the year 1982 whereas the first attempt for the *in vivo* electroporation was demonstrated in the year 1991. However, the method is known since 1960. The basic method for both *in vivo* as well as *in vitro* electroporation is the same. By applying the high electrical current for breaking the cell wall, pores are created on the surface of the cell.

The pores are formed within a second. The higher the pulse duration faster the pore forms. The duration of pulse and amplitude decides the permeability of the membrane for the gene transfer. The transgene can be delivered either through intramuscularly of through intradermally. Sometimes intratumoral gene transfer can also be performed in the case of cancer cells. The electroporation readily delivered plasmid DNA into the cell. The field strength and the pulse vary from tissue type. The *in vivo* electroporation method having a higher specificity and success rate directly injects the plasmid DNA into the target tissue. Contrary, the method is restricted to some of the tissues, it is not accessible to the internal organs. The *in vitro* electroporation is not as impressive as the *in vivo* therapy. The method is also known as an electro-gene injection, electro-gene transfer, or electrical mediated gene therapy.

Sonoporation: The method is often known as sonication, was first described in the year 1954 for the transdermal drug delivery. For the cellular DNA engulf, a temporary cell membrane permeability is created using the ultrasonic waves. After each round of sonication, the energy absorbed by the tissue results in the locally temporary heating that disrupts the cell membrane and produced holes.

The process of sonoporation mediated cavitation is called acoustic-mediated cavitation. Common ultrasound frequency for the gene transfer is 1-3 MHz with 0.5 to 2.5 W/cm2 intensity. The use of artificial microbubbles made up of the lipid layer and gas-filled core makes the method more advanced and efficient. The use of the surface stabilized such as synthetic lipid or polymers, phospholipid or albumin makes the microbubble more powerful for gene therapy. The transgene is introduced in the microbubbles by charge coupling, incorporating it into the shell or lumen. The Microbubbles are 1 to 6 µm in diameter having white blood cell-like resemblance.

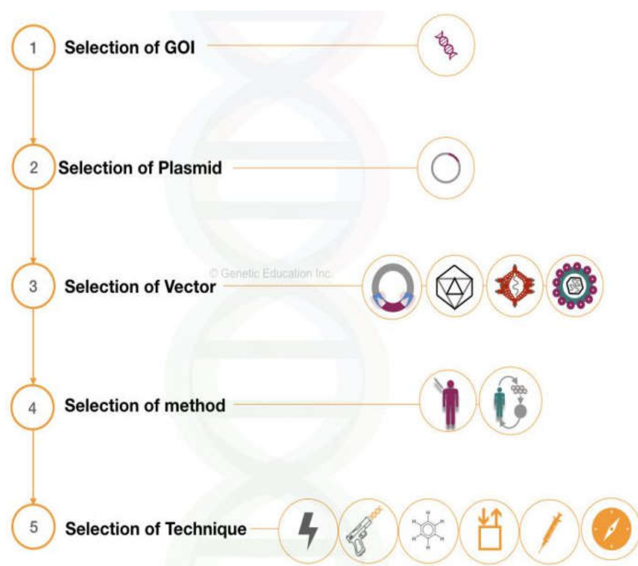

Fig .13. Steps in the process of gene therapy

The success rate of the sonoporation depends on the:

- The intensity of the ultrasound
- Frequency of ultrasound
- Size of DNA to transfer
- The structure of the microbubble
- Duration of sonication

The method is highly site-specific, non-invasive, and safer. Even the method is applicable for the internal organs also without any surgical operations. The method is readily available for the vast majority of tissue types such as muscles, heart, cornea, brain, and kidney tissues. Furthermore, along with the microbubbles using tissue-specific receptors, antibody and ligands increase the specificity of the method.

Gene Gun: The gene gun method is first introduced in the year 1987. The method is also known as ballistic DNA transfer, micro-projectile gene transfer or DNA-coated particle bombardment. Highly pressurized gas and metal ions are two components of the particle bombardment gene gun method. Also, instead of highly pressurized gas, electrical current or electrical discharge method can also be used. The microparticles made up of silver, gold or tungsten deliver the transgene under the speed of highly pressurized gas (helium). The Gene gun method is efficiently used for the intradermal, intramuscular or intratumor gene transfer. The success rate of the present method depends on the gas pressure and velocity, size of the microparticle, sie of the transgene, and the dose of injection. 1µm metal particles precisely transfer the transgene to the target. It is routinely used in ovarian cancer nowadays.

Photoporation: In the photoporation method, a transgene introduction is permitted by the laser-induced pores in the cell membrane. The success of the gene delivery depends on the frequency of laser light and the focal length. Due to the lack of documented evidence and research data, the method is not used so often in gene therapy. Although, it is as effective as electroporation.

Hydroporation: A large amount of DNA solution is directly injected via the hydrodynamic pressure. The pressure creates pore through which the DNA inserted into the host cell. The method is commonly for gene transfer in the hepatic cells.

Needle: The needle method is first used for naked DNA insertion thus it is more suitable for gene insertion in skin, muscle, liver and cardiac cells. The gene of interest injected directly through the needle without using any physical method. The method is simple yet effective. However, the efficiency is too low as compared to other gene therapy vectors.

Magnetofection: In this method, the magnetically charged particles copulated with the GOI. Under the higher magnetic field, the transgene inserted into the cell or cell line. The magnetofection method is more suitable for *ex vivo* applications. Some of the symbols used in this paper shown in Figure 12.

Chemical method: Here we are not discussing all the chemicals used in gene therapy. Gold particles, silver particles, silica and calcium phosphate are used as chemical agents for gene delivery. These particles efficiently transfer the gene into the cytosol by complexing with it or by protecting it from nucleases or other enzymes.

Synthetic Nanoparticles: Synthetic nanoparticles are another optional source for gene delivery that are safer and easy to use. Cyclodextrins like cyclic natural nanomaterials can interact with the DNA having low immunogenicity. Therefore it is one of the best naturally available nanomaterial for gene therapy. It is very essential for a foreign particle to escape from the endocytosis, the process that kills it. Polyethyleneimine (PEI) is a gene delivery vector that helps in escaping GOI from the endosome. However, due to the presence of high positive charges on it, the vector is less effective. Polyethyleneglycol (PEG) is one of the best nanoparticle used in gene therapy. It can efficiently transfer DNA to its target location. It is majorly used in the delivery of siRNA. Besides all these, Some of the peptides and proteins are also used for gene transfer. Other nano-particles such as dendrimers, polymethacrylate, chitosan, and cationic synthetic lipids are used as gene delivery vehicles too.

The process of gene therapy

Any of the gene therapy experiment can be divided into 5 different steps shown in the Figure 13.

- Selection of GOI

- Selection of plasmid
- Selection of vector
- Selection of method
- Selection of technique

Selection of GOI: One of the most crucial steps in the gene therapy experiment is selecting the gene of interest. We have to select the appropriate GOI based on the disease type.For instance, a single gene can be transferred efficiently rather than two or more genes. Therefore selecting monogenic disease yields more promising results. Furthermore, the size of the gene also matters a lot to succeed in the experiment. The gene transfer efficiency of smaller fragments is very good as compared with larger DNA fragments.

The GOI must have the following characteristic

- The gene must have high AT-rich sequences.
- GC content must be less than 50%
- The gene must not contain a large number of exons

Selection of plasmid: Plasmid plays an important role in delivering the gene of interest at its specific location into the host genome. The plasmid selected for the experiment must have a higher transgene carrying capacity. Also, it possesses some of the important sequences needed to insert the DNA.
It must have,

- Promoter sequences (specific to the gene of interest)
- ITR (inverted terminal repeats) which is needed for the recognition of the target site in the genome.
- An antibiotic resistance gene
- Control elements
- Genes essential for viral enzymes.

Selection of vector: Vector selection is another big factor for gene therapy. The vector selection process is based on the type of gene we want to insert. Although the viral vector gives excellent delivery rate and integration efficiency. It is not so safe. Furthermore, the selection of vector also based on the size of the GOI. For instance, if the size of the transgene >35 kb, the adenovirus should not be recommended because the gene carrying capacity of it is lower than this. Care must be taken while using the viral vectors especially, the retrovirus.

Selection of method: After selecting both GOI as well as the vector, decide the method of the gene transfer. If the target tissue is a somatic cell, use the somatic cell gene transfer method. Choosing *ex vivo* or *in vivo* is depends on the transgene which you want to insert. For example, if the transgene is for cystic fibrosis, *in vivo* gene therapy method works excellently, however, the same method does fail for DMD or AIDS. Contrary to this, the *ex vivo* gene therapy gives more precision for a disease like hemophilia.

The selection of the method is based on the:

- Type of disease
- Type of transgene
- Type of tissue
- Chance of infection

Generally, *ex vivo* gene therapy for AIDS does not recommend due to the high-risk factor associated with it.

Selection of technique: The fifth step of the gene therapy experiment is the selection of techniques. If the non-viral vector is selected as a method, you need one of the techniques from sonication, electroporation, magnetoception, gene gun or liposome. The DNA cannot directly be inserted into the host cell, so we need to create pores on the cell surface. Electroporation is the best technique for all types of gene transfer experiments with non-viral vectors. Even, it works more efficiently along with the liposome too.

Experimental set up: The experimental set up is as crucial as the above-listed steps. For the gene therapy experiments we need a highly contaminant-free, sterile, and sophisticated setup. A state of the art laboratory facilitating the gene therapy must be equipped with all the utilities and safety setups. Also, the experiments must be performed under the supervision of the experts. Steps of the gene therapy are shown in the below Figure 14.

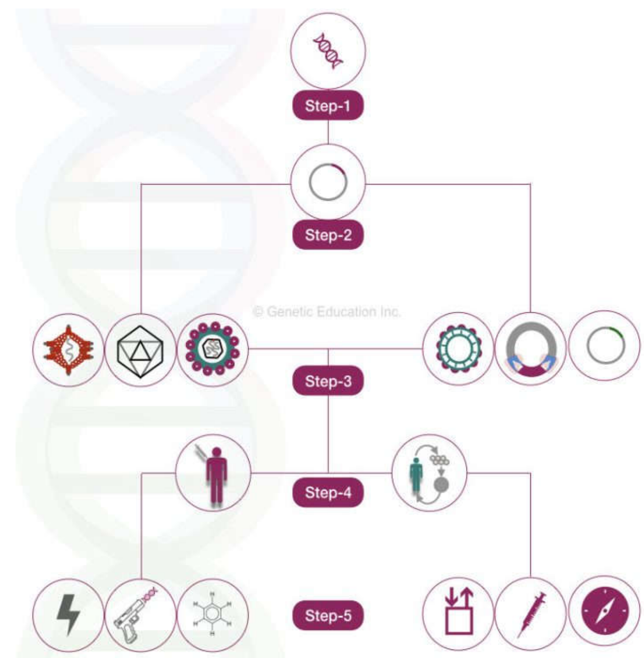

Fig 14. Experamental setup of Gene therapy

First gene therapy for glioblastoma (gbm) using retrovirus-producing cells

Glioblastoma multiforme (GBM) is the most common and most aggressive malignant primary brain tumor in adults. Gene therapy for glioma patients was initiated in 1992 at the National Institute of Health in United States (12). This clinical trial was based on suicide gene therapy, which utilizes the combination of the herpes simplex virus thymidine kinase (*HSVtk*) gene and the prodrug ganciclovir (GCV). It is now a well-known strategy and its efficacy has been confirmed (13-15). The bystander effect can be induced by optimizing the timing of GCV administration and therapeutic efficacy is increased in this system. The strategy of the clinical trial consists of GCV administration and the transduction of retrovirus vector harboring *HSVtk* gene, which induces the conversion of GCV to toxic metabolites. However, the retroviral titer was unfortunately too low in the clinical trial; therefore, virus-producing cells (VPCs) were transplanted into the tumor cavity to increase the transduction efficiency (Figure 15). This modified gene therapy was then applied to phase III clinical trials, but failed to provide efficacy (6).

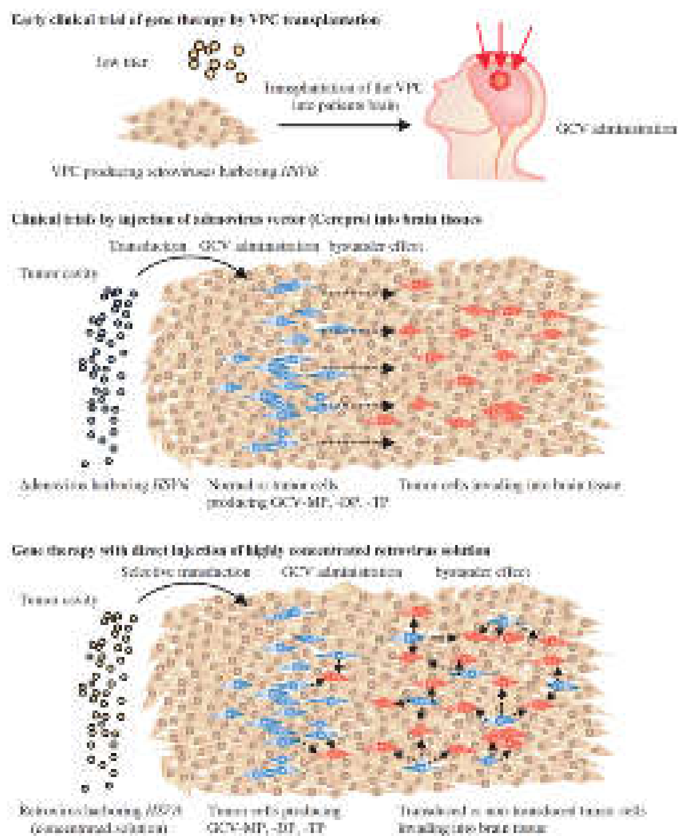

Fig .15. Strategy of suicide Gene therapy

One reason for the failure of this gene therapy is caused by the low transduction rate, probably caused by the low potential of VPCs to produce the retrovirus and the instability of the xenotransplanted mouse VPCs in humans.

Suicide gene therapy by the injection of adenoviral vector into the wall of the tumor cavity: Adenoviral vectors have been widely used in gene therapy studies (Gene Therapy Clinical Trials Worldwide, 2013). Because the genome of adenoviruses consists of a linear double-stranded DNA genome, the stability of the virus is relatively high, which allows for the preparation of high-titer virus solutions. Adenoviruses are able to transduce both dividing and non-dividing cells. In experiments, including those in which intra-cranial injection was sued for transplantation of retro- viral VPCs or adenovirus, much higher transduction rates were found in some areas of tumors in mice treated with an adenoviral vector (Puumalainen *et al*., 1998).

However, an important limitation in the use of adenoviral vectors has been the difficulty in obtaining expression stability, because the viral genome fails to replicate in the transduced cells. Adenoviral vectors were adapted to improve the transduction rate in clinical trials of suicide gene therapy using *HSVtk* (Sandmair *et al*., 2018; Trask, 2000; Eck, 1996; Smitt *et al*., 2003). In these clinical trials, adenoviruses were injected multiple times into healthy brain tissues in the tumor cavity following surgical removal of the solid tumor mass, which was then followed by GCV administration (Figure 15). An improvement in the median survival period of glioma patients treated with this vector has been reported (Smitt *et al*., 2003). The assessment of the safety of this suicide gene therapy was performed by serological assessment, *i.e.*, routine blood and urine analysis and the detection of anti-adenoviral antibodies. These assessments indicated that the therapy was well-tolerated and no major alterations in routine laboratory test results were observed. Recently, Ark Therapeutics completed three clinical trials with Cerepro, based on an adenovirus-mediated *HSVTk* gene therapy, and reported significant efficacy in recent phase III trials (Mitchell, 2010). However, the European Medicines Agency (EMA) has denied approval for Cerepro and further trials are required to meet regulatory requirements for approval (Mitchell, 2010).

Oncolytic viral therapy: Oncolytic viral vectors are based on the capacity of vaccinia, HSV, and Reo viruses to preferentially infect and lyse cancer cells (Wollmann, 2012). Different from genetically engineered replication-incompetent retro- and adenoviral vectors, oncolytic viral vectors destroy tumor cells through specific replication in these cells and expression of anti- tumor genes encoded by the vector. After lysis of infected tumor cells, thousands of viral progeny are released and spread to neighboring tumor cells. Because of the replication-competence of these viruses, the virus dosage required to achieve therapeutic efficacy seems to be low, compared with retro and adenovirus vectors. In addition, oncolytic viral vectors can also stimulate anti-tumor immune responses (De Siva, 2010; Melcher, 2011).

Held in 1991 at the National Institute of Health, USA. VPCs producing *HSVtk*-carrying retrovirus were transplanted into the patient's brain, followed by GCV administration. Middle, Adenoviruses harboring *HSVtk* are injected into the wall of the tumor cavity, formed after the removal of the tumor mass. Transduced normal or tumor cells surrounding the cavity converted the GCV to GCV-monophosphate (GCV-MP) via the catalytic activity of HSVtk. GCV-MP was converted to GCV-diphosphate (GCV-DP) and then finally to GCV-triphosphate (GCV-TP) by endogenous kinases inside transduced cells. GCV-TP inhibits DNA synthesis and leads to cell death. Because GCV-MP, GCV-DP, and GCV-TP disperse to neighboring cells through gap junctions, the non-transduced cells are also killed by this bystander effect. Tumor-specific cell death is based on the differential sensitivity to GCV-TP. Most normal cells that are no longer undergoing cell division are not affected by GCV-TP. Contrary to this, dividing tumor cells are susceptible to GCV-TP-induced apoptosis due to inhibition of DNA synthesis.

Bottom, A highly-concentrated retrovirus solution is injected into the wall of the tumor cavity, followed by GCV administration. Retroviruses selectively transduce to the tumor cells in the brain and stably express *HSVtk*. By allowing transduced tumor cells to migrate and disperse into tumor tissues, non-transduced cells distant from the cavity can be effectively killed via the bystander effect. The immune cells responsible for adaptive immunity are recruited to the site of the infection and participate in both the killing of virally infected cells and the production of antibodies against the foreign antigen, typically a tumor-specific antigen. In contrast, the innate immune system limits viral propagation, signals for maturation of antigen-presenting cells, and activates the adaptive immune response through antigen-specific T and B cell maturation. In recent years, clinical trials for glioma patients using oncolytic viral vectors have been performed and of these an HSV-based vector, HSV1716, has entered Phase III clinical trials in recurrent glioblastoma and we are currently awaiting the results.

Gene therapy with direct administration of a high-titer retrovirus solution: Due to a lower potential for viral particle production by retrovirus-packaging cells, clinical trials for malignant glioma by direct injection of retroviruses has never been performed. However, retroviral vectors have desirable features for use in gene therapy of malignant glioma. After retroviral infection, DNA reverse-transcribed from the retroviral RNA genome can integrate into the host genome only during mitosis. Brain tumor cells divide continuously, while most brain cells are differentiated and have ceased cell division. Thus, transduction and expression of toxic genes, driven by the integrated retroviral sequences, occurs only in tumor cells within the brain. Stability of viral vectors *in vivo* during gene transfer is a major concern for achieving high transduction efficiency. It is well-known that retroviruses are inactivated by human serum due to the triggering of the classical complement cascade (Welsh *et al*., 1975). Contrary to this, one study found that there was no inactivation of retrovirus in the cerebrospinal fluid of patients with glioma or unrelated disorders (Shimizu *et al*., 1995). These data suggest that gene transfer using retroviruses could be performed and directed into the tumor cavity after the removal of the tumor in glioma patients. To develop such a gene therapy, we established a retrovirus-producing cell line, PAMP51, that produces viruses (at a titer > 1×10^7 CFU/mL) by introduction of the polyoma early region into the retroviral genome (Yoshimatsu *et al*., 1998).

This method of virus production enables the injection of massive amounts of retrovirus directly into the patient's brain (Figure 15). If low-yield virus-producing cell lines, with titers of ~1×10^6 CFU/mL, were used for the therapy, more than a 100 L of virus solution would need to be administrated into the brain, to ensure that 1×10^{11} retroviruses are administered to the same number of cells, found in a tumor mass of a diameter of 5 cm. This is an unreasonable amount of viral solution to have to administer to patients. Thus, a highly concentrated viral solution greatly improves the likelihood and successful use of direct retroviral injection-based gene therapy. We reported that retrovirus solution could be readily concentrated to $1 \times 10^{11-12}$ CFU/mL by using high-yield virus- producing cells and low-speed centrifugation (Tamura *et al*., 2001). Because the polyoma early region enhances transcription of the retroviral genome from long terminal repeat (LTR) promoter, the production of empty viral particles without the RNA genome is dramatically reduced in the culture supernatant of PAMP51 cells. Empty viral particles can occupy the retroviral receptor on target cells and obscure the transduction of retroviral vector; moreover, the viral solution cannot be concentrated easily when empty viral particles are dominant in the solution. Thus, the concentrated retroviral solution prepared from PAMP51 cell culture supernatant is of high purity and appears to be safe for use in gene therapy. To evaluate the efficacy of the suicide gene therapy by direct injection of a highly- concentrated retrovirus solution, mouse glioma models were treated with the vector, followed by GCV administration (Tamura *et al*., 2001). In this model, the glioma was effectively cured by gene therapy using this technology. Also, by- stander effects were considered to be most important factor for this gene therapy. The timing of the viral injection and GCV administration were scheduled to allow for the distribution of transduced cells in the tumor mass and maximization of induction via the bystander effect. Be- cause the gene expression from the retroviral vector is very stable, even non-transduced cells distant from the tumor cavity were targeted via the bystander effect. In addition, the cells transduced with the retroviral vector have been suggested to prime the immune system. When mice once treated with the gene therapy were challenged again by subcutaneous injection of tumor cells, the grow- th of the tumor cells was completely suppressed (Tamura *et al*., 2001). Thus, not only does the reaction help reduce the tumor mass but it also has the potential to prevent tumor recurrence. In clinical trials for X-linked severe combined immunodeficiency (SCID) using a retroviral vector (30), leukemia occurred due to the insertion of the retroviral genome at the proto-oncogene *LMO2* (Hacein-Bey-Abina *et al*., 2003). The clinical trials of gene therapy using retroviral vector were suspended in 2003. However, the efficacy of this gene therapy had been confirmed and the development of leukemia was restricted to the youngest patients in the trial. The clinical trials for this disease were allowed to resume, but were resuspended in the next year. In suicide gene therapy for tumors, leukemogenesis is unlikely to be a concern in most cases, since transduced cells are killed by GCV administration. Furthermore, retroviruses are readily inactivated in serum as described above; there- fore, the incidence of retroviral infection in leukocytes *in vivo* is likely to be lower than in X-linked SCID treated using *ex vivo* gene therapy. Another safety issue in gene therapy with direct administration of a high-titer retrovirus solution is with the use of replication-competent retroviruses (RCRs). The adverse effects of retroviral propagation in the human body are still not known. Because a large amount of retroviral vector needs to be administrated to patients in this gene therapy, the assessment of the effects of RCRs has been performed according to the strict regulations set forth in Good Laboratory Practice Regulations in many countries. No RCR was detected in our high-titer retrovirus vector. In addition, oncogenesis and other side effects were not induced by the injection of a highly concentrated retrovirus (1×10^{11} CFU) into the brain of a primate model, the common marmoset, when assessed over 2 years (unpublished data). Further tests are still needed to ensure the safety of this gene therapy.

Transcriptional targeting of tumor cells is ideal in suicide gene therapy to reduce the side effects against normal cells. Tissue- and cancer-specific gene promoters have been utilized for restricted expression of toxic genes (Miyao *et al*., 1997; Shinoura *et al*., 2000; Komata *et al*., 2002). In addition, the LTR promoter of retroviral vectors is occasionally inactivated in transduced cells. Be- cause myelin basic protein (MBP) is mainly expressed in the brain, its promoter has been used to regulate *HSVtk* gene expression in retroviral vectors (Miyao *et al*., 1997). The combination of a retroviral vector and the brain-specific expression of *HSVtk* gene keeps normal cells in the brain from undergoing retroviral transduction-induced apoptosis. The SSX4 promoter was identified as a tumor-specific promoter, being active in most glioma cells and inactive in normal cells, and was used for controlling *HSVtk* expression in gene therapy (Yawata *et al*., 2011). The SSX4 gene is ex- pressed in all glioma cell lines and the utilization of its promoter is likely to be effective in most of the glioma patients. These tissue-specific promoters facilitate tissue or tumor-specific gene therapy using viral vectors and ensure the safety of the therapy.

Possible applications of gene therapy: The faulty or the mutated gene is replaced by the healthy gene using gene therapy method. The method can be used for the diagnosis of disease if approved globally.

It is used in the diagnosis of inherited diseases such as cystic fibrosis, Duchenne muscular dystrophy, muscular atrophy and hemophilia. Furthermore, efforts are being made to develop gene therapy against monogenic disorders such as thalassemia and sickle cell anemia. The *in vivo* gene therapy or viral vector-mediated gene therapy is a good option for treating diseases like Parkinson's disease, Alzheimer's disease, and brain tumors. Nowadays gene therapy opened doors for dentistry. It is used to grow new teeth, bone repair and teeth regeneration. DNA vaccines are another futuristic application of gene therapy, a naked DNA is directly injected for the production of therapeutic proteins such as insulin and thrombotic factors. Treating cancer using transgene is another utilization of gene therapy in which the expression of an oncogene is suppressed by using a transgene. Also, the infected cancerous cells are likewise killed by expressing a toxic producing gene into those cells. Consequently, it also promotes apoptosis of the infected cells. In addition to this, cardiac disorders, neurological disorders, infectious disorders and autoimmune disorders can be treated using gene therapy methods.

Limitations of gene therapy: Each type of gene therapy has different problems, therefore, fully functioning gene therapy is still not available for the clinical trial on humans. The viral vectors used in gene therapy can infect the host cells and produce a strong immune response against it. Furthermore, the transgene is not expressed all the time. Due to the short-lived nature of transgene, the success rate of gene therapy is too low. Gene transfer experiments are restricted to live human embryo because of the ethical issues associated with it. Rapid integration of transgene is not possible, therefore, we don't know when will the transgene be expressed. Further, not all the cells of particular tissue accept the transgene. Random integration to other locations can produce an adverse effect, gene activation/deactivation or oncogene activation. The cost of gene therapy is very high, at approximately $ 100,000 per therapy (the insurance company doesn't cover it).

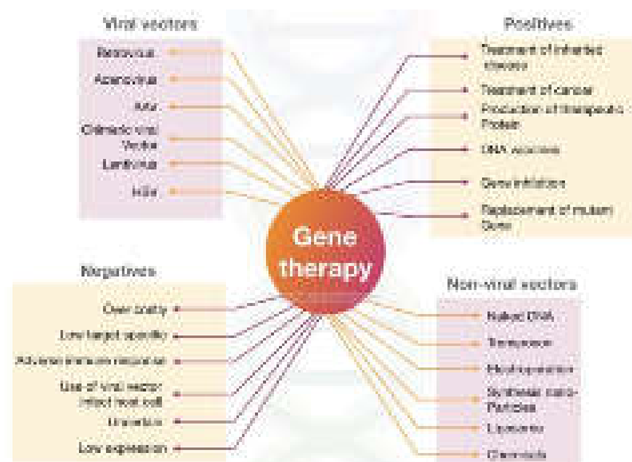

Fig .16. Gene therapy limitations

Challenges & future aspect of gene therapy: Undoubtedly, gene therapy is an effective way to prevent any disorder but with it, so many challenges are involved. Safety is one of the first challenges associated with it. The viral vector-based gene transfer is more effective but can infect the host cell or stimulate an immune response. Therefore it is very important to design a safer vector. Since non-targeted insertion can cause serious health problems, the GOI must be incorporated at a specific location. It is also a challenging task to insert the gene at a specific location. The gene must be inserted at a particular location, just switch on the normal function of it and does not interrupt in other gene's function. The new gene can perhaps raise the oncogene expression which results in carcinoma. Therefore, the insert should not be involved in the oncogenic activity. Besides all these technical issues, the cost of gene therapy is a bigger challenge in commercializing it. It costs more than $ 100,000 per gene therapy. Predicting the future of gene therapy is quite difficult because the results are uncertain and scattered.

Notwithstanding it will be a dream comes true for us if we succeed. Many inherited diseases can be treated alongside life-threatening disorders like cancer. Scientists are very close to treating monogenic disorders like cystic fibrosis and thalassemia. Furthermore, aggressive strategies for the DMD and other related disorders suggest that there will be a lot of progress observed in the coming years. However, gene therapy can be also be practiced incorrectly. Gene therapy can be misused in enhancing athletic performance, increase longevity, stopping the aging process, and to gain more power (superhuman capability). All these activities are un-natural and can unbalance the natural phenomenon, therefore, we have to use it from discretion.

Conclusion

Inserting a transgene or replacing a faulty gene is a tedious phenomenon, which, requires, advance instrumentations, precisions, and expertise. All actions are subjected to total aseptic conditions, a minor contamination/infection with a virus can contaminate the entire cell line and the patients too. Altering the embryo and changing the genetic composition is unethical and against the natural laws. In broader prospects, the manifestation is through nature only, henceforth, the human rights wing and government both are on VETO for this. Many viral vectors have been developed to treat malignant glioma; however, current gene therapies are not sufficiently efficacious.

Our strategy of gene therapy, using a high-titer retroviral vector, has advantages of both retroviral and adenoviral vectors. At present, retroviral vectors can be concentrated to 1 × $10^{11\text{-}12}$ CFU/mL in our system. Moreover, high transduction rates can be achieved with retroviral and adenoviral vectors, ultimately leading to a higher efficacy of gene therapies. To perform clinical trials of this gene therapy for malignant glioma, preparation of large quantities of retroviral vectors is further required. Suspension cultures of VPCs were established to scaleup retroviral production. Improvements in retroviral production systems and confirmation of the safety and efficacy of suicide gene therapy will enable us to conduct clinical trials in and/or treat more patients.

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