

Recent developments in the Adeno-Associated Viral Gene Therapy for Non-Small Cell Lung Cancer during the past 20 years

A project submitted

By

Amrin Islam Razna
ID -13346019
Session- Summer 2013

To

The Department of Pharmacy

in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy (Hons.)



Inspiring Excellence

Dhaka, Bangladesh
September, 2018

Certification Statement

This is to certify that the project titled “To study the progress of Adeno-associated virus gene therapy for non-small cell lung cancer in the last 20 years” submitted for the partial fulfillment of the requirements for the degree of Bachelor of Pharmacy from the Department of Pharmacy, BRAC University constitutes my own work under the supervision of Rubayat Islam Khan, Senior Lecturer, BRAC University. Throughout the project I have given appropriate credit where I have used the language, ideas or writings of another.

Signed

Counter signed by the supervisor

Dedicated to my parents

Acknowledgements

This paper has become a reality with the kind support and help of several individuals. I would like to extend my sincerest gratitude to all of them.

I would like to express how grateful I am to the Almighty for keeping me in good health and allowing me to pursue my ambitions through the knowledge I have acquired in life.

First and foremost, I would like to express gratitude towards my parents for their constant efforts in giving me support and inspiring me to pursue my dreams. Without whom I would not be the person I am today.

I am immensely grateful towards to the most esteemed Professor Eva Rahman Kabir, Ph.D (Chairperson, Department of Pharmacy, BRAC University), who provided constant guidance whenever I needed it. Her constant effort, kind words, encouragement and enthusiasm towards my research project allowed me to grow as a research scientist. Her linguistic skill helped me express myself in an ordered and collected manner. She has constantly reassured me that my paper showed what I had hoped to express as a research scientist.

I would like to express my gratitude towards my respected supervisor Rubayat Islam Khan (Senior Lecturer, Department of Pharmacy, BRAC University), who helped me reach a decision regarding the field of work I would go into. His valuable insight has helped me whenever I was unsure of my work while his suggestions gave me a new perspective.

Lastly I would like to thank all the people who have helped me to their best abilities whenever possible.

ABSTRACT

Gene therapy has a diverse plethora of applications. Many genetic diseases – including both dominant gain-of-functions diseases and recessive conditions have an equal opportunity to be cured by it.

In this study, the treatment of non-small cell lung cancer with the help of gene therapy using adeno-associated virus (AAV) vector is the focal point. Non-small cell lung cancer requires multiple drugs for effective treatment. In spite of this the survival rate is low compared to other forms of cancer. In addition to this, the choice of drugs and methods of treatment greatly depends on the health of the patient. Gene therapy is considered to be an ideal treatment in this study. It not only cures the condition from the root level but also can be applied to all patient types. AAV is used in place of adenovirus, in the light that even though its onset of action is slow, its duration of action is greater.

Many techniques of gene therapy have been covered in this review. These include CRISPR-Cas9 technology, zinc-finger nuclease, splicosome-mediated RNA trans-splicing, RNA interference technique, antisense-oligonucleotide therapy. The future of gene therapy can be promising if these techniques could become more developed by thorough and rigorous experimentation to refine their methodologies.

CONTENTS

<u>Sl. No.</u>	<u>Heading</u>	<u>Page No.</u>
1.	Acknowledgements	i
2.	Abstract	ii
3.	List of abbreviations	v
4.	List of figures	vii
5.	List of tables	ix
6.	CHAPTER- 1 : Introduction	1
7.	CHAPTER - 2 : Purpose of the study	4
8.	CHAPTER -3 : Benefits of Gene Therapy	6
9.	3.1 : Splicing up the affected DNA and then repairing it	7
10.	3.2 : Blocking a diseased gene from functioning	9
11.	3.3 : Boosting up the immune system	11
12.	CHAPTER - 4 : Classification	13
13.	4.1 : <i>In-vivo</i> gene therapy	15
14.	4.2 : <i>Ex-vivo</i> gene therapy	16
15.	CHAPTER -5 : The use of adeno-associated virus in gene therapy	17
16.	5.1 : Adeno-associated virus [AAV]	18
17.	5.2 : Role of adeno-associated virus [AAV] in gene therapy	19

18.	5.3 : Vector design	22
19.	CHAPTER- 6 : Molecular anomalies for non- small cell lung cancer	26
20.	CHAPTER -7 : Gene therapy for non-small cell lung cancer	34
21.	7.1 : CRISPR/CAS9 as therapeutic equipment	39
22.	7.2 : Zinc – finger Nuclease as a Therapeutic Weapon	42
23.	CHAPTER -8 : Concluding remarks	50
24.	CHAPTER - 9 : References	54
25.	CHAPTER - 10 : Appendix	60

LIST OF ABBREVIATIONS

1. AAV- adeeno-associated virus
2. NSCLC- non-small cell lung cancer
3. SMaRT™ - Spliceosome-Mediated RNATrans-splicing
4. CRISPR-Cas9- Clustered Regularly Interspaced Short Palindromic Repeats- associated protein9
5. gRNA- guide RNA
6. ASO- Antisense oligonucleotide therapy
7. ssDNA- single-stranded DNA
8. ORF- open reading frames
9. ITRs - Inverted Terminal Repeats
10. CMV –cytomegalovirus
11. EGFR - epidermal growth factor receptor
12. ALK – anaplastic lymphoma tyrosine kinase
13. STK11-serine/threonine11
14. TP53- tumour proteinp53
15. RB1- retinoblastoma1
16. KEAP- kelch- like ECH associated protein gene
17. NFE2L2- and nuclear factor erythroid 2 like 2

18. NF1- neurofibromin1
19. LOH- loss of heterozygosity
20. HD- homozygous deletion
21. rAAV- recombinant AAV
22. RNAi- RNA interference
23. PAM- proto-spacer adjacent motif

LIST OF FIGURES

<u>Figure No.</u>	<u>Name of Figure</u>	<u>Page No.</u>
Figure 3.1	The use of CRISPR-CAS 9 in the permanent cure for mutated genes	8
Figure 3.2	The triple-helix forming oligonucleotide method prevents the transcription of mutated genes	10
Figure 3.3	The Rybozymes technology is used for blocking the production of mutated protein	10
Figure 4.1	Different methods of transferring genes by <i>in-vivo</i> method	15
Figure 4.2	<i>Ex-vivo</i> gene therapy procedure	16
Figure 5.1	Diagrammatic representation of the genome of AAV	20
Figure 5.2	Different forms of AAV genome	21
Figure 5.3	Vector development	22
Figure 5.4	Production of AAV vector	25
Figure 6.1	Adenocarcinoma and Squamous cell carcinoma	28
Figure 6.2	Signal transduction pathway in the activated HER receptors	29
Figure 6.3	Genomic status of the LKB1/STK11 gene	30
Figure 6.4	Localization of FOXO1 in NSCLC, with and without FBS	31
Figure 6.5	AKT expression in NSCLC after knock-down using RNA interference	32
Figure 6.6	Visualization of apoptosis – using TUNEL staining	33
Figure 7.1	Comparison of infecting abilities of different serotypes	35
Figure 7.2	Quantitative analysis of infecting ability of different serotypes	36

Figure 7.3	Different types of recombinant AAV therapies	38
Figure7.4	CRISPR Cas- 9 can be used for inhibiting PD-1 gene and reactivate the immune system	39
Figure 7.5	Different methods of using CRISPR – CAS 9 for genome editing	40
Figure 7.6	Structure and function of zinc finger nuclease	43
Figure 7.7	Up- regulation of ZBTB20	44
Figure 7.8	The role of ZBTB20 in cell multiplication	45
Figure 7.9	ZBTB20 suppresses the FOXO1 function	46
Figure 7.10	Comparison of mRNA levels and protein levels of FOXO1 in affected and healthy cells.	46
Figure7.11	ZBTB20 regulates FOXO1 promoter activity – luciferase reporter analysis of human FOXO1 promoter	47
Figure 7.12	A series of deletions were done to pinpoint the transcriptional region	47
Figure 7.13	ChIP assays to show the recruitment of ZBTB20 onto FoxO1 promoter	48
Figure 7.14	FoxO1 re-introduction or knockdown weakens the multiplication powers of ZBTB20. Protein level of FoxO1 was determined by Western blot in A549 cells (B)	48

LIST OF TABLES

Table 1: Tissue specification of different types of AAV.....	21
---	----

CHAPTER - 1

INTRODUCTION

Gene is a specific piece of DNA containing hereditary information, which control particular traits in living organisms. Chromosomes are pieces of DNA with associated nucleotides – these specific stretches of DNA forms genes. The nucleotides serve as foundations for production of proteins by transcription and translation. The proteins are the structural and functional units of cells.

So if the nucleotide sequence is disrupted, it produces non-functional or malfunctioned proteins. This is termed as mutation. This mutation leads to conditions like cancer. However each organism carries two copies of each gene - maternal or paternal. If one copy is defective, the other can provide some protection against genetic conditions. Each individual carries about 6 defective genes in general.

Gene therapy is a type of treatment where the patient is given the corrected or healthy gene that will supplement or obscure the functions of a malfunctioned gene. The healthy gene will normally get incorporated into the genetic profile of the patient. The therapeutic gene is delivered by using specialized carriers called vectors. The vectors can incorporate the therapeutic gene within them and then deliver the gene to a specific site by binding to specific receptors.

There are numerous future prospects of using gene therapy in the prevention and cure of diseases where current medical knowledge still proves deficient. Most of these conditions are caused by either single or multi gene mutations that render the normal development of the individual. The basic principle of gene therapy is very simple. The faulty gene is identified at first. Several methods using cutting-edge technology has been recently developed to identify genes. Genetic mapping using DNA sequencing is one of them, DNA screening is another method. Then the therapeutic gene needs to be identified and isolated. Isolation of the desired

gene can be done by attachment of a marker to the specific gene and then separating it using restriction enzymes. The restriction enzyme will cut the gene only at fixed locations. The isolated gene is then replicated using PCR. This is a technique which incorporates additional nucleotides and replicating enzymes to replicate a particular sequence. Next the gene is introduced into the vector and delivered to the patient. Usually two types of delivery techniques are used- *in vivo* and *ex-vivo*. Different types of vectors are used- viruses, liposome and direct injection. This approach is different from drug therapy, because drugs can only improve symptoms but may not correct the cause. (“What is gene therapy?,” n.d. ; Learn.Genetics, n.d.-b)

So it can be deduced that gene therapy is of considerable importance in the fight against diseases caused by mutations. This is because genetic mutations are hardly treatable by current drug regiments. Altering the genome at the molecular level is the desired norm in these diseases. CRISPR-Cas9, SMaRT™, ASO, Rybozymes gene therapy are very few of the current techniques that have been currently been widely studied in order to provide the desired genetic therapy goals.

CHAPTER - 2

PURPOSE OF THE STUDY

The aim of this project is to study the progress of adeno-associated viral gene therapy for non-small cell lung cancer during the past 20 years. Many diseases are caused by mutated genes which do not function properly or sometimes doesn't function at all. It could be possible to replace this mutated gene with a normal one, so that the development of the disease is prevented. Many types of cancer are caused by mutation of the p53 gene suppresses tumor growth. This could be made possible by a very recently developed technology to edit DNA molecule. The method is known as genome editing.

Technically genome editing refers to any procedure that aims to identify and accurately edit the base sequence of the DNA. There are at least 8000 diseases that are caused by genetic mutations. One of the major milestones of gene editing was the discovery of site-specific DNA double-stranded break (DSB) in the target gene. This could make it possible to introduce homologous recombination like insertions or deletions. This procedure follows the principle that once a break is made, the cell's repair mechanism will correct it.

This DNA double-stranded break can be specifically targeted and manipulated by using various genome-editing tools. They not only create the required niche but also incorporate desired sequence into the genome. If the desired genome is the correct sequence for a medical condition, it will, in theory, cure the disease. CRISPR-Cas9, SMarT™, ASO, Rybozymes gene therapy are the genome editing tools that are showing promising results. In order to proceed further, it can be suggested that experiments can be designed to make the techniques more site-specific and reactive. The techniques also need to be safer and more effective in terms of administration, as stated by American Society of Gene & Cell Therapy (2017).

CHAPTER - 3

BENEFITS OF GENE THERAPY

There are proposed 3- fold benefits of gene therapy. These are replacing a diseased gene by removing it and placing a healthy normal gene, by muting a mutate gene from functioning and increasing the immune system's response to abnormal cells. Among all the methods, replacement and silencing are showing promising results.

3.1 Splicing up the affected DNA and then repairing it

The mutated gene can be replaced by many different techniques. One such method is the zinc- finger nuclease. The nuclease will recognize and bind to the mutated gene, and then edit the sequence. The zinc fingers will guide the DNA to the specific site, and then the nuclease will cut the strand. But the DNA is designed in such a manner so that it will automatically seal any breaks. This problem can be overcome by custom-building a sequence as close as possible to the patient's genome, so that the cell can be tricked into considering it as its own part. It is mainly used to treat conditions involving improper regulation of genes, as seen in (CRISPR THERAPEUTICS, 2017)

Another method is the SMaRT™ (Spliceosome-Mediated RNA Trans-splicing). In this method, the messenger RNA, which codes from the defective protein, is targeted and rectified. It does not repair the entire gene, but only the specific part containing the mutation only. Viral vectors are used to directly repair the genome. It also uses special enzymes to cut the faulty part and paste the functional copy of genes, as stated by (Learn.Genetics, n.d.-b ; Learn.Genetics, n.d.-a)

CRISPR-cas9 is a relatively new and promising gene editing method. It can selectively delete, modify or add parts to the DNA to correct disease. CRISPR means Clustered Regularly Interspaced Short Palindromic Repeats, which is found in bacterial genome. Cas9 is an

endonuclease of bacteria associated with CRISPR. The cas9 acts as biological scissors to cut mutated genes. It can easily be designed to edit a mutated genome. A specifically designed guide RNA takes the cas9 to the locus of the mutated gene.

Guide RNA consists of both crRNA and tracrRNA. Once the repair is done, the cell's own healing mechanism and correct version of DNA will rejoin the cut and finally correct proteins will be synthesized. All the above techniques are currently focusing on monogenic conditions. But CRISPR-cas9 is a promising field which can correct both dominant and recessive mutations in the patient's cell, as shown in ("New gene therapy boost immune system to cure cancer," 2002)

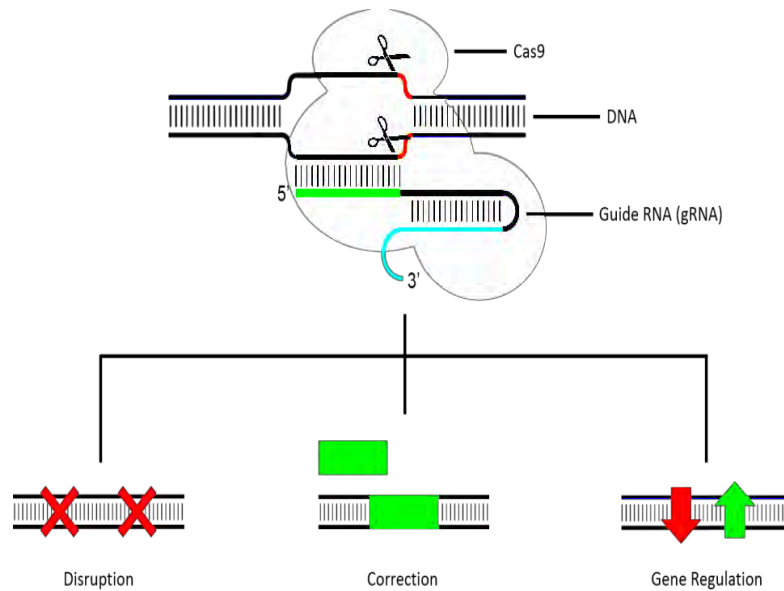


Figure 3.1: The use of CRISPR-CAS 9 in the permanent cure for mutated genes. – adapted from (Learn.Genetics, n.d.-a)

3.2 Blocking a diseased gene from functioning

Many dominant-types of diseases are proposed to be targeted by this method. In this type of mutation, the diseased mutation is the genetically dominant one, so it will be always expressed in the genotype. As a result, any other methods of altering the genome will not effectively. So the most effective way would be to completely switch off the malfunction gene so that it would not produce harmful protein.

One way of doing it is the triple -helix –forming oligonucleotide method. In this process, the main target is the mutated gene’s DNA sequence. A specialized piece of short DNA stretch is synthesized- oligonucleotide- which will specifically bind to the gap between two strands of the mutated DNA and prevent it from transcribing.

Next is the RNA interference technique. This technique employs the knowledge that the body will destroy the double-stranded RNA of viruses. This process involves the building of an RNA molecule with a base sequence complementary to that of the mutated gene. It will find and bind to the mutated region. So it will form a double-stranded RNA and will get destroyed.

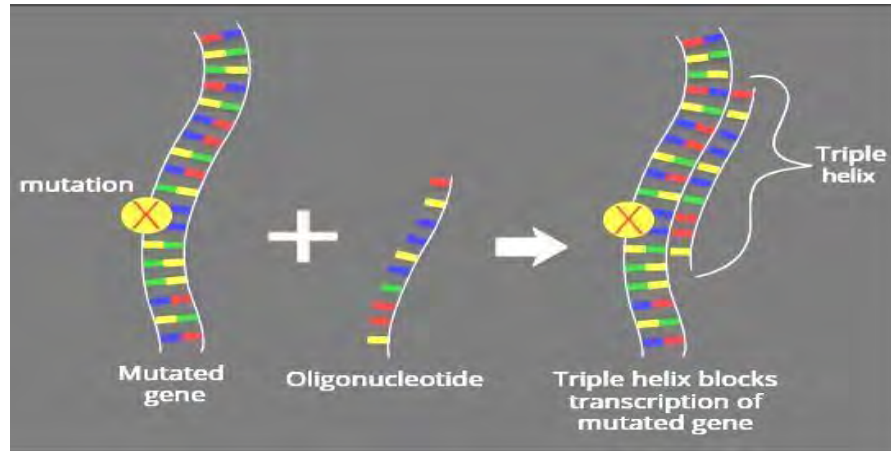


Figure 3.2: The triple-helix forming oligonucleotide method prevents the transcription of mutated genes. – adapted from (Learn.Genetics, n.d.-a)

Rybozymes gene therapy targets the mRNA transcript copied from defective genes. Rybozyme is an RNA acting as an enzyme. They work as molecular scissors to cut RNA. The rybozyme is designed to recognize and cut RNA from mutated genes, so that defective protein is not produced, as stated by (Learn.Genetics, n.d.-a)

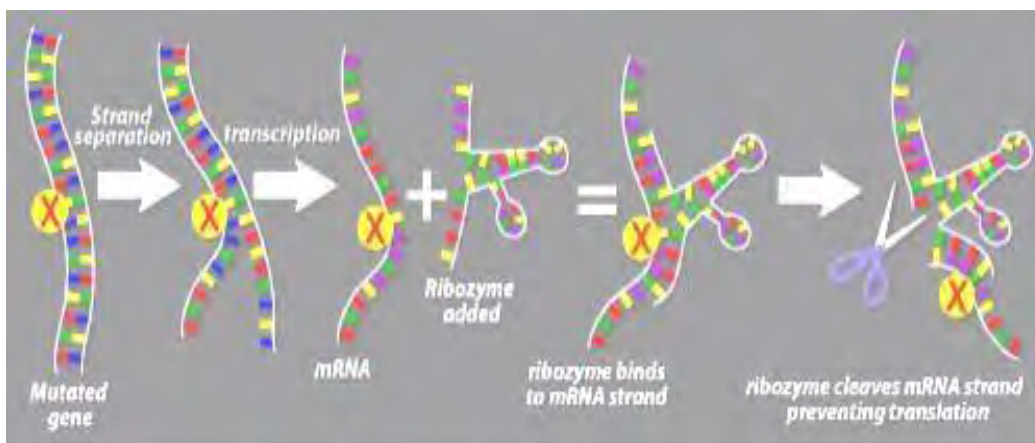


Figure 3.3: The Rybozymes technology is used for blocking the production of mutated protein. - adapted from (Learn.Genetics, n.d.-a).

Antisense oligonucleotide therapy (ASO) is a gene silencing technique that aims to prevent the faulty gene's expression. ASO works by suppressing the translation step so that mutated protein is not formed. Short single-stranded pieces of modified bases – oligonucleotides- are specifically designed to recognize and bind to the faulty mRNA in the cell. Inside the cell the nucleotides inhibit protein synthesis by binding to RNA or producing an enzyme- RNase H to degrade the RNA.

The above techniques all work in more-or-less the same manner. But the mechanism is different from each other. This aspect of gene therapy is very valuable because it can be used to silence most of the mutations that causes different types of cancer. So both dominant and recessive mutations can be targeted by these techniques, as shown by (Learn.Genetics, n.d.-b ; CRISPR THERAPEUTICS, 2017)

3.3 Boosting up the immune system

One of the reasons for why mutagenic cells are not destroyed by immune system is because the immune cells cannot recognize them. Using gene therapy, the immune cells' genome's pathogenic database can be enhanced by adding the mutagenic cell's genome. In this way the immune system will recognize the mutagen as non-self and destroy the cell, as shown in Mayo Clinic, (n.d.) .Studies show that scientists have found a way to isolate the white blood cells of the patient. Then they modify the genome of white blood cells by adding the antigen from cancer cells. After re-introduction of the leukocytes in the body, the modified leukocytes can destroy cancer cells, as shown in (Learn.Genetics, n.d.-a)

Cross & Burmester, (2006) stated that “the immune system can also be enhanced by specifically designed molecules that have immunogenic properties. Viral or nonviral vectors deliver these immunogens to the surface of tumors. These molecules are genetically engineered to be recognized by leukocytes. A recently developed process uses gene therapy to make immune cells not to be affected by transforming growth factor – beta, which is an immunosuppressant that helps cancer cells to stay hidden from immune cells. A mutated version of the gene was inserted into the bone marrow and affected site of test animals which showed 70% and 80% recovery rates. It has been suggested that the signaling of this gene in immune cells is blocked”. It has been also found that the metastasis stages are also eradicated. Even in its early stage of development this technique is showing promising results.

Another technique using gene therapy for enhancing the immune system is by creating recombinant vaccines. These vaccines are not for preventive purpose, they are proposed to have stimulative effects on the immune system. A neoplastic gene or antigen is added to specific leukocytes. These cells will produce immune response when introduced into the body. The gene can also be directly introduced into the body through vectors. Mouse models whose colon adenocarcinoma cells have been modified with human carcino-embryonic antigen showed decrease in tumor size and long-lasting immunity. An engineered vaccinia virus had been used as a vector, as shown in (“Types of Gene Therapy,” n.d.)

CHAPTER - 4

CLASSIFICATION

Gene therapy is mainly classified into two types, on the basis of site of action of the desired genes. They are germ-line therapy and somatic cell therapy. Germ-line therapy is the permanent way of providing therapy. In this process, the genes of the gametes are altered. The therapeutic gene is inherited from parent to child, and the effect will be present in all cells. A successful gene therapy not only abolishes the diseases from future generation, but also from the whole population.

Somatic cell therapy is a temporary method of gene therapy. It involves normal body cells, so it cannot be inherited. It is restricted to only the patient and his affected cells. But this therapy has its own set of shortcomings. Somatic or body cells have a short life-span; they become old and are shed, causing loss of genes that are inserted and making this therapy short-term. In addition to this, the affected organ's cells are regenerating continuously, and which will also contain the defective gene. So repeated therapy is also required. This has the effect of consuming more time, energy and money on the patient's side. In spite of this, this technique has shown modest results now-a-days. Clinicians have also been able to perform this therapy to fetuses, which had eradicated serious conditions in the neonate that could hamper its growth. So this would achieve the effects of germ-line therapy.

Somatic cell therapy is further classified into 2 types, based on whether the therapy is done inside or outside the body.

4.1 In-vivo gene therapy

(Canver, 2009) stated that “*In-vivo* therapy is done inside the body”. Many physical processes are used - increasing the permeability of the cell and uptake of DNA. It involves mechanical, electrical, ultrasonic, hypo dynamic or laser therapy. This method also has specific tissue sites- skin, lung, colon, muscle, pancreases, liver, bone marrow, spleen and brain. It is less invasive, safer and simpler. But there is no way to monitor the progress of the therapy inside the body. In addition to this, the therapy is non-specific and can cause systemic effects. Viral and non-viral vectors are used , as shown by (PHARMACOLOGY, 1998; Amer, 2014). The vectors may get attacked by immune cells, making the therapy short-lasting.

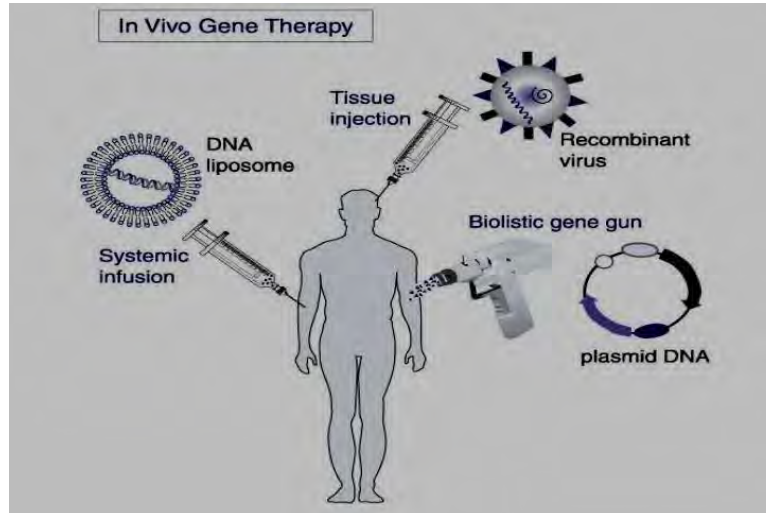


Figure 4.1: Different methods of transferring genes by *in-vivo* method. – adapted from (Jain KK, 1998)

4.2 Ex-vivo gene therapy

According to (Nayerossadat , Maedah, & Ali, 2012; Amer, 2014) , “*ex-vivo* therapy is performed outside the body”.) The patient’s affected cells are removed, mixed with vectors and after successful transduction, re-implanted. As the patient’s own cells are used, tissue rejection is eliminated. The whole method is monitored and screened, and the collected cells are homogenous, infected and grown in the same conditions for better results. But the therapy is temporary and requires continuous supply of cells. During each therapy, the patient needs to undergo surgery and cell removal, followed by grafting. Apart from this, only transplantable cells can be used this technique targets bone marrow stem cells, liver cells, blood vessel smooth muscle cells and tumor- infiltrating lymphocytes for cancer treatment, as stated by (Nayerossadat , Maedah, & Ali, 2012)

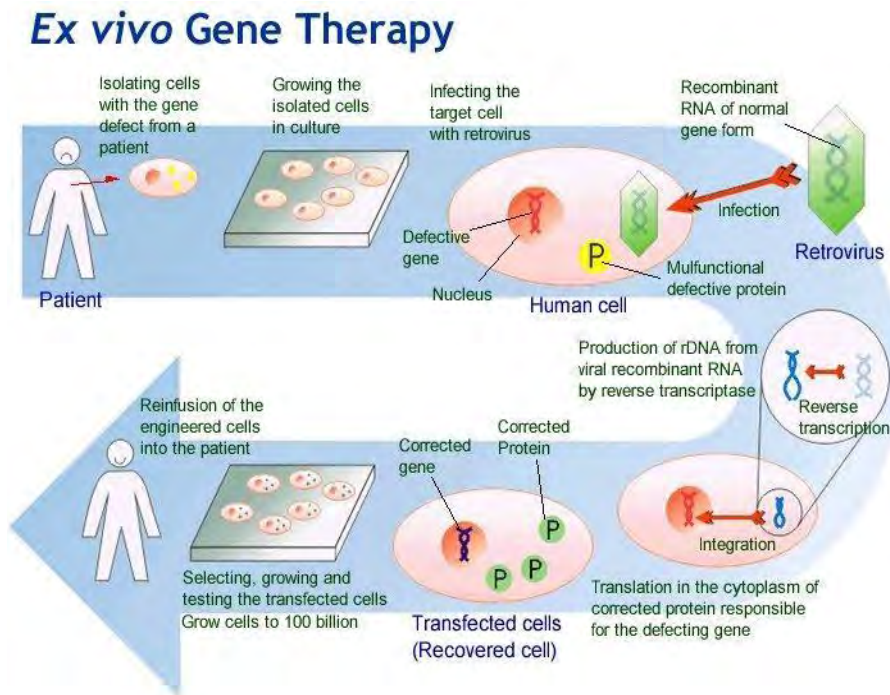


Figure 4.2: *Ex-vivo* gene therapy procedure. – adapted from (Jain KK, 1998)

CHAPTER - 5

THE USE OF ADENO-ASSOCIATED VIRUS IN GENE THERAPY

5.1 Adeno-associated virus[AAV]

Coura & Nardi, (2008) showed that “adeno-associated virus is a small member of the parvovirus family. The wild form of this virus is present in humans in a commensalisms relationship. These viruses require a co-virus to replicate. They are classified as DEPENDO-VIRUSES. Since it biologically needs a partner for replication, it can be referred to as a satellite virus. It is the only virus which can incorporate at a specific site in the human genome.”

Adeno-associated viruses have a jelly-roll β -barrel, with anti-parallel beta sheets. The beta barrels have icosahedral arrangement. There are 3 clustered peaks, each around the icosahedral structures for rotation. There is a sub-loop at the centre of each peak, and it is between two other loops. It contains single-stranded DNA [ssDNA]. AAV can insert genetic material with high precision at any specific site on chromosome 19. The virus is non-pathogenic, as it is present inside the human body in a harmless way. So it does not activate the immune system. AAV can infect both dividing and non-dividing cells. The genome of the adeno-associated virus consists of either positive-or-negative sensed ssDNA, and is 4.7 kilo bases long as shown by (Coura & Nardi, 2008 ; Xie et al., 2002)

Xie et al., (2002) reported that “at both ends of the DNA, there are Inverted Terminal Repeats [ITRs], in between them are open reading frames [ORFs] - rep and cap. Rep contains 4 overlapping genes for the Rep proteins. Cap consists of overlapping nucleotides for capsid formation. ITR consists of 145 bases. They have the ability to form a hairpin, which leads to self-priming or primase free second strand synthesis. ITR is also involved in the integration of AAV DNA into the host cell.”

The ITR needs to be delivered in the cis configuration, whereas the rep and cap parts can be in trans-configuration. The adeno-associated virus also contains two promoters – p5 and p19. These promoters can be used to synthesize different types of mRNAs with overlapping sequences. The mRNA contains introns which can be removed as well, and they can be used for making the packaging rep proteins- rep78, rep68, rep52 and rep40. Rep 78 and 68 can combine with the hairpin bend and assists in self-priming. The p40 promoter encodes for capsid proteins- vp1, vp2 and vp3 which are all synthesized from a single mRNA. They can be spliced at two sites – longer intron or shorter intron.

The primary receptors for adeno-associated virus are heparin sulphate proteoglycan, but secondary receptors also used. Mutations at the ligands will affect AAV –receptors ability to bind with the virus. Majority of the mutations occurs within the peaks at the capsid, another mutation occurs at the capsid surface, and the next at under the surface peak, which has indirect binding to receptors. (Xie et al., 2002)

5.2 Role of adeno-associated virus [AAV] in gene therapy

Adeno-associated viruses can be of many different types. But the most useful type for gene therapy is serotype 2 AAV. The virus first binds to its serotype-specific receptor, then enters the cell by endocytosis. It evades the cell's defense mechanism, unsheathes itself and enters the nucleus. Inside the nucleus it forms double – stranded intermediates, which incorporate into chromosome 19. The virus remains in a dormant state in the nucleus. When a co-infection by helper virus occurs, it starts to replicate. The ITRS are the replication zones, rep78 and 68 are specific DNA-binding proteins, rep 52 and 40 helps in replication. The AAV

can also gain access to the nucleus by nuclear pores due to its small size. The AAV vector has 145 bp inverted terminal repeats, which facilitates the activation of the transgene. All the important functions are already present for vector preparation, the open-reading frames, rep and cap proteins can be replaced by desired genes. The rep and cap proteins are present in a different plasmid, as shown by (Novus Biologicals, 2016)

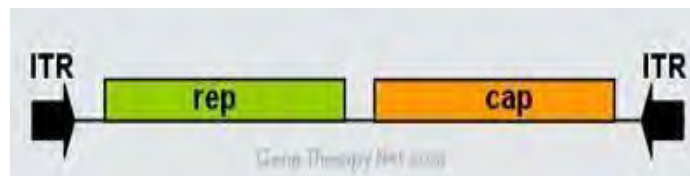


Figure 5.1: Diagrammatic representation of the genome of AAV– adapted from (“Types of Gene Therapy,” n.d.)

Papadakis et al., (2004) has shown that “AAV can be used to introduce genes to both dividing and non-dividing cells, but it does not alter the integrity of the DNA. They have large half-life and greater concentration than other viral vectors, produces mild immune response, and can infect a wide range of cells”.

(Novus Biologicals, 2016) has shown that “although AAV can integrate into chromosome 19, it requires the assistance of the rep proteins, which are absent in current vectors. So only a small fraction successfully integrates into host DNA”. Gill et al , (2011) stated that “without helper virus, the AAV enters the nucleus and binds to DNA regions which are uncoated. The integrated genome can be withdrawn by helper virus infection. The latent infection mainly occurs at tandem repeats. A research using the AAV sequence was used as an instrument to study AAV infections, with the results showing that infected cells showed the same sequence as AAV.”

Table 1: Tissue specification of different types of AAV

AAV 1	Smooth muscle, CNS, lung, retina, pancreas, heart, liver
AAV 2	Smooth muscle, CNS, lung, retina, kidney
AAV 3	Smooth muscle
AAV 4	CNS, lung, retina, kidney
AAV 5	Smooth muscle, CNS, lung, retina
AAV 6	Smooth muscle , heart , lung, liver, adipose
AAV 7	Smooth muscle, retina, CNS, liver
AAV 8	Smooth muscle, CNS, retina, liver, pancreas, heart, kidney, adipose
AAV 9	Smooth muscle, heart, lung, liver, pancreas, CNS, retina, testes
AAV 10	Smooth muscle, heart, lung, liver, pancreas, CNS, retina

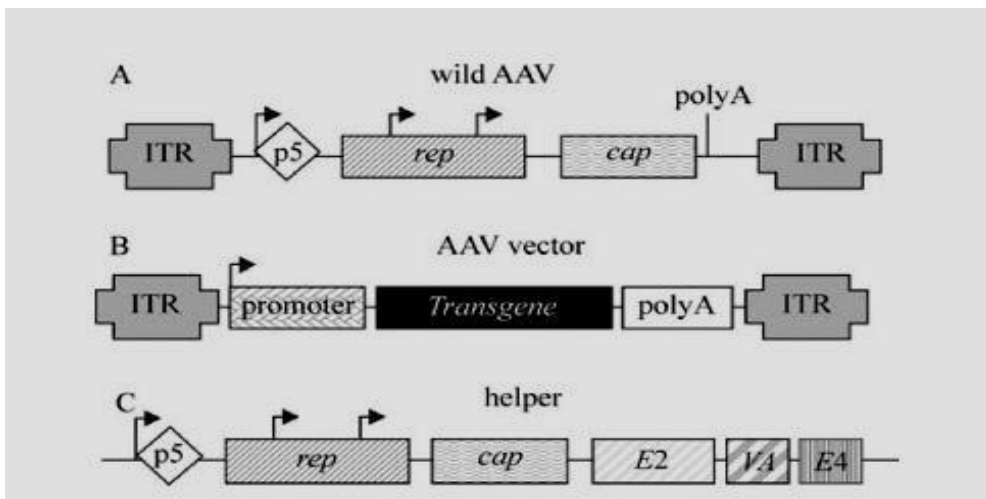


Figure 5.2: Different forms of AAV genome- a) wild type ; b) AAV vector with the wild type ITR , transgene and promoter ; c) AAV helper cassette with rep and cap genes for packaging and E2, VA , E4 for replication. – adapted from (Novus Biologicals, 2016)

5.3 Vector design

Viral promoters are needed to induce successful transduction of the vector into host cells. They induce successful propagation of virus and higher transcription rates. Since the adeno-associated virus depends on another virus, a combination of cytomegalovirus [CMV] and AAV is used. It has been shown that CMV/AAV combination can directly integrate into the host cell. The admixed vectors were also shown to provide a sustained action. A study has shown that using two serotypes of AAV can be used. One serotype contained the transcription factor; the other had the therapeutic gene activated by promoters. A 1:1 ratio of the viruses increased the effects. (Naso , Tomkowicz, Perry III., & Strohl, 2017; Chandler et al., 2015)

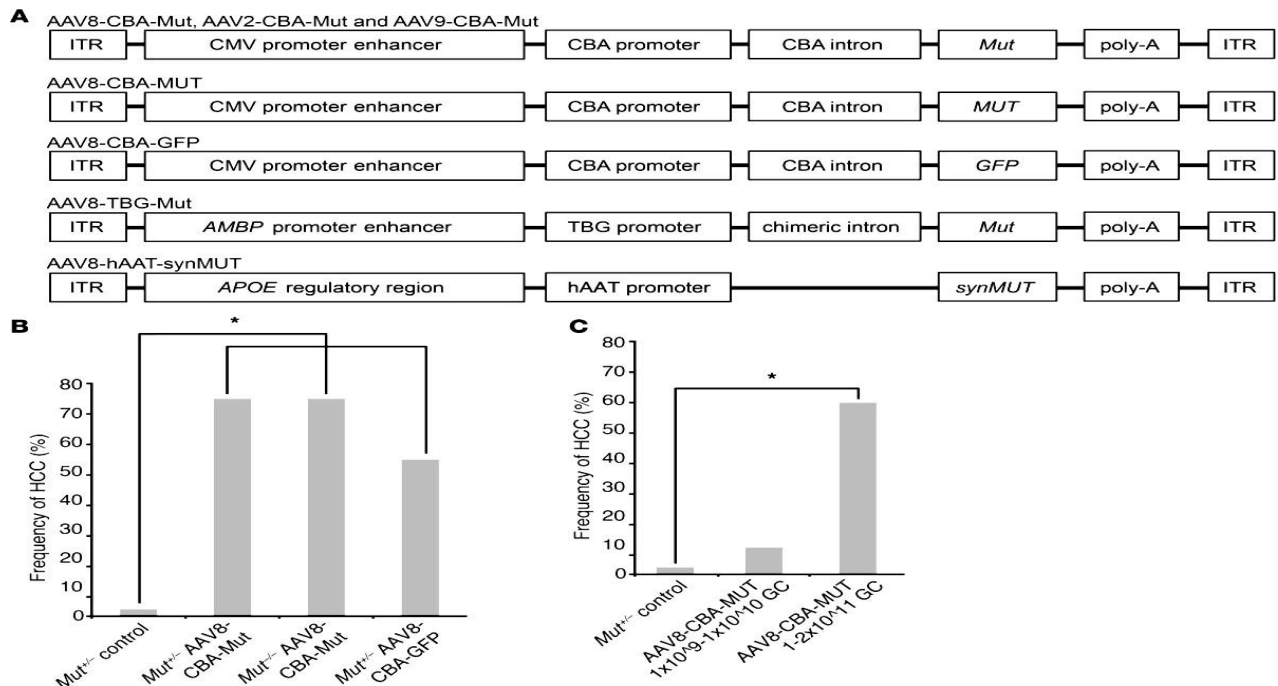


Figure 5.3: A) Packaging of AAV vectors in serotypes – 2, 8 and 9. B) Transgene expression compared to the development of carcinoma. C) Correlation between dose and frequency of developing carcinoma after therapy. – adapted from ((Naso Michael F et al., 2017)

Faruki et al.,(2017) states that “the rational design of the vectors is determined by the packaging size of cassette placed between two ITRs. The size of the cassette should not be greater than 5kb, or it will cause reduction in the reproduction rate and therapeutic effect. This problem can be solved by the addition of double overlapping vectors. The formation of the double strand is the rate-limiting step of the process”. A self-complementing AAV can be used, which contains the single-strand’s packaged genome, and forms a double-strand by itself. AAV enters cells through interactions with sialic acid, galactose and heparin sulphate. If the choice of binding is altered by altering the DNA, then new cell- specific AAV can be produced. Also this factor can be exploited by means of selecting the virus which shows tropism for a particular tissue receptor, then using it to infect specific tissues. Studies have shown unique sequences for capsid specificity. Methods to reshuffle the capsid sequence, inserting additional bases and directional growing can create AAV with useful properties

The RNA polymerase II controls the expression of genes in eukaryotes. The mechanisms for transcription are directed to the core promoter structure. The RNA polymerase and transcription factors are found within the -35 to + 35 regions. The [TATAA/TA] TATA box is at -25, but could be absent for many genes. The initiator is at the transcription start site, -3 or +5, and can start transcription without TATA box. There is also a downstream promoter element at +30 is another region to function independent of TATA box. Many factors are needed by the RNA polymerase to find the location of the promoter and create a suitable pre-transcription intermediate. Transcription factors are required for gene activation. They manipulate the activities of the transcription organelles, and regulate gene expression, by causing the RNA polymerase to stop at sites. New transcription factors are created by manipulating the way the DNA-binding and activation regions work. Cancer-specific promoters are synthetically-

designed transcription factors that will incorporate into cell life-cycle. Heterochromatin separates the promoters from transcription sites, so any transgene will have to avoid the heterochromatin or it will be converted into heterochromatin itself and be silenced. This is called positional effect variegation [PEV]. So any new genetic material needs to be provided in an expression cassette, which would retain the matter in the actively- transcribed euchromatin , according to (Chandler et al., 2015)

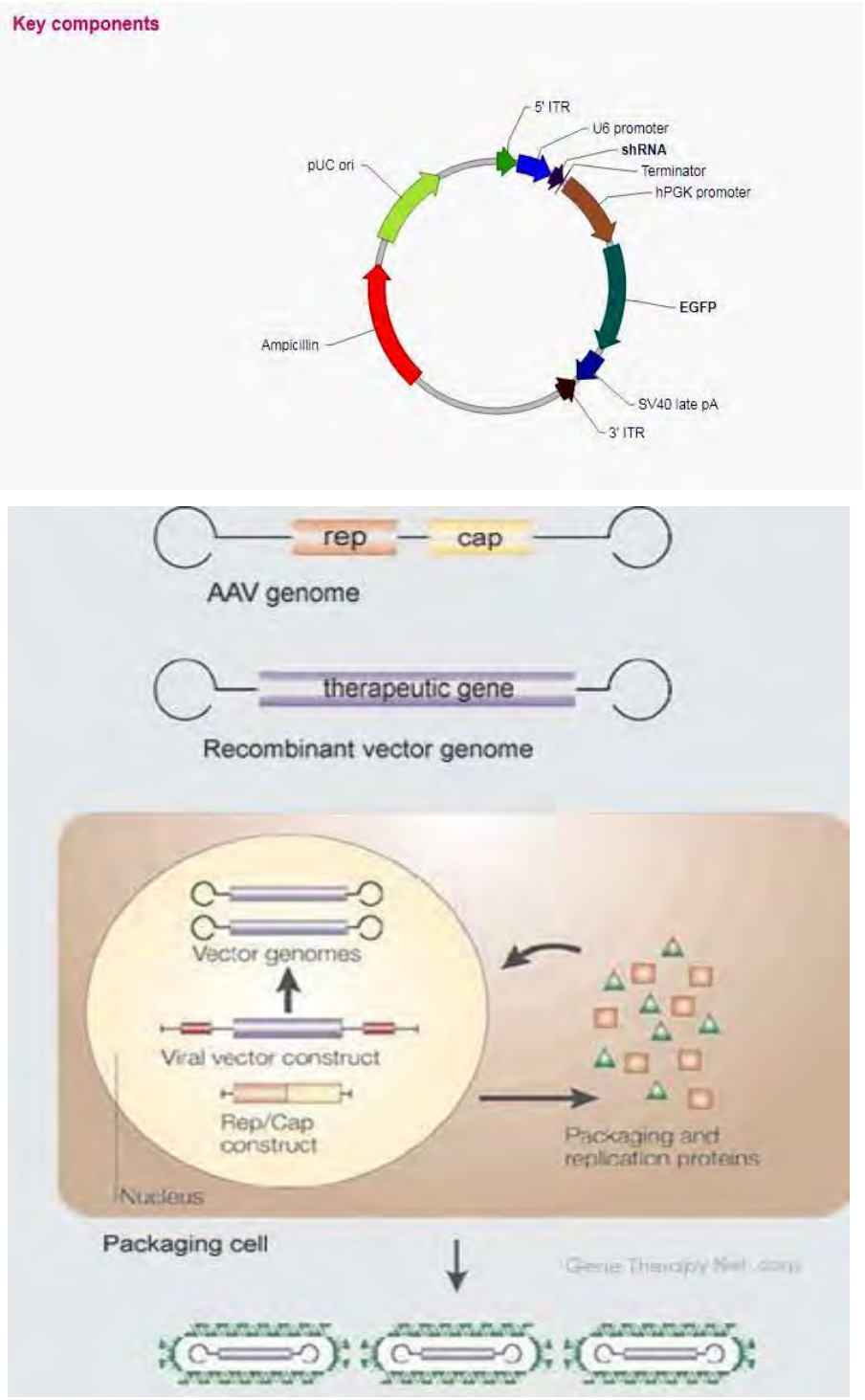


Figure 5.4: Production of AAV vector – adapted from (“Types of Gene Therapy,” n.d.)

CHAPTER - 6

MOLECULAR ANOMALIES

IN NON-SMALL CELL

LUNG CANCER

Non-small cell lung cancer [NSCLC] is broadly classified into adenocarcinoma and squamous cell carcinoma. Tumour adenocarcinoma showed low immune cell expression, and squamous cell carcinoma showed higher immune cell expression. Adenocarcinoma showed mutations in the epidermal growth factor [EGFR], anaplastic lymphoma receptor tyrosine kinase [ALK] genes, serine/threonine11 gene [STK11] deletion, high proliferation, brain metastases and tumour protein p53 gene mutation [TP53]. Squamous cell carcinoma expressed retinoblastoma 1 gene deletion [RB1] in affected cells, oxidative stress damage in kelch like ECH associated protein gene [KEAP] and nuclear factor erythroid 2 like 2 [NFE2L2] genes, inflammation and neurofibromin 1 [NF1] gene mutations. EGFR, TP53 and SKT11 gene inactivation in also found in adenocarcinoma, RB1, NFE2L2 and NF1 gene mutations associated with T-cell overexpression were found in squamous cell carcinoma. Harmful mutations occurred in the TCGA domain, with TP53 gene mutations as the most common one , as agreed by (Vijayalakshmi & Krishnamurthy, 2011)

Wu, Zhao, Li, Zhu, & Yin, (2007) has agreed that “another common mutation that occurs is of the KRAS gene. Apart from this, other mutations can also occur, for example - translocation of the echinoderm microtubule-associated protein-like 4 [EML4] fused with anaplastic lymphoma kinase [ALK] to form the oncogene EML4-ALK, mutations or deletions causing loss or gain of functions – BRAF, phosphatidylinositol-4,5- bisphosphate 3-kinase, catalytic subunit alpha[PIK3CA], protein kinase B[AKT1] and mitogen- activated protein kinase [MEK] and gene amplifications of Myc, Cyclin D1 and HER2”.

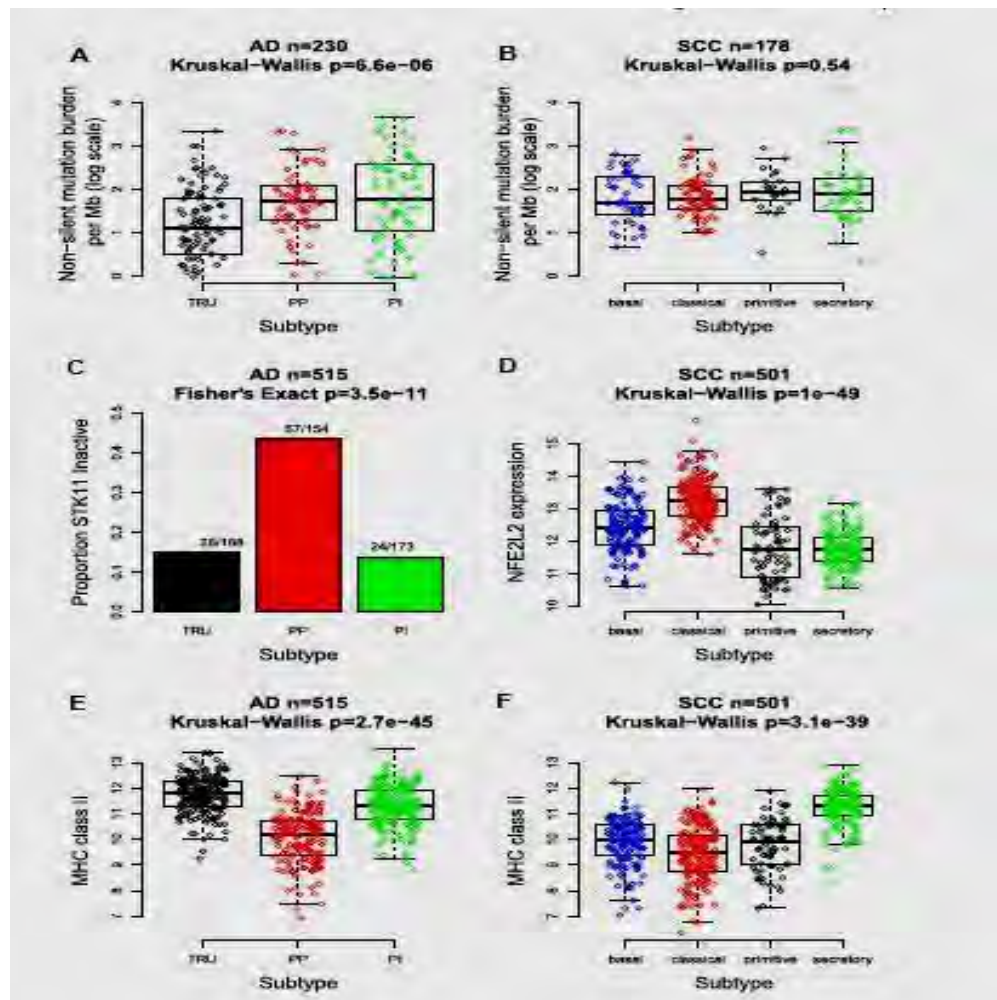


Figure 6.1: Adenocarcinoma and Squamous cell carcinoma – non-silent types {A, B}, SKT-11 gene in activation in adenocarcinoma {C}, NFE2L2 expression in squamous cell carcinoma {D}, MHC II signatures with associated test p-values {E.F}– adapted from (Vijayalakshmi R & Krishnamurthy Arvind, 2011)

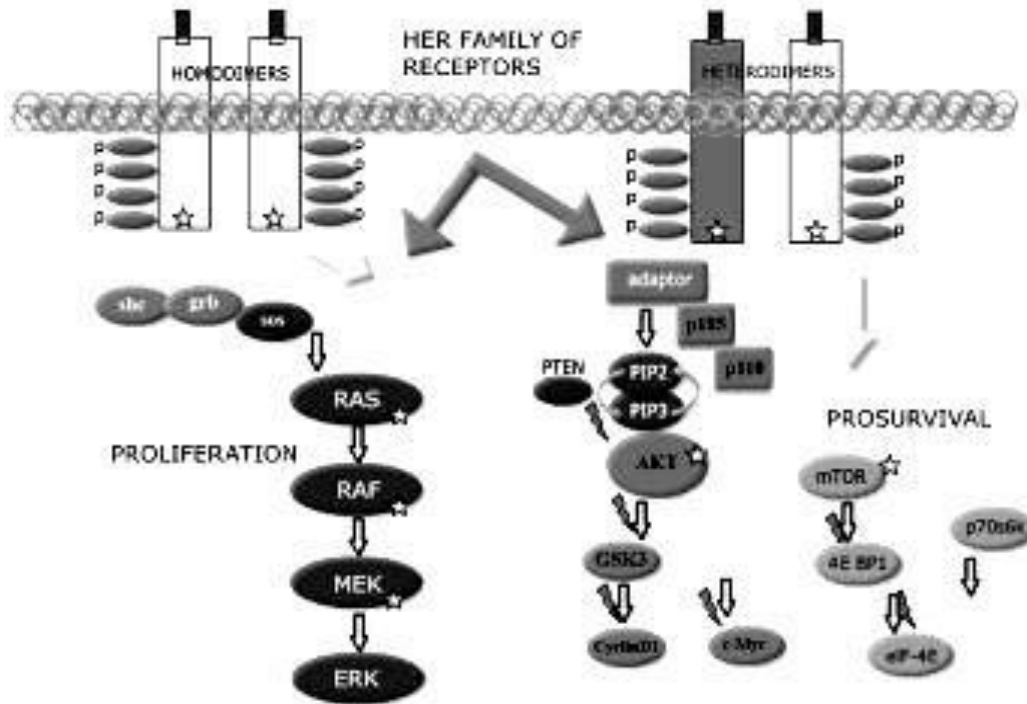


Figure 6.2: Signal transduction pathway in the activated HER receptors– adapted from (Wu Jian-Qing et al., 2007)

The tumour-suppressor gene SKT11 is located on chromosome 19. It controls the energy metabolism of cells and cell's polarity through the activation of AMP – activated protein kinase [AMPK]. This molecule restores the ATP level by increasing catabolic processes and increasing anabolic processes. This gene has many roles in the prognosis of lung cancer. First of all, chromosome 19 is the secondary target region which is lost during lung cancer. Next, a significant percentage of patients are shown to contain this chromosomal mutation, causing SKT11 to inactivate due to frameshift of non-sense mutations. In addition to this, the gene is also responsible for tumor proliferation and metastasis. Last but not the least, it affects tumour polarity. A recent research has shown that chromosome 19 showed loss of heterozygosity [LOH] /allele imbalance in non-small cell lung cancer. Even though LOH was less predominant,

homozygous deletion [HD] was mostly found to be the reason of gene inactivation, as shown by (Takara Bio 2017, 2018)

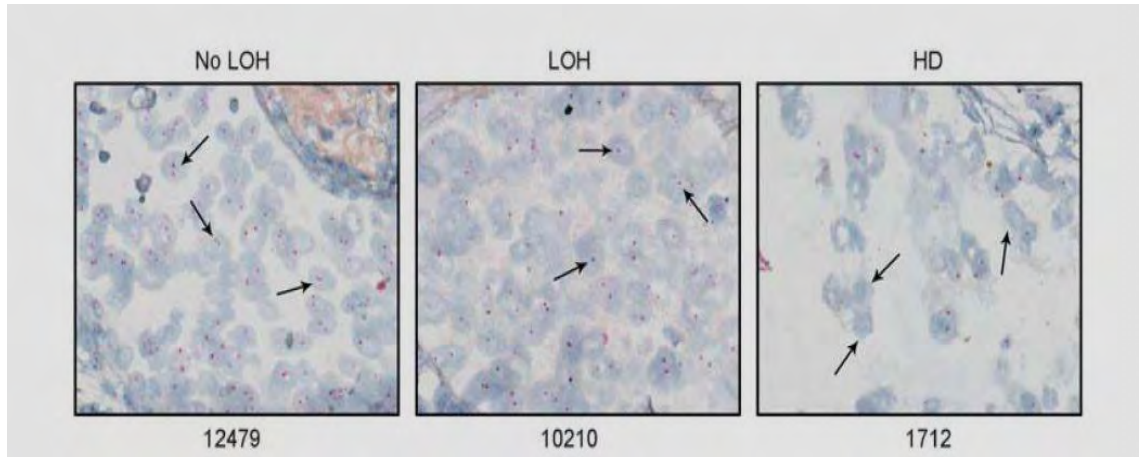


Figure 6.3: Genomic status of the LKB1/STK11 gene. Hybridization signals for the LKB1/STK11 gene in normal and tumor lung tissues. Distribution of the hybridization signals determines the genetic status of the gene in the tumor as no LOH , LOH or HD (#1712). – adapted from (Gill RK, Yang SH, Meerzaman D, Mechanic LE, Bowman ED, Jeon HS, Roy Chowdhuri S, Shakoori A, Dracheva T, Hong KM, Fukuoka J, Zhang JH, Harris CC, 2011)

Another anomaly that has been found is that of the transcription factor FOXO1. FOXO1 are fork-head box proteins that are essential molecules regulating the cellular life cycle and pre-apoptotic signaling. During NSCLC, phosphorylation of AKT diminishes the activity of FOXO1. The molecule is usually located in the nucleus of healthy cells and aids in the programmed death of mutated cells. Cells with NSCLC exhibited that the FOXO1 was continuously present in the cytoplasm, instead of the nucleus. AKT is a molecule that promotes cell growth and multiplication by disrupting the normal mechanisms and controls the pre-and post-apoptotic signal regulators. AKT negates the activity of FOXO1 in addition to neutralizing the effects of chemotherapy and radiation.

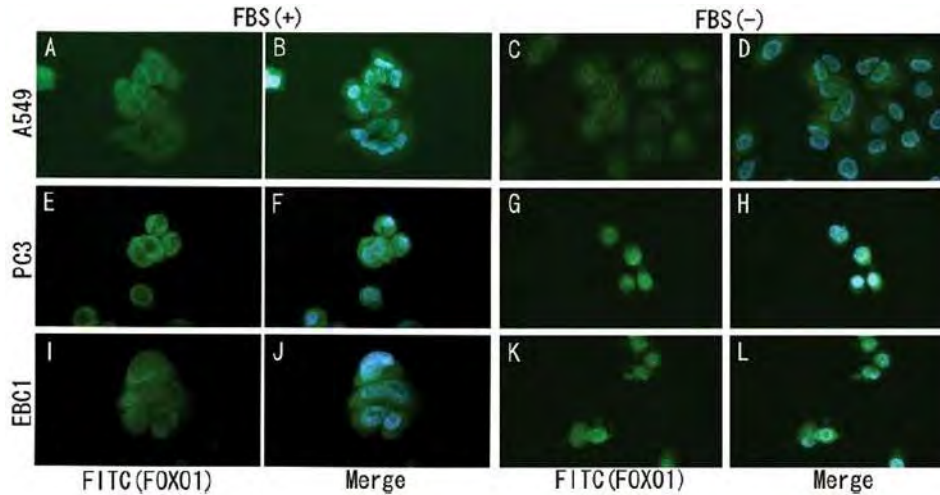


Figure 6.4: Localization of FOXO1 in NSCLC, with and without FBS. In presence of FBS, FOXO1 is present in the cytoplasm [A, B, E, F, I, J]; without FBS it is present in the nucleus [C, D, G, H, K, L]. – adapted from (Maekawa T, Maniwa Y, Doi T, Nishio W, Yoshimura M, Ohbayashi C, Hayashi Y, 2009)

Research conducted using different types of NSCLC cells using fetal bovine serum [FBS], which is used as a differentiating agent, and without FBS. Cells grown in FBS showed migration of FoxO1 in the cytoplasm, while those without it. In order to prove that the relocalization was due to AKT activation, RNA-interference was used to suppress the activity of AKT. Analysis by Western blotting provided evidence that the levels of AKT were significantly lower in all cells.

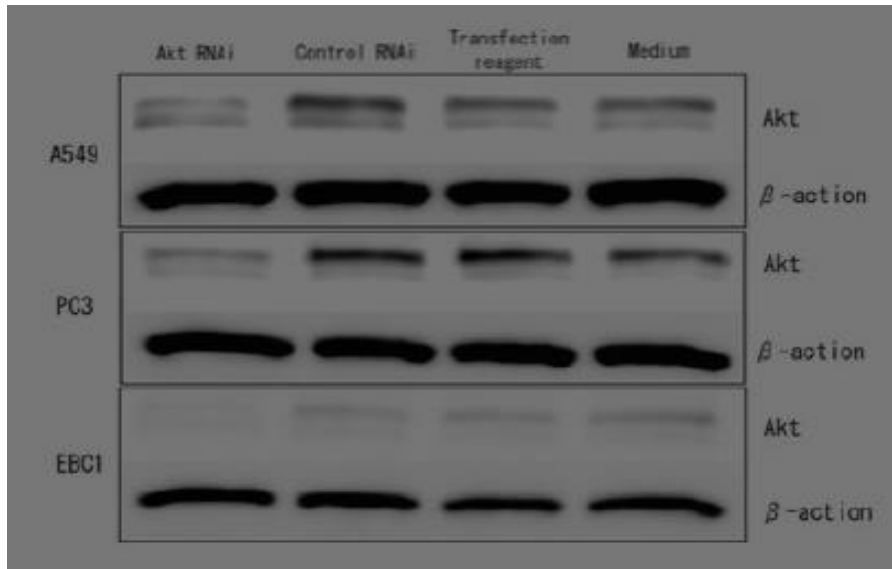


Figure 6.5: AKT expression in NSCLC after knock-down using RNA interference. After RNA interference , the AKT expression was remarkably decreased in the cells. – adapted from (Maekawa T, Maniwa Y, Doi T, Nishio W, Yoshimura M, Ohbayashi C, Hayashi Y, 2009)

When studying apoptosis of these cells, it was shown that the control cells showed irregular patterns of apoptosis, whereas the RNA-interference cells had a more predictable rate of apoptosis.

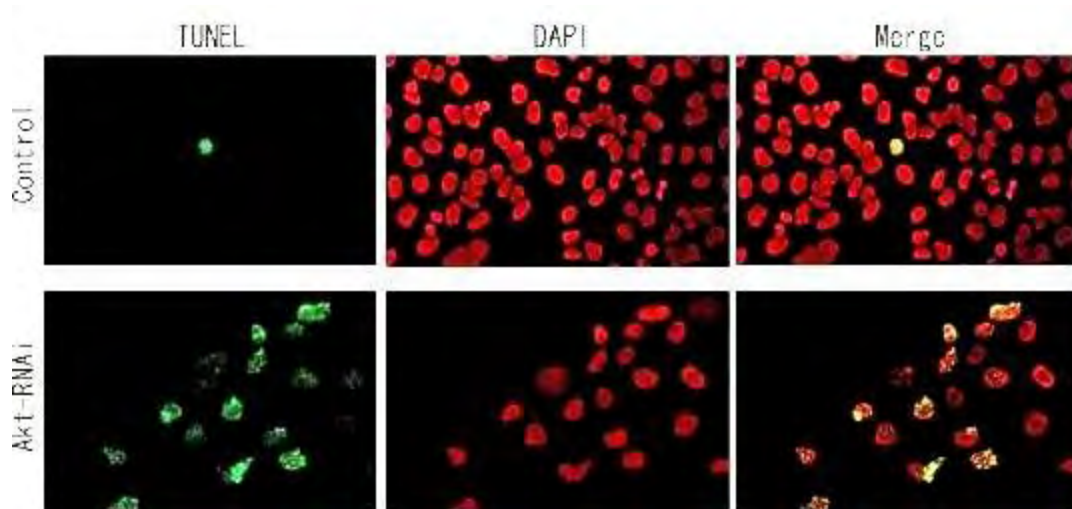


Figure 6.6: Visualization of apoptosis – using TUNEL staining, the control hardly showed apoptosis, using RNA interference, apoptosis accelerated remarkably. – adapted from (Maekawa T, Maniwa Y, Doi T, Nishio W, Yoshimura M, Ohbayashi C, Hayashi Y, 2009)

So it can be deduced that when external transcription factors were excluded from the culture medium, the FoxO1 was located inside the nucleus. But in presence of transcription factors were present, FoxO1 relocated in the cytoplasm. It is also seen that the AKT pathway is triggered by transcription factors, which eventually leads to translocation of FoxO1, as stated by (Porteus , 2015)

CHAPTER - 7

GENETHERAPY FOR NON-SMALL CELL LUNG CANCER

Gene therapy is a promising field for the treatment of non-small cell lung cancer because the lungs can be accessed by the airways, provides a large surface area for infection, has a local action and reduces systemic toxicity. Vettami & Claudio, (2004) has agreed that “immune response for cancer requires cross-presentation of tumor antigens by antigen presenting cells. They must undergo maturation by stimulation of CD40 by the CD40 ligand. So the transduction of CD40 ligand is considered an essential part in gene therapy. AAV 2 is the most useful vector for transduction of genes in primates. The presence of other forms of serotypes – AAV1 to 10 – has made this virus a potential candidate for gene therapy. Various combinations of serotypes have shown higher success rates compared to single serotypes. The combination AAV2/5 has shown promising results”.

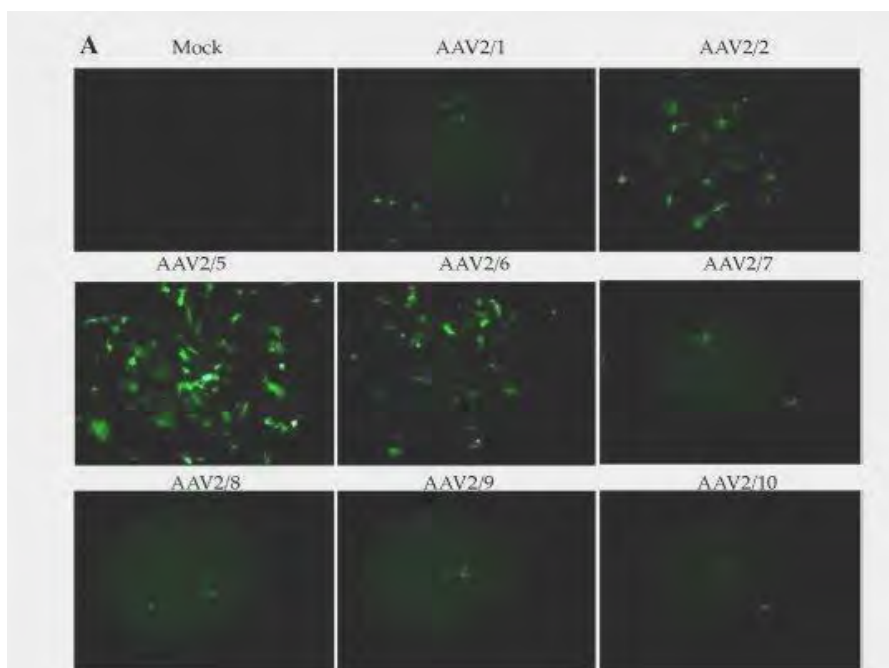


Figure 7.1: Comparison of infecting abilities of different serotypes. – adapted from (Wu Jian-Qing et al., 2007)

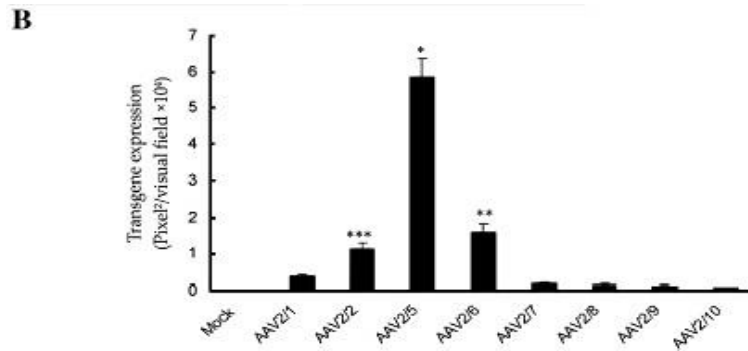


Figure 7.2: Quantitative analysis of infecting ability of different serotypes – adapted from (Wu Jian-Qing et al., 2007)

This method uses recombinant AAV, which lacks the viral RNA. So these are in fact nanoparticles which can cross the cell membrane and effectively enter the nucleus and deliver the therapeutic gene. IRT activated transgenes within rAAV DNA forms plasmid-like structures inside nucleus. It will stay separated from host DNA as episomal DNA, and will be diluted as the cell multiplies, with the loss of the therapeutic effect. This property will be useful where short- term therapy is needed, as shown by (Faruki et al., 2017). The tumour-suppressor gene p53 can be reintroduced into the cell, as suggested by a recent study. The treatment was carried out using other viruses and it reduced the rate of tumour development and enhanced apoptosis. In the absence of co-infection, rAAV integrates the gene into host cells, producing a dormant infection. But if this virus is further engineered, it loses its ability to infect at a specific site and can hold less amount of DNA. Another possible method is to integrate adenoviral DNA with rep and cap proteins in a plasmid that will be able to generate AAV genome, as stated by (Castillo, 2016).

RNA interference [RNAi] technique remains a very powerful tool for cancer treatment. It is a technique to block a dominant gene from functioning. By utilizing a complementary sequence that acts as an in-between state between the target sequence and the mRNA transcript, scientists have recruited RNA – induced silencing complex that will block

the mutated gene. This method could utilize either oligonucleotides or templates from which AAV hairpin regions are synthesized. This technique is mainly employed for gain-of-function mutations. It also has the advantage a single dose administration produces a continual basis of therapeutic effect, and the effect increasing with time.

Tenenbaum et al., (2003) has shown that “zinc-finger nuclease is a technique that can perform a cut on double-stranded DNA, which can then be resealed using the template of the normal therapeutic sequence. This technique is not very popular now because it is very difficult to identify and edit the mutation without a reference sequence. A possible way to tackle this problem is to incorporate the enzyme alongside AAV infection site on chromosome 19”.

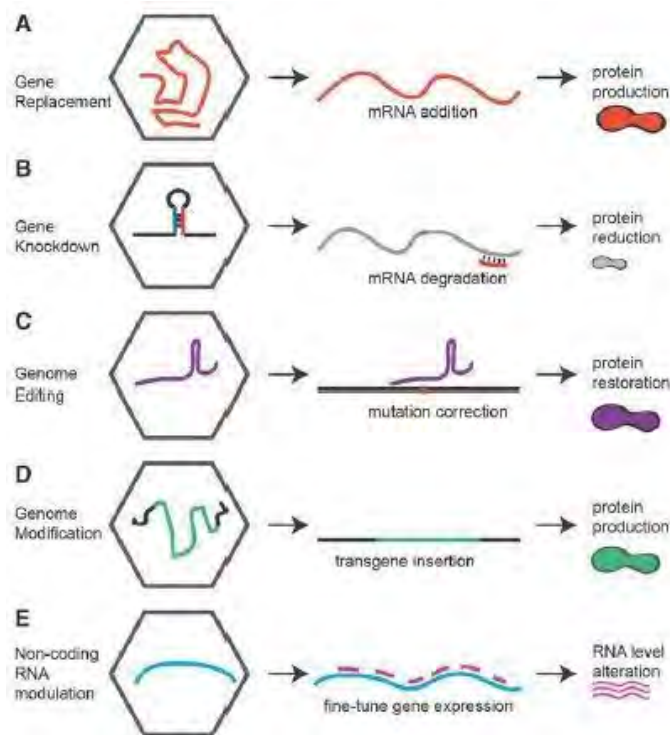


Figure 7.3: Different types of recombinant AAV therapies: (A) Gene delivery using a cDNA to replace a lost or mutated gene, driven by an exogenous promoter. (B) Gene knockdown through small interfering RNAs that can disintegrate a mutated gene or infectious virus. (C) Genome editing by zinc finger nucleases, transcription activator-like effectors nucleases, or the clustered regularly interspaced short Palindromic repeat (CRISPR)/Cas9 system to introduce or correct mutations in the host or virus genome or introduce transcripts at double-stranded genome breaks. (D) Genome modulation through introduction of DNA sequences with homology to the host genome to correct a mutation or provide a therapeutic gene. (E) Non-coding RNA modulation or sequestration to alter transcript and protein levels, for example through introduction of sequences with engineered binding sites. – adapted from (Valdmanis Paul N & Kay Mark A, 2017)

Anti-sense therapy is used when dominant genes are needed to be silenced. This can take place at many stages. Transcription of the oncogene can be blocked by anti-sense oligonucleotide that binds to transcription starting sites. Translation of the oncogene can be blocked by anti-sense nucleotides that bind and degrade the transcript. The mutated protein can be blocked by using specific antibodies. Another method is to induce the tumour cells to kill themselves, by using a prodrug that will be converted into the drug inside the tumour cells. Another way is to deliver genes that will have an anti-angiogenesis effect directly to the tumour vessels. Introducing a cytokine gene to enhance detection by lymphocytes or allows antigen presentation, as stated by (Castillo, 2016).

7.1 CRISPR/CAS9 as therapeutic equipment

Valdmanis & Kay, (2017) has agreed that “CRISPR/Cas9 is an acceptable, rapid and precise method to alter, correct or remove mutations in a cell. A recent study has shown that the lymphocytes, edited by this method, when reintroduced, destroyed tumour cells. The gene coding the PD-1 [programmed death 1] protein was switched off to enhance immunity to cancer. The CRISPR system can be engineered to be made to act on double-stranded DNA at a 5'-NGG proto-spacer adjacent motif [PAM].”

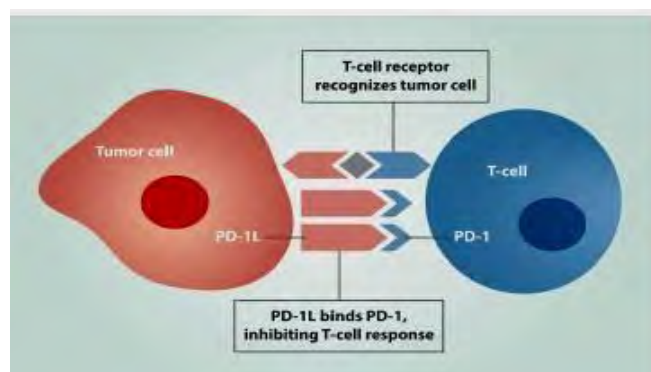


Figure 7.4: CRISPR Cas- 9 can be used for inhibiting PD-1 gene and reactivate the immune system. – adapted from (Castillo Andres, 2016)

The CRISPR RNAs guide the endonuclease Cas9 to the specific site of the DNA. Normally when a DNA is cut, it will tend to heal itself by rejoining the break and the CRISPR manipulates this healing by inserting the gene of interest inside the gap. When the process is correctly done, a frame shift mutation interrupts the functions of mutant gene.

Tenenbaum et al., (2003) have shown that AAV and CRISPR/Cas9 can be utilized together and showed promising results. This combined technique can also be used for studying and developing treatment for multi-mutation conditions like lung cancer.

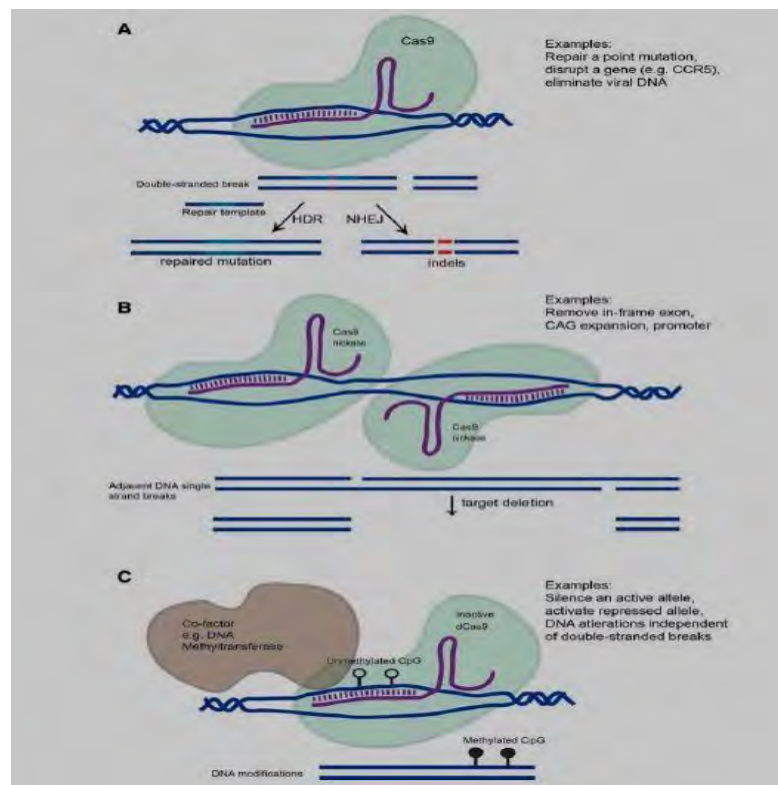


Figure 7.5: Different methods of using CRISPR – CAS 9 for genome editing. – adapted from (Valdmanis Paul N & Kay Mark A, 2017)

A recent technique utilizing CRISPR/Cas9 and the AAV is one which uses AAV-split-Cas9. The method provides positive results for distribution of viruses in target cells,

expressing antigens, immunologic response against target cells and editing power in affected cells. Of the many different orthologs, the Cas9 from *Streptococcus pyogenes*, SpCas9 is chosen as the desired Cas9 type. This Cas9 has fewer shortcomings, resulting in its wide range of site of action. This enzyme can be split at sites which have high mutation tendencies will retain the packaging properties and reduce the ability to get inactivated after reaching host cells. The minute amount of Cas9 inactivation caused by splitting is counteracted by adding another Cas9 from *Rhodothermusmarinus*. This has the additional benefit of increasing the size of AAV by greater than 2kb. The split Cas9 remains viable in target cells and effectively modifies the mutation.

Moreover the therapy is dose-dependent, where higher concentrations provide greater editing rates. Furthermore, transcription-activator fusion domains can also be incorporated into the virus. The same virus can be used for both editing and activation of genes, which only depends on the gRNA spacers' length. In addition to this, another major advantage is that AAV does not provoke an immune response, compared to Cas9 when used singly. Normal AAV-CRISPRCas9 induces immune response in host by making the host more susceptible to infections. This property can be eliminated in the split Cas9. It also reduces the level of perforin from T- lymphocytes and NK-cells, which is a potent chemical for cell death.

In addition to this, CRISPRCas9 is also used in the detection of resistant mutated non-small cell lung cancer cells. This process is known CRISPR-barcoding. The mutated cells are unresponsive to EGFR-inhibitors. The researchers have used a specialized guide-RNA and an oligonucleotide to detect differences in cancer cells. KRAS-G12D and EGFR-T790M were used together as biomarkers to detect the carcinomas. Another procedure involved the marking of EML4- ALK mutation also shows promising results. This study has allowed

scientists to understand that the protein kinase and tyrosine kinase inhibitors in non-small cell lung cancer are susceptible to chemotherapy. This study has also provided the evidence that bypassing the tyrosine kinase inhibitors will provide the ultimate cure for non-small cell cancer. It could be also used for evaluation of the effectiveness, response and resistance of standard drug therapy of the condition, as shown by (Khan, 2012)

7.2 Zinc – finger Nuclease as a Therapeutic Weapon

Zinc – finger nucleases are a group of fusion proteins that have different individual parts bonded together. The entity consists of a specific region for DNA binding and a non-specific region for nuclease activity. The DNA-binding region is encoded by zinc molecules which are site-specific. The zinc molecules are bound to endonuclease part of the FokI. The term Zinc-finger is used because the molecule is stabilized by a zinc ion. They are broadly categorized into 8 groups depending on their structural integrity. The format for designing a zinc-finger nuclease is C₂H₂-two cysteine and two histidine molecules which are bound to the zinc particles. The molecule is usually 20-30 amino acids long, with antiparallel β -sheets with cysteine and α helix with histidine molecules. The fingers have a triplet code for binding with complementary amino acids. FokI enzyme is an endonuclease mainly found in the bacterium *Flavobacterium okeanokoites*. The enzyme has a structure which contains a binding region at the N-terminal and a cleaving part at the C-terminal. The binding region is specific and has a particular complementary sequence to which it binds while the cleaving part is non-specific and cuts the phosphodiester bonds.

The zinc-finger nuclease [ZFN] is a very effective tool to detect specific sequences within complex genomes. When two ZFNs bind to each other in a particular orientation, the cleaving part of the molecule forms a dimer and makes an opening in the DNA double helix. When the template DNA is absent, NHEJ repairs the break and can cause frameshift

mutations. This technique can be exploited to silence dominant mutagenic alleles. A special advantage of this treatment is that that vector does not persist in parent cells, but the therapeutic effect is permanent and carries on to future generations, as shown by (Khan, 2012)

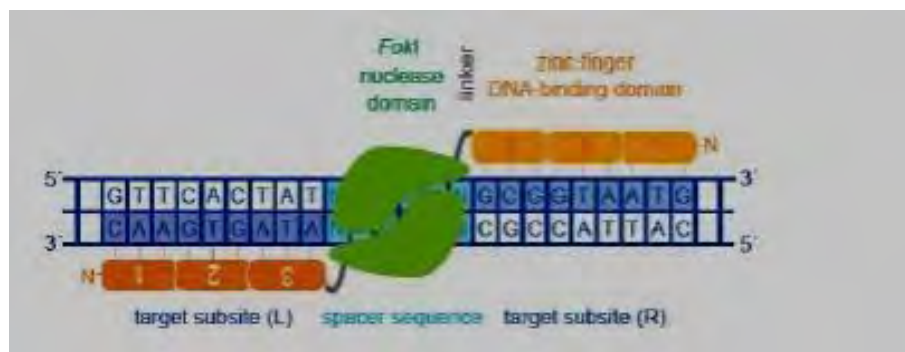


Figure 7.6: Structure and function of zinc finger nuclease – adapted from (Khan, 2012)

A promising technique is the Zinc finger protein ZBTB20. ZBTB20 consists of an intact N- terminal with a BTB region and a C-terminal zinc finger domain. This molecule is a transcription repressor of the POK (POZ and Krüppel) family. It has been shown that the repressor gene expression was significantly higher in NSCLC cells than healthy cells, about 6-times than that of normal cells. Examination by Western blotting also confirmed the presence of the suppressor protein in malignant cells in increasing amounts. Apart from this, it has also shown enhanced inflammatory signaling that led to improved activities of the suppressor genes.

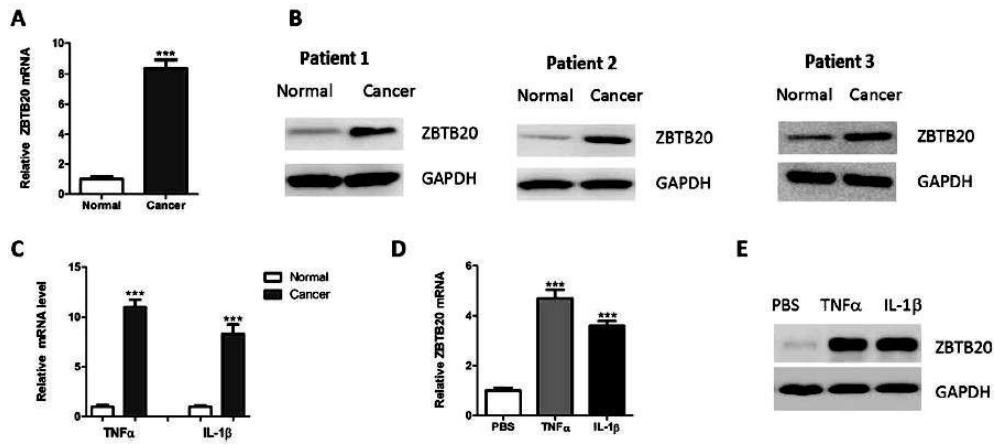


Figure 7.7: Up- regulation of ZBTB20- the m-RNA and protein analyzed by PCR [A] and Western blot [B] , comparison between affected and normal cells [C] , mRNA levels of TNF α and IL-1b in NSCLC or adjacent normal tissues. (D and E). – adapted from (Zhao JG, Ren KM, & Tang J, 2014)

In order to determine the functional effectiveness of the gene, placebo vectors and repressor vectors were prepared and inserted into the treated cells. So an over expression of ZBTB20 genes and the number of cells expressing them was found in the some examined cells, while others showed no improvement.

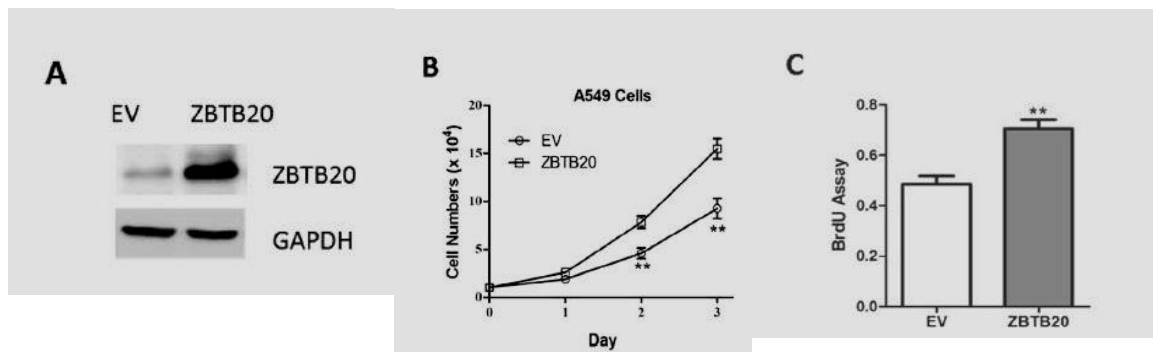


Figure 7.8: The role of ZBTB20 in cell multiplication – A] Western blot analysis of the protein expression in cells infected by placebo and therapeutic vector. B] Comparison of the growth curves of placebo and therapeutic vector. C] The cell proliferative potential (BrdU) of placebo and therapeutic vector. – adapted from (Zhao JG et al., 2014)

Certain cell cycle regulators are controlled by some tumor suppressor genes and oncogenes. These are FoxO1, P53, Stat3, E2F1, b-Catenin and c-myc. The gene that was most affected by ZBTB20 was the mutated FoxO1. Over expression of ZBTB20 suppressed the activity of FoxO1, while silencing the treatment increased the target gene's protein levels.

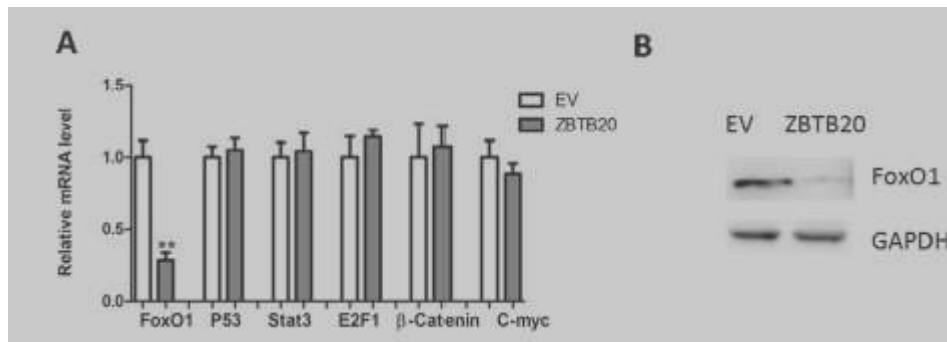


Figure 7.9: ZBTB20 suppresses the FOXO1 function – mRNA and protein levels of FoxO1, P53, Stat3, E2F1, b-Catenin and C-myc were analyzed by real-time PCR (A) and Western blot (B) in A549 cells transfected with empty vector (EV) or ZBTB. – adapted from (Zhao JG et al., 2014)

The amount of diseased FoxO1 RNA and protein found in the cytosol of cancer cells was also decreased after treatment, compared to that of normal cells. Repression of the FoxO1 gene was to increase the effects of the treatment to three fold.

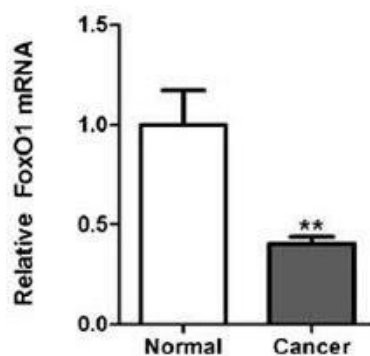


Figure 7.10: Comparison of mRNA levels and protein levels of FOXO1 in affected and healthy cells. – adapted from (Zhao JG et al., 2014)

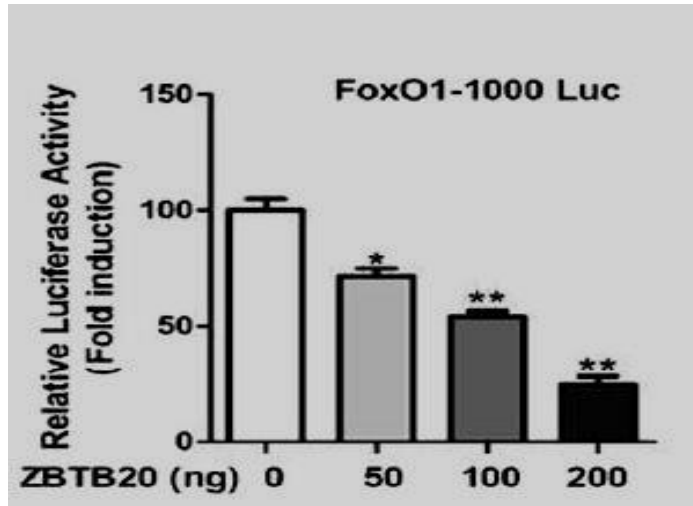


Figure 7.11: ZBTB20 regulates FOXO1 promoter activity – luciferase reporter analysis of human FOXO1 promoter. – adapted from (Zhao JG et al., 2014)

The promoter region of the affected FoxO1 gene provides some interesting information about its relationship with ZBTB20. The promoter region is from -1000 bp to +10 bp. Deletion of this region eliminates the therapeutic effects of ZBTB20. This indicates that the responsive region is located in the promoter locus at in the +200 bp to- 100 bp zones. Analysis by chromatin immunoprecipitation (ChIP) also revealed ZBTB20 attaches to this promoter region.

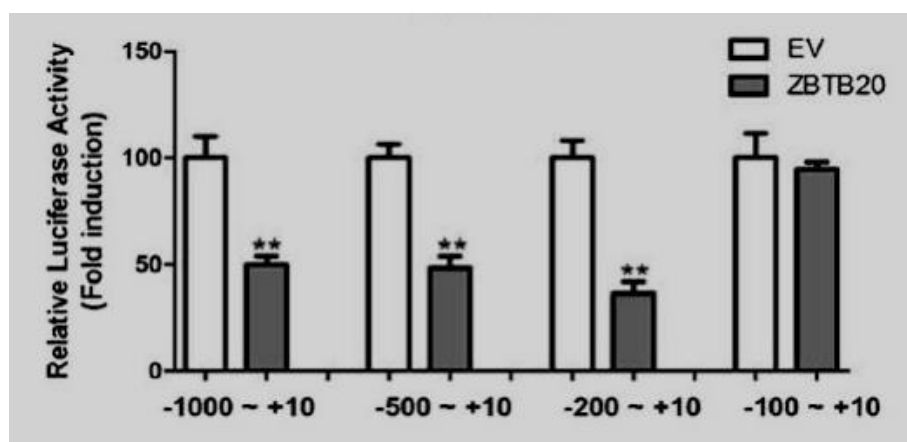


Figure 7.12: A series of deletions were done to pinpoint the transcriptional region – adapted from (Zhao JG et al., 2014)

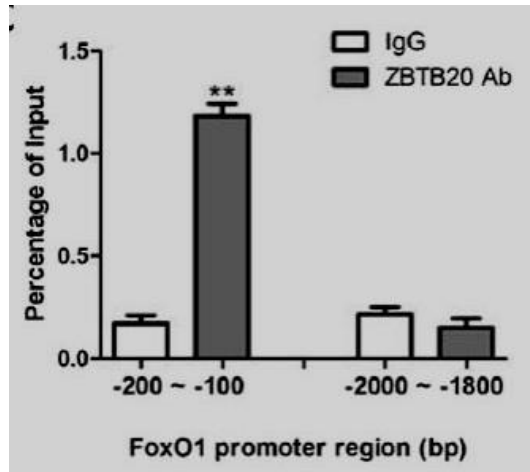


Figure 7.13: ChIP assays to show the recruitment of ZBTB20 onto FoxO1 promoter. – adapted from (Zhao JG et al., 2014)

In order to illustrate the relation between immunity and ZBTB20, some cell samples were re- infected with FoxO1 and empty vectors. The cells which already contain the gene over expressed them, but the cells where the activity of FoxO1 was depressed, showed normal results. These cells showed improved immune response which further activated the activities of ZBTB20.

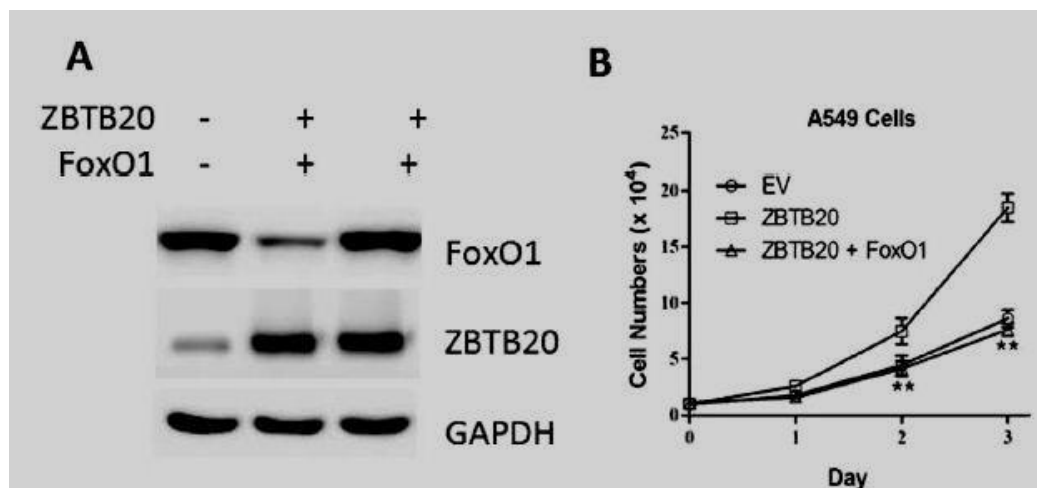


Figure 7.14: FoxO1 re-introduction or knockdown weakens the multiplication powers of ZBTB20. Protein level of FoxO1 was determined by Western blot in A549 cells (B). – adapted from (Zhao JG et al., 2014)

Uncontrolled cellular multiplication occurs when proto-oncogenes are activated and tumor suppressor genes are inactivated. One of the most common mutations is that of the tumor-suppressor gene p53. This gene is located at 16-20 kb on chromosome 17. The gene is responsible for checking the growth of cells and inducing apoptosis. It has been found that the wild-type p53 induces growth arrest by cyclin- dependent kinase inhibitor p21, but the regular p53 gene uses the bcl-2 and bax. The wild-type AAV is 4860 nucleotides long with the coding zone at 145 nucleotides at ITRs containing units in the cis-configuration, as deduced by (Zhao et al., 2014)

CHAPTER - 8

CONCLUDING REMARKS

The use of gene therapy in the fight against hard-to-cure diseases and conditions is of highest concern now. Apart from this, gene therapy can also be used to cure minor to moderate ailments as well. Gene therapy can be of many different types. It can consist of procedures that will remove the affected area of DNA and maybe replace it with healthy genes. It can be a method to introduce a loss-of-function property to a dominant harmful protein by blocking the functions of the harmful gene. It can also be used as an aid to boost up the immune system, so that erroneous mutations can be detected and deleted. As the name suggests, gene therapy is of prime importance in the treatment of genetic diseases. One of the most beneficial uses of gene therapy is for the innate disease SCID – Severe Combined Immunodeficiency. The patient suffering from this disease lacks a properly functioning immune system. They need to be solitarily confined to an aseptic environment during their lifetime to prevent them from catching diseases. This is because even the slightest infection can be life-threatening for them. SCID was treated using the therapeutic gene ADA, by placement of the gene into the bone marrow removed from the patients, then transplanting the treated bone marrow back to into the patients. It did not produce any noticeable side-effects and the patients were able to lead a normal life. Chronic Granulomatus Disorder (CGD) is another similar disease which shows promising results using gene therapy. In this disease the body loses its ability to fight bacterial and fungal infections. This can be quite life-threatening for the patients. Using similar processes as for SCID, patients for CGD can also be treated for long- term. Apart from genetic disorders, gene therapy is also used for the treatment of acquired disorders. One of the most important classes of diseases is cancer. Several treatment strategies have been developed to fight this incurable disease. Cancer is a condition that is mainly caused by a series of genes, so it is quite difficult to treat. However, many targeted cancer therapies have been developed. Examples include vaccines, suicide therapies, viral therapies, etc. Majority of clinical trials for cancer are at Phase III, the rest are at Phase I and

II. Another group of disease showing promising results is neurodegenerative disorders caused by inherited dominant gain-of-function mutations. Parkinson's disease and Huntington's chorea are the prime examples. These diseases can be treated by blocking the harmful gene from functioning or by removing the harmful gene and replacing it with corrected gene. Clinical trials are set to be at Phase I levels for neurodegenerative disorders.

In case of short-term therapy, AAV2-AAV5 therapy using raw genetic material can be considered a choice of therapy. For a more successful long-term therapy, permanent methods are required. It has been seen that for treatment using CRISPR/Cas9, there have a broad spectrum of therapeutic approaches. Immunity for cancer is increased by turning off the PD-1 gene. The CRISPR/Cas9 system can also be employed in the AAV/AAV5 system, thus converting it efficiently into a permanent therapy. Simply by switching from one source of Cas9 to another, the plethora of potential effects can increase. Another successful method used is the ZNF. Experiments have shown that it can decrease the level of harmful suppressor gene in NSCLC by significant levels. This process also controls the levels of harmful gene production by decreasing the amounts of mRNA transcribed from it. In addition to this, CRISPR/Cas9 can also be used as a diagnostic agent for cancer. They can be used for detection of specific mutation regions in cancer cells by employing site-specific markers. Gene therapy has already started showing promising results. One of current techniques showing promising result is an rAAV based technique that is fused with the synthetic micro RNA [miRNA] technique.

This process is mainly used for the treatment of dominant gain-of-function diseases like Huntington's disease and Parkinson's disease. The synthetic mRNA uses the metabolic pathways as endogenous RNA.

It has been successfully used to decrease the production of harmful proteins from mutated genes. Trials are being carried out where this therapy is also used for targeting both gain-of-function and loss-of-function diseases. One of the diseases that are targeted next is the disease caused by the deficiency of alpha1-antitrypsin. The deficiency not only causes liver damage but also emphysema. Knockdown of the gene that causes the deletion or reduction of this protein greatly reduces the incidence of developing these conditions. AAV-based therapies are in the end stages of approval by FDA and already approved by EMA. CRISPR/Cas9 and synthetic RNA-based technologies are next in line to be tested in clinical trials. Both techniques need considerable amounts of exploitations in order to develop efficient vectors that will not be carcinogenic and specific. This is because both of these processes involve the introduction of exogenous materials into the body. Another field that needs to be considered is the site-specific delivery of genes. This will lead to the more efficient development of vector system and delivery methods. In addition to this, the biochemical nature of the target cell must be clearly understood.

Apart from this, the current methods of gene delivery also have poor expression rates of desired genes. Effective methods need to be developed so that the therapeutic gene is permanently expressed every time the cell divides. More exhaustive research is needed in the fields of cellular enhancers and viral gene promoters that can be used for effective therapy. One method for permanent expression of genes is to extract the transcription factor and mechanism of a common gene and use it for the beneficial gene. This can be done by combining the gene of interest – without promoter – to the promoter of the common gene in the downstream region.

CHAPTER - 9

REFERENCES

- Adeno-Associated Viral Vectors. (n.d.). Retrieved September 26, 2017, from <http://www.genetherapynet.com/viral-vector/adeno-associated-viruses.html>
- Amer Magid H. (2014). Gene therapy for cancer: present status and future perspective. *Molecular and Cellular Therapies*, 4(27), 2: 27. <https://doi.org/10.1186/2052-8426-2-27>
- Canver Matthew C. (2009). *Evaluation of the Clinical Success of Ex Vivo and In Vivo Gene Therapy*. *Journal of Young Investigators*. University of Pennsylvania. Retrieved from <https://www.jyi.org/2009-january/2017/10/2/evaluation-of-the-clinical-success-of-ex-vivo-and-in-vivo-gene-therapy>
- Carroll Jeff Dr. (2011). Cut-and-paste DNA: fixing mutations with “genome editing.” Retrieved September 26, 2017, from <https://en.hdbuzz.net/038>
- Castillo Andres. (2016). *Gene editing for the treatment of lung cancer (CRISPR-Cas9)*. Universidad del Valle. Retrieved from <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5335857/>
- Chandler Randy J, LaFave Matthew C, Varshney Gaurav K, Trivedi Niraj S, Carrillo-Carrasco Nuria, Senac Julien S, ... Venditti Charles P. (2015). Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy. *The Journal of Clinical Investigation*, 125(2), :870-880. <https://doi.org/https://doi.org/10.1172/JCI79213>.
- Chew WL, Tabebordbar M, Cheng JK, Mali Wu EY, Ng AH, Zhu K, W. A. C. G. (2016). A multi-functional AAV-CRISPR-Cas9 and its host response. *Nature Methods*, 13(10), 868–874. <https://doi.org/10.1038/nmeth.3993>.

- Coura Renata dos Santos, & Nardi Nance Beyer. (2008). A role for adeno-associated viral vectors in gene therapy. *Genetics and Molecular Biology*, 31(1), 1–11. <https://doi.org/http://dx.doi.org/10.1590/S1415-47572008000100001>
- CRISPR THERAPEUTICS. (2017). Our Programs. Retrieved September 26, 2017, from <http://www.crisprtx.com/our-programs/crispr-cas9-gene-editing.php>
- Cross Deanna PhD, & Burmester James K. PhD. (2006). Gene Therapy for Cancer Treatment: Past, Present and Future. *Clinical Medicine and Research*, 4(3), 218–227. Retrieved from <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1570487/>
- Faruki Hawazin DrPH, Mayhew * Gregory M. DrPH, Serody, Jonathan S, M., Hayes, D. Neil, M., Perou Charles M PhD, & Lai-Goldman Myla, M. (2017). Lung Adenocarcinoma and Squamous Cell Carcinoma Gene Expression Subtypes Demonstrate Significant Differences in Tumor Immune Landscape. *Journal of Thoracic Oncology*, 12(6), 943–953. <https://doi.org/10.1016/j.jtho.2017.03.010>
- Gene Therapy for Diseases*. (2017). 555 East Wells Street Suite 1100 Milwaukee, WI 53202. Retrieved from https://annualmeeting.asgct.org/about_gene_therapy/diseases.php
- Gill RK, Yang SH, Meerzaman D, Mechanic LE, Bowman ED, Jeon HS, Roy Chowdhuri S, Shakoori A, Dracheva T, Hong KM, Fukuoka J, Zhang JH, Harris CC, J. J. (2011). Frequent homozygous deletion of the LKB1/STK11 gene in nonsmall cell lung cancer. *Oncogene*, 30(35), 3784–3791. <https://doi.org/10.1038/onc.2011.98>
- Jain KK. (1998). *PHARMACOLOGY*. (Harry Majewski, Ed.). Oxford: EOLSS.

- Khan, K. (2012). *Establishing a zinc finger nuclease platform for targeted genome editing in human cell lines, primary keratinocyte stem cells and cardiomyocytes in vivo*. Hannover Medical School Institute of Experimental Hematology.
- Learn.Genetics, G. S. L. C. U. of U. (n.d.-a). Approaches To Gene Therapy. Retrieved September 26, 2017, from <http://learn.genetics.utah.edu/content/genetherapy/approaches/>
- Learn.Genetics, G. S. L. C. U. of U. (n.d.-b). What is Gene Therapy. Retrieved September 26, 2017, from <http://learn.genetics.utah.edu/content/genetherapy/intro/>
- Maekawa T, Maniwa Y, Doi T, Nishio W, Yoshimura M, Ohbayashi C, Hayashi Y, O. Y. (2009). Expression and localization of FOXO1 in non-small cell lung cancer. *Oncology Reports*, 22, 57–64. https://doi.org/10.3892/or_00000406
- Mayo Clinic. (n.d.). Gene therapy. Retrieved from <https://www.mayoclinic.org/tests-procedures/gene-therapy/about/pac-20384619>
- Naso Michael F, Tomkowicz Brian, Perry III William L., & Strohl William R. (2017). Adeno-Associated Virus (AAV) as a Vector for Gene Therapy. *BioDrugs*, 31(4), 317–334. <https://doi.org/10.1007/s40259-017-0234-5>
- Nayerossadat Nouri, Maedah Talebi, & Ali Palizban Abas. (2012). Viral and nonviral delivery systems for gene delivery. *Advanced Biomedical Research*, 1(27). <https://doi.org/10.4103/2277-9175.98152>
- New gene therapy boost immune system to cure cancer. (2002). Retrieved September 26, 2017, from https://www.eurekalert.org/pub_releases/2002-12/nu-ngt121202.php

- Novus Biologicals, L. (2016). How Adenovirus and Adeno-Associated Virus Work as Gene Therapy Vectors. Retrieved September 26, 2017, from <https://www.novusbio.com/antibody-news/antibodies/how-adenovirus-and-ado-associated-virus-work-as-gene-therapy-vectors>
- Papadakis E.D., Nicklin S.A, Baker A.H., & White S.J. (2004). Promoters and Control Elements: Designing Expression Cassettes for Gene Therapy. *Current Gene Therapy*, 4(1), 89–113. Retrieved from <https://www.ncbi.nlm.nih.gov/pubmed/15032617>
- Porteus Matthew H. (2015). *Towards a new era in medicine: therapeutic genome editing*. Stanford University. <https://doi.org/10.1186/s13059-015-0859-y>
- Takara Bio 2017. (2018). Helper virus-free preparation of AAV particles to deliver Cas9 and sgRNA. Retrieved November 1, 2017, from http://www.clontech.com/US/Products/Genome_Editing/CRISPR_Cas9/AAV_Systems?sitex=10020%3A22372%3AUS
- Tenenbaum L, Lehtonen E, & Monahan PE. (2003). Evaluation of Risks Related to the Use of Adeno-Associated Virus-Based Vectors. *Current Gene Therapy*, 3(6), 545–565. <https://doi.org/10.2174/1566523034578131>
- Types of Gene Therapy. (n.d.). Retrieved September 26, 2017, from <http://www.genetherapynet.com/types-of-gene-therapy.html>
- Valdmanis Paul N, & Kay Mark A. (2017). *Future of rAAV Gene Therapy: Platform for RNAi, Gene Editing, and Beyond*. Stanford University. <https://doi.org/10.1089/hum.2016.171>

- Vettami, E., & Claudio, P. P. (2004). Gene therapy for lung cancer: practice and promise. *Annali Italiani Di Chirurgia*, 75(3), 279–289. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/15605515/?ncbi_mmode=std
- Vijayalakshmi R, & Krishnamurthy Arvind. (2011). Targetable “Driver” Mutations in Non Small Cell Lung Cancer. *Indian Association of Surgical Oncology*, 2(3), 178–188. <https://doi.org/10.1007/s13193-011-0108-0>
- What is gene therapy? (n.d.). Retrieved September 26, 2017, from <https://www.esgct.eu/Useful-Information/Introduction-to-Gene-Therapy.aspx>
- Wu Jian-Qing, Zhao Wei-Hong, Li Yan, Zhu Bei, & Yin Kai-Sheng. (2007). Adeno-associated virus mediated gene transfer into lung cancer cells promoting CD40 ligand-based immunotherapy. *Virology*, 368(2), 309–316. <https://doi.org/10.1016/j.virol.2007.07.006>
- Xie Qing, Bu Weishu, Bhatia Smita, Hare Joan, Somasundaram Thayumanasamy, Azzi Arezk, & Chapman Michael S. (2002). *The atomic structure of adeno-associated virus (AAV-2), a vector for human gene therapy. Proceedings of the National Academy of Sciences of the United States of America PNAS*,. Florida State University. <https://doi.org/10.1073/pnas.162250899>
- Zhao JG, Ren KM, & Tang J. (2014). Zinc finger protein ZBTB20 promotes cell proliferation in non-small cell lung cancer through repression of FoxO1. *FEBS Letters*, 588(24), 4536–4542. <https://doi.org/10.1016/j.febslet.2014.10.005>

CHAPTER – 10

APPENDIX

A1: Gene Therapy - an experimental method of treatment where normal genes are used to subdue or block the actions of mutant genes that causes genetic disorders.

A2: Genome editing – the use of biotechnological techniques to make particular changes in the specific DNA sequence of a living organism’s genome.

A3: Adeno-associated virus- a small single-stranded virus of the Parvovirus family which can infect animal cells with the help of adenovirus and introduce genetic material on the chromosome 19 of humans.

A4: Non-small cell lung cancer – a type of cancer which affects the epithelium of lungs. They are non-respondent to chemotherapy and are generally treated by surgery.

A5: p53 gene – a gene that is responsible for apoptosis, controlling cell cycle, suppressing tumor, and stopping cell division when DNA is damaged.

A6: Double-stranded DNA breaks – a break in the DNA that occurs when both strands of the DNA double helix is cut.

A7: CRISPR-Cas9- clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9. It is a genome –editing system that is present in bacteria, which functions as their immune system. The bacteria use it to capture and remember portions of viral genome and then use the Cas9 to cut the invading viral DNA.

A8: SMaRT- Spliceosome-mediated RNA trans-splicing- this is a technique that edits the pre- mRNA by suppressing cis-splicing and enabling trans-splicing between the pre-mRNA and its target.

A9: RNA interference technique- RNAi is a natural process by which cells use short, double-stranded RNAs (dsRNAs) to recognize messenger RNAs (mRNAs) with exquisite specificity,

leading to their enzymatic destruction and preventing their translation into a protein. Therefore, they inhibit gene function

A10: Antisense oligonucleotide therapy- Treatment with antisense oligonucleotides. These are small pieces of DNA or RNA that can bind to specific molecules of RNA. This blocks the cell's ability to use the RNA to make a protein or work in other ways

A11: Inverted Terminal Repeats - The terminal inverted repeats are usually composed of special sequences at the extreme tips of the transposon that signal exactly where that breakage and joining should occur, plus more internal binding sites for the transposase.

A12: Open reading frames - A reading frame is a sequence of nucleotide triplets that are read as codons specifying amino acids; a single strand of DNA sequence has three possible reading frames. Open reading frames (ORFs) are parts of a reading frame that contain no stop codons.

A13: FOXO1 - Members of the class O of fork head box transcription factors (FOXO) have important roles in metabolism, cellular proliferation, stress resistance, and apoptosis. The activity of FOXOs is tightly regulated by posttranslational modification, including phosphorylation, acetylation, and ubiquitylation. Activation of cell survival pathways such as phosphoinositide-3-kinase/AKT/IKK or RAS/mitogen-activated protein kinase phosphorylates FOXOs at different sites which regulate FOXOs nuclear localization or degradation.

A14: Zinc – finger nuclease- Zinc finger nucleases (ZFNs) are a class of engineered DNA-binding proteins that facilitate targeted editing of the genome by creating double-strand breaks in DNA at user-specified locations.