Prevalence and application of an evolving genome editing tool “CRISPR/Cas System” in cancer research and treatment

A thesis submitted to the Department of Mathematics and Natural Science in partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

Department of Mathematics and Natura Sciences
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By

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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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Approval

The thesis/project titled " Prevalence and application of an evolving genome editing tool "CRISPR/Cas System" in cancer research and treatment " submitted by Mahdi Mubin Shaikat (21176005) of Spring, 2021 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Masters of Science in Biotechnology on December, 2022.

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Ethics Statement

This study does not involve any kind of animal or human trial.

Abstract

One of the biggest causes of mortality worldwide is cancer. Cancer is one of the most challenging diseases to cure because of its capacity to spread quickly, metastasis, and create diverse tumors as a result of mutagenesis events. The gene editing tool Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems and CRISPR-associated (Cas) enzymes have demonstrated enormous potential in the treatment of genome-based disorders, particularly cancer, due to their great precision and efficiency. CRISPR-Cas systems can find fresh targets for brand-new prospective anti-cancer therapies thanks to their capacity to purposefully activate or repress genes. The mechanisms of the various kinds of CRISPR-Cas systems are outlined in this paper. This study also covers a number of additional uses for CRISPR-Cas systems in the realm of cancer, including as diagnostic and therapeutic uses.

Keywords: CRISPR/Cas9; genome editing; Cancer, vectors, onco genes, cancer treatment

Dedication

Dedicated to my beloved Parents

Acknowledgment

All praise and glory to Almighty Allah (SWT) who gave enormous courage, knowledge, wisdom and patience to carry out and complete this thesis. Peace and blessing of Allah be upon last Prophet Muhammad (Peace Be upon Him).

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Abbreviations:

AAV Adeno-Associated virus

ABL1 Abelson Murine Leukemia 1

ALK Anaplastic Lymphoma Kinase

APC Antigen Presenting Cell

AV Adenovirus

AuNP Gold Nanoparticle

B-ALL Adolescent B Lymphoblastic Leukemia

BCR Breakpoint Cluster Region

BL Basal-Like

CAR Chimeric Antigen Receptor

CARF CRISPR-associated Rossman fold

CDK Cyclin-Dependent Kinase

CDKO CRISPR-based Double Knock-Out

CIMP CpG Island Methylator Phenotype

CIN Chromosomal Instability

CPP Cell-Penetrating Peptide

CRC Colorectal Cancer

CRISPR Clustered Regularly Interspaced Short Palindromic Repeats

CRS Cytokine Release Syndrome

CSC Cancer Stem Cell

CTL Cytotoxic T Lymphocyte

CTLA-4 Cytotoxic T lymphocyte-associated Antigen-4

CrRNA CRISPR RNA

DETECTR DNA Endonuclease-Targeted CRISPR Trans Reporter

DLBCL Diffuse large B-cell lymphoma

DMD Duchenne Muscular Dystrophy

DNMT DNA Methyltransferase

DSB Double-Strand Break

EBV Epstein–Barr Virus

EFGR Epidermal Growth Factor Receptor

ER Estrogen Receptor

ERK Extracellular-signal Regulated Kinase

GM-CSF Granulocyte-Macrophage Colony-Stimulating Factor

GO Graphene Oxide

HBV Hepatitis B Virus

HCC Hepato-Cellular Carcinoma

HCMV Human Cytomegalovirus

HCV Hepatitis C Virus

HD Hydrodynamic Delivery

HDAC Histone Deacetylase

HEPN Higher Eukaryotes and Prokaryotes Nucleotide-binding

HER2 Human Epidermal Growth Factor Receptor 2

HGF Hepatocyte Growth Factor

HHV-8 Human Herpes Virus-8

HLA Human Leukocyte Antigen

HO-1 Heme Oxygenase-1

HPV Human Papilloma Virus

HSPC Hematopoietic Stem and Progenitor Cell

HTLV-1 Human T-lymphotropic Virus 1

HUDSON for Heating Unextracted Diagnostic Samples to Obliterate Nucleases

KIKO Knock-in and Knock-out

KSHV Herpes Virus associated with Kaposi’s Sarcoma

LNP Lipid-based Nano Particle

LV Lentivirus

MAPK Mitogen-Activated Protein Kinase

MCPyV Merkel Cell Polyoma Virus

MELK Maternal Embryonic Leucine Zipper Kinase

MET Mesenchymal Epithelial Transition

MFN2 Mito fusion 2

MHC Major Histocompatibility Complex

MSI Microsatellite Instability

NSCLC Non-Small Cell Lung Cancer

ORF Open Reading Frame

PAM Protospacer Adjacent Motif

PD Programmed Cell Death

PDX Patient-Derived Xenograft

PEG Polyethylene Glycol

PEI Polyethylene Imine

PR Progesterone Receptor

RNAi RNA interference

RNP Ribonucleoprotein

RPA Recombinase Polymerase Amplification

SCLC Small Cell Lung Cancer

SHERLOCK Specific High Sensitivity Enzymatic Reporter UnLOCKing

SLICE sgRNA Lentiviral Infection with Cas9 protein Electroporation

TALEN Transcription Activator-like Effector Nuclease

TCR T-Cell Receptor

TIL Tumor-Infiltrating Lymphocyte

TKI Tyrosine Kinase Inhibitor

TME Tumor Microenvironment

TNBC Triple-Negative Breast Cancer

TNF Tumor Necrosis Factor

TNM Tumor-Node-Metastasis

TSG Tumor Suppressor Gene

TracrRNA Trans-activating CRISPR RNA

VEGF Vascular Endothelial Growth Factor

WHO World Health Organization

ZFN Zinc-Finger Nuclease

lncRNA long-coding RNA

siRNA small interfering RNA

Chapter 01

1.1 Introduction

Cancer has permeated the globe because it is more competent and developing proclivity to metastasis to diverse tissues via unregulated multiplication. The etiology of these kinds of diseases or conditions is exceedingly complicated. Yet, environmental and hereditary factors remain the main two risk factors among the numerous risk factors that perpetuate the disease or condition. Inactivating cancer progression suppressors, upkeep of proliferative signals, avoidance of cell death, the extension of cell cycle life, support of angiogenesis, and facilitation of metastasis are the primary causes of carcinogenesis and tumour progression (Kozovska et al., 2021). Accurate investigation and identification of the underlying reasons can greatly aid scientists and doctors in developing the most effective treatment techniques. They have presented various new hallmarks, such as mutation on genes, instability of genes, inflammation, evasion of immunogenicity, unregulated physiological energetics, and tumour heterogeneity in some cells, and offered an extended detailed investigation of their prior features (Rahman & Tollefsbol, 2021).

As science and medicine have progressed, small ligands or molecules and antibodies have been developed to interfere with the cell signalling of different types of carcinogenic genes. There are some approved treatments, such as Sacituzumab govitecan, which is an antibody-drug conjugate that targets anti-Trop-2 mAb (hRS7) protein for HER2 breast cancer (X. Li et al., 2020), other types of treatment; anticancer agents like Paclitaxel which prevents microtubules from becoming disassembled and keep the polymer together, however, many alternative therapy alternatives are still inconsequential whenever it comes to offering effective therapies or fighting resistance pattern due to a lack of in-depth understanding in cancer (Barbour et al., 2021). Numerous genome-editing methods have been developed as an evolving part of modern technology, and they may now be used to generate genetic maps of phenotypes for a wide variety of disorders. ZFN and TALEN are two of the several genome editing tools that may target and alter genomic regions. Due to the necessity for customized modification of every targeted DNA, these techniques were arduous and expensive(Meeske et al., 2020). The development of exceptionally adaptive genome-editing approaches in current times has enabled scientists to quickly and economically insert particular sequence modifications into the genomes of many cell types. A natural adaptive immune defense

system of bacteria; CRISPR/Cas (clustered regularly interspaced short palindromic repeats) is one of the cutting-edge genome editing tools in the genetic sector due to its wide acceptance and success stories(Wang et al., 2021). The CRISPR/Cas system has revolutionized every part of bioscience, from its unanticipated discovery as an adaptive immunity response from bacterial origin to being one of the most efficient gene-editing tools with a wide variety of uses. Cancer treatment is one of the most significant areas of CRISPR technology, and this technique is very effective in cancer research and treatment strategies because it may be used to induce genetic, transcriptional, and epigenetic alterations and assess the subsequent unhealthy phenotype (Ghorbani et al., 2021).

1.2 Aim of the study

The main goal of this study is to explain how important CRISPR/Cas is; as an efficient and valuable genome editing tool for diagnosing and treating cancer. Notably, the review wants to find out what causes tumours to grow and what treatments are already available. It also wants to understand how the CRISPR/Cas tool works in genome editing, explain its uses and different ways to deliver it, show how CRISPR-Cas systems have helped diagnose different types of cancer, and discuss about its restrictions and potential future opportunities.

1.3 Justification for the Study

Genome editing has been proposed as the cancer treatment industry's upcoming big thing. Further revolutionizing cancer study, diagnosis, and treatment, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems technology will play a very significant role. CRISPR systems are accessible, flexible, and inexpensive compared to other genome editing technologies like ZFNs and TALENs(Sánchez-Rivera & Jacks, 2015). Genomic editing at several chromosomal target sites is made possible by the various CRISPR-Cas systems, which have implications for the treatment of a broad range of genetic diseases. This review discusses the function and applications of several CRISPR technologies. The role of CRISPR in cancer diagnosis and treatment, as well as various CRISPR/Cas delivery strategies, including vectors and other physical or chemical processes, are also addressed. In closing, this study highlights its significance in cancer and its potential for treating various cancer types.

Chapter 02

Methodology

The pioneering CRISPR/Cas technology and its recent revolutionary implications in the diagnosis and treatment of cancers are discussed in this study. Original primary and secondary research publications indexed in databases like, PubMed, Scopus, Springer and Science Direct were used to assemble all the information and material for this comprehensive study. Journals such as Frontiers, Nature, Nature Biotechnology, Immunity, Science, etc., were mined for publications for this review. The papers that met the inclusion criteria were evaluated and analyzed for content. There are additional references to the articles mentioned at the conclusion of the article. Before the final draft of the study was submitted, identical materials were also deleted by hand. The purpose of this study is to provide a concise summary of recent research on the use of CRISPR/Cas technology for cancer diagnosis and therapy.

Chapter 03

3.1 CRISPR/Cas Technology

Specific acquired immunity against abnormal mutagenic genes or genetic elements like plasmids and viruses has been revolutionized by the discovery of clustered regularly interspaced short palindromic repeats (CRISPR) in Escherichia coli and CRISPR related proteins (CRISPR/Cas) from genes neighboring to CRISPR sequences as a responsive prokaryotic immune response(Fuziwara et al., 2022). Originating in the Escherichia coli genomes and discovered in 1987, CRISPRs are made up of 29 nucleotide repetitive sequences that are divided by 32 sequences of a nucleotide molecule known as Spacers(Miller et al., 2017). Only when a phage DNA emerges and comes into touch with a host cell do these spacer sequences become active. CRISPRs have been detected in archaea and in the genomes of bacteria and archaea with widely varying evolutionary origins; around 84% of CRISPRs are found in archaeal genomes and 45% in bacterial genomes(Brinkman & van Steensel, 2019).

Mechanisms similar to RNA interference (RNAi) mechanisms in eukaryotes are at the basis of how CRISPR/Cas systems protect prokaryotic cells' acquired immune function from foreign genetic material. Lactic acid of bacteria Streptococcus thermophilus, when infected with any bacteriophages, integrates new Spacers sequences from the exogenous nucleic acid segments of viral phage, resulting in a changed resistant phenotype(F. Li et al., 2020). Specific resistance to a certain phage was achieved by editing the CRISPR/Cas system by removing or adding Spacer sequences corresponding to the infecting genetic traits. Consequently, CRISPRs that are structurally similar to this system provide the exact mechanism in developing immunity; a typical CRISPR/Cas system is composed of two parts: a guide RNA (gRNA) molecule containing spacer sequences corresponding to infecting genetic traits or typical genetic region, and Cas enzymes, which are RNA-protein structures operating in dsDNA endonuclease interaction(Zhao et al., 2019).

For the most part, spacer sequences help by providing sequence specific information that may be used to mount a directed defence against the introduction of external genetic material. CRISPR is transcribed and translated into crRNA, a type of CRISPR system RNA, throughout the immune response process. These crRNAs direct the Cas endonuclease enzyme to the corresponding nucleic

acid of unfamiliar DNA so that it may cleave it. These molecular techniques have impressive capabilities in cancer, where they may be used to manipulate the faulty genome transcriptome, epigenome, and immune-associated cells in various ways. Multiple clinical trials using modified T-cells (allogeneic or autologous) for immunotherapy have used some of these approaches. Targeted gene selection, delivery techniques, and CRISPR/Cas techniques improvement without unintended off-target consequences are essential for the CRISPR/Cas systems to be clinically applicable in cancer treatment(Wegner et al., 2020).

There are three phases to the processes of the bacterial CRISPR/Cas system: adaptation, maturation (crRNA-expression or pre-CRISPR RNA) and interference. In the adaption phase, a complex combination of Cas proteins is expressed through the CRISPR/Cas loci; the Cas proteins then attach to the particular sequences of the DNA, resulting in duplet double-strand breakage. The Protospacer adjacent-Motif, a small sequence of two to four bases, is crucial for Cas protein identification of their target areas (PAM). Once the double helix is broken, a fragment of targeted DNA called a protospacer is generated and inserted between dual repeat regions in the CRISPR assemble, where it functions like a spacer-sequence for only that targeted DNA. After the CRISPR array regions have been produced, a single lengthy pre-crRNA is transcribed and develops into crRNA with the support of Cas protein molecules and other auxiliary components(Lentsch et al., 2019). Mature crRNAs have spacers that are virus-specific and duplicate sequences on either side. During the interference phase, the developed crRNA acts as a guide RNA, identifying comparable patterns in the entering chromosomal RNA, like the virus-associated RNA, and permitting breakage and silencing by Cas proteins, defending the host cells from the penetrating disease. Every CRISPR/Cas system has its own distinct expression as well as interference phases. Two spacers from similar mobile genetic components may be incorporated into the adaptable and hereditary defensive measure of prokaryotes using Type I-E, II, and III CRISPR/Cas systems (Figures 1 & 2). When the CRISPR system does not have enough knowledge about its intended target, naive acquisition happens(Carusillo & Mussolino, 2020).

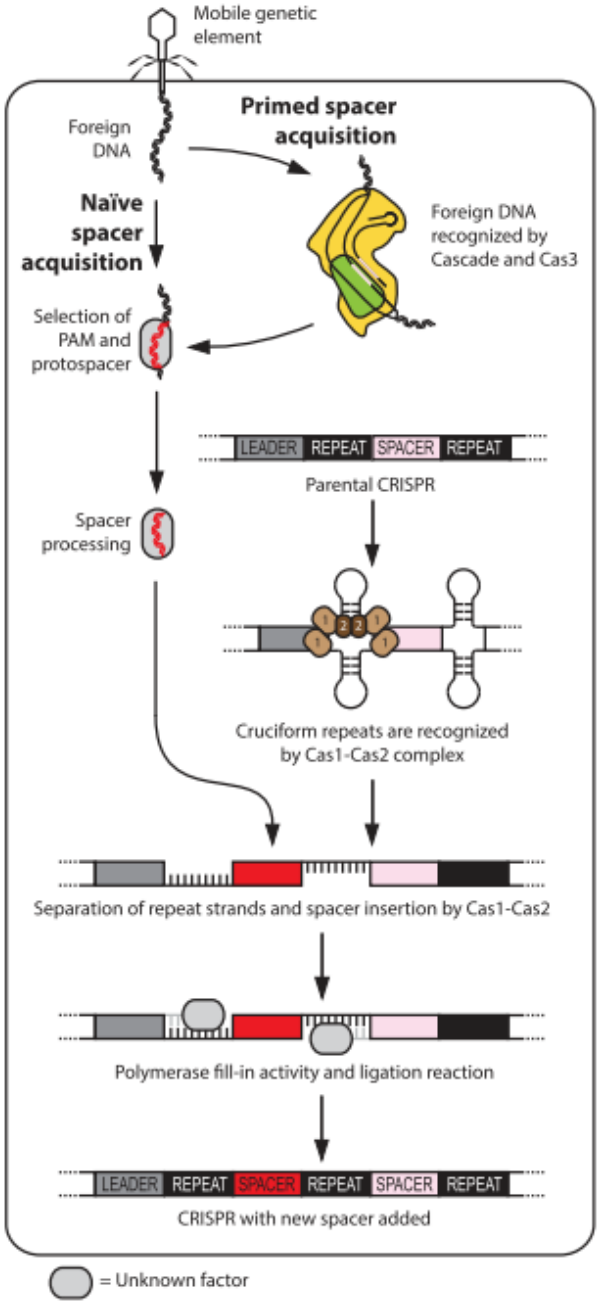

Figure 1: Illustration of the adaptation mechanism in the Type I-E system. There are two types of acquisition phase: primed and naïve, both requiring a PAM (Protospacer Adjacent Motif) and being dependent on Cas1-Cas2 complex. The Cas1-Cas2 complex is responsible for CRISPR array recognition and is known to prepare it for spacer integration. Primed acquisition requires CRISPR locus spacers that match the target DNA and a Cascade and Cas3 complex, wherein multiple19

spacers from the same mobile genetic element can be integrated. Naïve acquisition occurs when there is a lack of information regarding the target in the CRISPR (Adapted from Ratan et al., 2018).

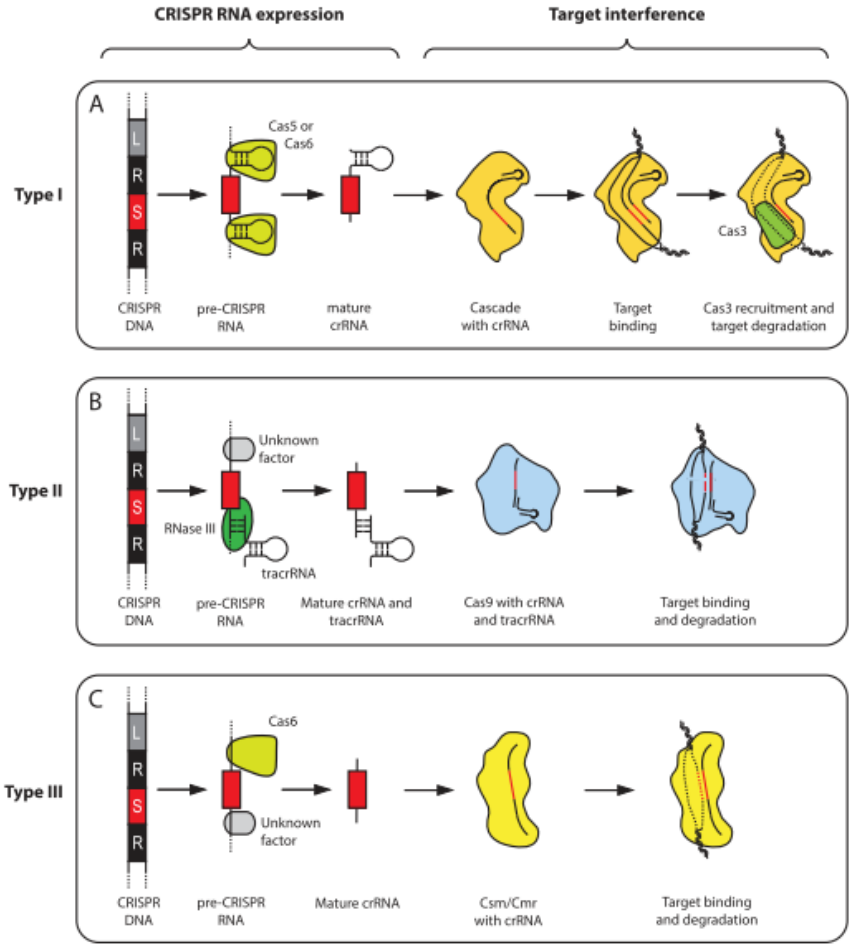

Figure 2: Illustration of the crRNA processing and interference. (A) In the Type I system, Cas5 or Cas6 is used to process the pre-crRNA. Moreover, the target interference utilizes Cas3 proteins including the Cascade and crRNA. (B) In the Type II system, RNase III and tracrRNA are used for processing the crRNA. An additional unknown factor that can perform 50 end trimming is also utilized. For Cas9, the DNA targeting is done in a crRNA-guided manner. (C) In the Type III system, Cas6 and an additional unknown factor capable of performing 30 end trimming is used for crRNA processing. Here, Type III Csm/Cmr complex is used to target the DNA, including RNA in some cases (Adapted from Ratan et al., 2018).

Although spacer and repeat lengths may vary between CRISPRs at the similar or separate genomes, they are highly conserved and unique within a given particular CRISPR locus(Bender et al., 2021). Variable in length from 23 to 55 nucleotide bases, CRISPR system repeat sequences all have palindromic sequences that fold into hairpin shapes. Similarly, the length of spacers ranging from 21 to 72 nucleotide bases. CRISPR systems are a versatile tool for genetic modification due to the wide availability of Cas proteins that can attach to nucleic acids. CRISPR/Cas methods are more user-friendly, quicker, and economical than their predecessors like ZFN and TALEN, and CRISPR has a greater targeting effectiveness that results in less negative effects than those technologies(Kong et al., 2021). As a result of their ability to detect a 3 to 4 base pair based DNA, ZFNs are able to utilize site-specific tracking to create DSBs at precise locations due to its zinc finger protein sequence that helped to get amalgamated nuclease enzyme forming methods. Since the FokI nuclease resides as a dimer, DSBs are possible. Similar to ZFN in structure and dimerization, TALEN is able to recognize particular DNA base pairs by use of TALE proteins, but with more precision(Akhtar et al., 2021). Off-target mutations are a limitation both of the ZFNs and TALENs. In addition, each time a unique sequence in the particular DNA is desired, a unique protein must be synthesized.

CRISPR/Cas technology namely CRISPR-Cas9 comprising the Cas9 enzymes, only require modification in the short-sequence of gRNA for site-specific breakage, in contrast to TALENs and ZFNs. Incorporating RNA changes directly into the gene code increases its efficiency. Because numerous gRNAs may be inserted into the CRISPR system simultaneously, the method makes it easy to simultaneously modify many genes. It is because many gRNAs may be designed for a single target thanks to the targeted sequences often located directly ahead of the PAM-sequence, which are short regions that exist once per eight base pairs. In addition, while TALENs and ZFNs use a protein-DNA-binding mechanism, where CRISPR/Cas9 systems recognize DNA via Watson-Crick complementary base(Akhtar et al., 2021).

Human CRISPR genome editing, whether in-vivo or in-vitro, requires many tools and two major repair mechanisms to successfully insert or delete genes at the desired location. CRISPR-Cas9 is one of the most commonly employed systems in 21 treating numerous human illnesses, and its process consists of three main steps: identification, cleavage, and repair, as described by Mengstie and Wondimu (2021). Using Watson and Crick base pairing, the developed specific particular

single gRNA (sgRNA) recognizes the target site sequence. The 5'-NGG-3' PAM motif activates Cas9, which breaks down proteins the site of action 3 bp ahead of the PAM sequence to generate DNA double-strand fractures (DSBs). Cas9's HNH domain pierces the complimentary thread of the targeted genome, whilst the RuvC region splits the non-complementary strand(Chandrasekaran et al., 2022).

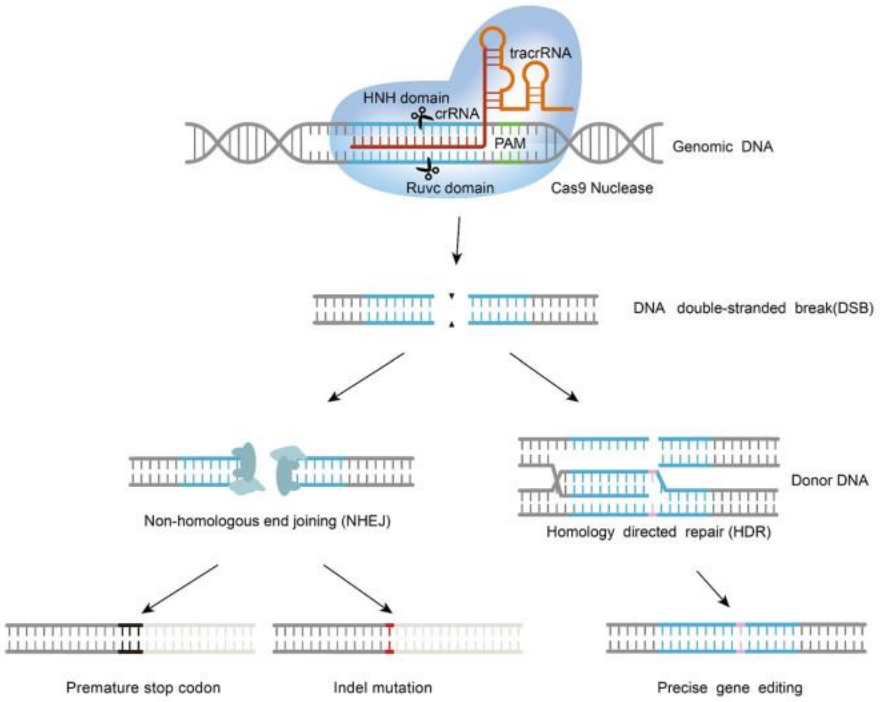

Figure 3: Mechanism of action of the CRISPR-Cas9 system. The sgRNA guides the Cas9 endonuclease to the target site and binds to the complementary DNA region upstream of the PAM sequence by forming a Cas9-sgRNA or RNP complex. The nuclease domain of the Cas9 cleavesthe double strands at the target site which results in the formation of DSBs. These DSBs are repaired by either non-homologous end joining (NHEJ) pathway whereby indels are inserted into the site for gene knockouts, or by homology-directed repair (HDR) pathway whereby donor sequences are knocked into the site with the help of a donor DNA template (Adapted from Moses et al., 2018)

In higher eukaryotes, double-strand breaks (DSBs) are corrected by either nonhomologous end joining (NHEJ) or homology-directed repair (HDR). Besides allowing for genetical mutations to be induced without the need for an external homologous pattern, the NHEJ pathway's enhanced activity during 90% of the cell life also allows for the emergence of spontaneous deletions or

insertions (indels) in the target location, resulting in a greater in the frequency of these mutations. Due to the potential for additional frameshift alteration or prematurely stop codons to occur as a consequence of these indels, the targeted gene may be rendered inactive(van Dongen et al., 2020). Since this system has the ability to induce failure mutations, it is well-suited for applications that require immortalized ceill-lines, such as cancer therapy. Using an exogenous homology repair pattern, the HDR method can deliver extremely precise alterations at the specific location, such as gene implantations or substitutions. The cell cycle's late S and G2 phases are when this mechanism is most prominent. This means that NHEJ and HDR can cause the precise disruptions and incorporation of genes that are needed. Figure 3 depicts a schematic of the CRISPR-Cas9 system and its main workings (Kwon et al., 2022).

3.2 Classification of CRISPR system

Classifying CRISPR/Cas genome editing system according to their gene sets and repeat sequences reveals a wide range of diversity. Cas9, Cas12a, Cas13, and their orthologues are only a few of the many distinct nucleases that may be utilized to specifically cut off certain sections of DNA. Different systems' focused cleavage is a result of distinct structures of effector module incorporating distinct Cas proteins. Class I and class II are the two classes of CRISPR/Cas systems. Class I includes Types I, III, and IV, whereas Class II has Types II, V, and VI. Each of these 6 main categories is subsequently broken down into further 33 subcategories(Xu et al., 2021). All of these Cas proteins are characterized by the presence of helicase domains. nuclease motifs and RNA-recognition motifs that are specific to each protein. Up to this point, Cas 1 and Cas 2 have already been ubiquitous nucleases across a wide range of genome editing systems, while Cas3, Cas10, and Cas9 have been isolated to just a few distinct categories. The selectivity and efficacy of this types of systems can be affected by a wide variety of parameters, including secondary structure, gene sequence, and gRNA length. The efficacy of CRISPR/Cas systems also depends on factors like as the targeted gene locus, the accessibility and permeability of chromatin, the number of nucleosomes, the gRNA sequence region, and the components surrounding the binding sites. The specificity and selectivity of a gRNA is determined by its ability to attach to the region of the targeted gene; this binding takes place between the PAM region and the first 10 to 12 nucleotides of the gRNA at the 3' end. Table 1 provides a quick summary of the features of several CRISPR/Cas systems(Karimian et al., 2019).

A crRNA and numerous protein sub-components make up the ribonucleoprotein (RNP) complexes in a class I system, whereas a crRNA and a single protein subunit make up the RNP complex in a class II system whilst targeting the virus RNAs. Class II - Cas9 proteins digest pre-crRNA in conjunction with tracrRNA and RNase III, on the other hand class VI Cas13 proteins and type V Cas12 proteins do this task alone. Cas12a and Cas12b proteins break double-stranded targeted DNA upon identification by the matured crRNA, where Cas13 fragments the corresponding ssRNA. Cas13 proteins can work with or without PAM in the targeted RNA sequence, whereas Cas12 proteins need it when working with dsDNA. Complimentary interaction between the target RNA and the crRNA activates Cas13, leading to the degradation of the collateral ssRNA. Because of this trait, RNA viral infections may be detected and treated(Shi et al., 2021). Cas12 proteins, on the contrary, exhibit comparable action, enabling the identification of ssDNA viruses. As initiated before, all CRISPR/Cas systems have similarities during the adaption stage, but each has its own quirks throughout the expression and inhibition phases. Using Cas proteins and auxiliary factors, a CRISPR array comprising intruding spacers are transcribed and matured into crRNA, which is then integrated into RNP complex. RNP complexes are responsible for recognizing and binding to crRNA-encoded sequence complements in cellular nucleic acids. In response to this identification, the interference phase is triggered, leading to the degradation of the detected nucleic acid.

Table 1: Characteristics of the different types of CRISPR-Cas systems (Adapted from Song et al., 2021 and Xu & Li, 2020)

CRISPR/CAS System		**Subtypes**	**Effector Protein**	**Nuclease Domains**	**Target**	**PAM Requirement**	**tracrRNA**
Class 1	Type 1	A, B, C, D, E, F	Cas3	HD	DNA	-	Not Needed
		A (Csm), B (Cmr), C	Csm3, Cmr4	Autocatalytic	RNA	-	Not Needed
	Type 3		Csm6, Csx1	HEPN	-	-	Not Needed
	Type 4	D	Cas10	HD	DNA	-	Not Needed

		A, B	Csf1	-	DNA?	-	Not Needed
Class 2	Type 2	A, B, C	Cas9	RuvC, HNH	DNA	High	Needed
	Type V	A (Cpf1), B (C2c1), C (C2c3), D (Cas Y), E (Cas X)	Cas12	RuvC	DNA	Medium	Needed by subtypes B and C
	Type 6	A (Cas 13a), B, C, D	Cas13	HEPN	RNA	Low	Not Needed

3.2.1 Type 1 system

Cas3 proteins, which are members of the Type I system, have DNase domains and both helicase and contribute to target degradation in several ways. All Type I systems, with the exception of Cas1, Cas2, and Cas3, generate a Cascade-like complex; the amount of Cas nucleotides varies between Type I to A and Type I to F. Nevertheless, the Cas3 is an integral element of the Type I-A system's cascading complex(D. Zhang et al., 2022). After crRNA synthesis is complete, the cascades aid in the interaction of crRNA and the identification of targeted genetic information. Additionally, in some forms, it improves spacer absorption as well. In type I systems, the pre-crRNA is mostly processed by Cas6-like endonuclease to generate matured crRNAs bordered by a brief 50 tags. This is true for the vast majority of variants or sub-types. When it comes to incorporating foreign spacers into to the host genome, the CRISPR arrays of type I ability to control PAM and depends on the cooperation of many Cas protein subunits. The effector Cas enzyme is a helicase-deficient (HD) nuclease that lacks a target-binding-critical Cas3 helicase domain. During the Type I system interference phase, the crRNA directs the cascading nucleolytic activity by attaching to the external targeted DNA on a sequence region-specific foundation and recruiting Cas3 for destroying any of the relocated strands via exonuclease activities. In order to disseminate novel spacer uptakes, the type I-F CRISPR-Cas system needs

interfering machinery, meanwhile the type I-B subtype just needs Cas1 and Cas2 for adaptability. However, the pre-crRNA is digested by the encoded Cas5d in type I-C systems since they do not encode for Cas6 enzymes(D. Liu et al., 2020).

3.2.2 Type 2 System

Cas1, Cas2, Cas9, and sometimes Csn2 or Cas4 are the proteins encoded by the Type II system. There are three subtypes of the Type II system: II-A, II-B, and II-C. Type II-A and Type II-B systems share the CSN2 and CAS4 genes, which generate proteins involved in the adaptation process, but the Type II-C system lacks the fourth gene. Cas9, the hallmark protein molecule, plays a role in adaptation by helping to digest crRNA and fragment the targeted DNA with the assistance of tracrRNA. CRISPR/Cas9 has greatly accelerated the creation of innovative research and biotechnological tools. The fact that only one Cas protein is needed for genome-editing contributes to its ease of use. Spacer regions help in targeting, with Cas9 enzymes managing spacer recruitment and phage resistance. At least three components make up the type II systems. These are the Cas endonucleases, the matured crRNA, and the tracrRNA(W. Xue et al., 2014). There are numerous Cas protein loci throughout the various subtypes, but only Cas9 has been shown to have endonuclease function. During the process of adaptation, the CRISPR array is bordered at one end by repeats from an invadinthe invasion of virus or plasmid. The CRISPR arrays then undergo pre-crRNA transcription during the expression stage. In order to generate matured crRNAs that are coupled to Cas9 and tracrRNA, RNase III is used to cleave the hybridization crRNA-tracrRNA and remove the 5′ end of each spacer region during the interference step. Cas9's crRNA instructs it to activate its endonuclease function and nick the desired DNA sequence. With the aid of PAM in brief trinucleotide identification, CRISPR uses Watson and Crick base matching to connect to the corresponding target DNA. Without PAM, the CRISPR microarray will not be able to identify the target gene that is complimentary to the crRNA, and as a result, CRISPR will entirely ignore these regions(Wu et al., 2020). R loop creation between the the Cas9 and target DNA enables the two strands to split upstream of PAM. Evidence from a single research suggests that the degree of similarity between the PAM-distal targeting and the 5' crRNA region is more important than Cas9 engagement alone in optimizing the nuclease catalytic performance. Cas9's ability to catalyze the breaking of the targeted DNA strand relies on two different endonuclease regions, the HNH and the RuvC/RNase H-like regions. The double-stranded DNA-breaking Cas9 is transformed into a nickase by the HNH nuclease region primarily slicing the targeted strand corresponding to the

crRNA spacer region, while the RuvC-like domain required to perform non-target strand. Additionally, the Cas9 endonuclease fixes the ends after chopping the virus or plasmid DSB by nonhomologous end rejoining, which might result in frameshift changes due to deletions or insertions. A guide RNA scaffold made from crRNA and tracrRNA is used to prompt site identification prior to the targeted DNA breakage. Building a system consisting of Cas9 and a guiding RNA scaffold into which a 20-nucleotide-long targeted region designed alongside a 5′-NGG-3′ PAM sequence may be incorporated is necessary for therapeutic uses of CRISPR-Cas. Off-target breakage may be avoided in CRISPR/Cas systems by carefully designing them to be as particular as possible. The CRISPR/Cas9 systems have the ability to trigger either the stimulation or repression of a target gene. Genetic variations at Cas9's spacer region render nuclease sites like RuvC and HNH inactive, protecting DNA that has been coupled to Cas9. The resultant Cas9 protein, known as deceased Cas9 (dCas9), binds to the target gene region but lacks the cleavage capacity of wild-type Cas9. These dCas9 are capable of forming dCas9 protein interactions, which attach to transcription sites and modulate activator-like stimulation or repressor-like repression of the targeted gene, respectively, in conjunction with proteins such as transcriptional activators or repressors. Together with other proteins, like as TET1 and p300, epigenetic manipulation of chromatins is possible using Cas9 enzymes(W. Liu et al., 2021).

3.2.3 Type 3 System

There are four subtypes of the Type III CRISPR-Cas system, all of which fall under class I: A (Csm), B (Cmr), C, and D. Cas10 proteins with a palms domain as well as an HD-type nuclease region is characteristic of this mechanism. Cyclase activities inside the palm domain is absent in type C systems, while type D systems lacking an HD domain region altogether. The RNP complex in such a situation is comprised of 2 filaments, one of which is composed of 6 Cas7 members and the other of which is made up of 3 subunits of a Cas11 homologue, either Csm2 or Cmr5. Cas5 and Cas10 proteins cap the 5' end of the crRNA molecule that encloses these filaments, providing space for the string handles. This system typically has two main phases throughout its development or expression phase. Upon exposure to Cas6 enzymes, the pre-crRNA transcript is cleaved into a single spacer region. Cas6 variations are involved in crRNA digestion in a subset of type III settings, wherein secondary processing generates length-diverse crRNA protein variations. One unique aspect of this mechanism is that it possesses 3 different nuclease functions. The first step is the breakage of the target RNA inside the RNP, which is facilitated by cas7 proteins accelerating

the breakage at region-specific locations(Luthra et al., 2021). This 'ruler' mechanism generates RNA fragments 6 nucleotides in length and is unique to type III systems. It was shown that incorrect pairing between the targeted genome and crRNA had no effect on nuclease function. The HD region of Cas10 proteins is a metaldependent DNase that is necessary for the nucleases' second function, which is the non-specific ssDNA breakage of protospacer sequences (PSSs). The HD region of the Cas10 enzymes inside the RNP complex is initially exposed to the antisense DNA strand by the Polymerases of RNA, initiating transcription. The activation of the Cas10 protein is reliant on the initial homology between the targeted RNA as well as the crRNA. Inactive Cas proteins result from mismatches between the 3' targeted RNA protospacer domain and the 5' end of the complementary crRNA. This safeguard prevents the host's CRISPR array from becoming accidentally targeted and severed. As a final step, nuclease activities degrade RNA without selecting for specific sequences. The palm domain causes ATP to be converted into cyclical oligoadenylate, that activates Csm6 or Csx2 enzymes, as Cas10 proteins break the desired location. Nonspecific RNA breakdown, like ssDNA breakage, is triggered by the attachment of an RNP complex to the targeted RNA in the absence of compatibility between both the crRNA and the targeted RNA(Ghaffari et al., 2021). The CRISPR-associated Rossman fold (CARF) at the N-terminus of Csm6 or Csx2 in RNP aggregates recognizes the cyclical oligoadenylate generated by Cas10, and the greater eukaryotes as well as prokaryotes nucleotide-attaching (HEPN) region at the C-terminus breaks the RNA. This multiple nuclease activity may be useful since it might trap the evasive variants before they can spread.

3.2.4 Type 4 system

The CRISPR/Cas loci in Acidithiobacillus ferrooxidans ATCC 23270 is representative of the Type IV CRISPR/Cas that are present in a number of bacterial genome sequences, most commonly on plasmids. This method, like subtype III-A, often does not include CRISPR arrays and missing the cas1 and cas2 proteins. In fact, no other CRISPR/Cas systems are present in the genomes of several bacteria, making this one the unique CRISPR/Cas system in those organisms. The effector complex of the type IV systems a highly reduced big subunit (csf1), two genomes for RAMP enzymes of the Cas5 (csf3) as well as Cas7 (csf2) families, and, in certain circumstances, a protein for an anticipated complementary strand. This system may have a hallmark gene, and it appears to be csf1(Falzone et al., 2021). A DinG family helicase, csf4, is present in one subgroup of type IV complexes, whereas the other category, which excludes DinG but often has a genes for a tiny

alpha-helical enzyme, likely a tiny subunit, is present in the other. Like type III systems, type IV CRISP/-Cas systems would be portable components that, once accessible, could use crRNA from a variety of CRISPR arrays. We cannot rule out the possibility of other processes, like the synthesis of crRNA straight from foreign RNA, without the insertion of spacers in CRISPR cassettes.

3.2.5 Type 5 system

Cas12a enzymes also referred to as Cpf1, are the most well-known components of the Type V system. A (Cpf1), B (C2c1), C (C2c3), D (Cas Y), and E are some of the subgroups that make up the system (Cas X). Breaking the targeted gene and the PAM region upon DNA absorption causes a staggering end when using Cas12a as a single cRNA-guided protein, as opposed to the blunt endpoints generated by Cas9. Cas12a enzyme variations execute pre-crRNA autonomously, targeting a broad variety of loci upon that targeted DNA thanks to their ability to recognize and bind to certain PAM regions. Due of the diversity of malignancies, this has exciting implications for cancer treatment. And because ssDNA has a non-specific breakage capability, the CRISPR/Cas12a methods may be used to show how they can be put to use in the identification of the viral DNA. Cpf1 performs breakage via a T-rich PAM and a RuvC-like region. As a result of an alteration, the Ruv-C-like region loses all of its functions(Rosenblum et al., 2020). Cpf1 is distinct from Cas9 in several key respects, including its ability to mature crRNA independently of tracrRNA, its ability to generate staggered DSBs in DNA, and its ability to cleave target DNA just next to a short rich PAM.

3.2.6 Type 6 system

The major Cas enzyme of the type VI system that focuses on the invasion of RNA is called Cas13a or C2c2. Cas13a, upon engaging its targeted RNA, initiates collateral breakage at non-target RNAs. This system's capacity to regulate both coding, as well as non-coding RNA components, has been established in biomedical contexts, such as the identification of particular viral RNA regions and tumour RNA in sufferers. However, certain Cas enzymes are transformed into nonspecific ssRNase or ssDNase proteins, breaking any ssRNA or ssDNA together with targeted nucleic acids, once the matured crRNA identifies and attaches to the corresponding targeted sequences near the surrounding PAM repeats(Rahimi et al., 2019). The structural shift of the RNP complex is also triggered by the interaction of crRNA and the target RNA. This causes the two HEPN domains to coalesce into a single catalytic unit. Breakage of ssRNA and even

ssDNA occurs with the breakage of the targeted nucleic acid when this enzymatic site is located close to the crRNA-target RNA or DNA adjunction. Collateral breakage is the off-target cleavage that occurs without intent and can be used for in vitro identification of nucleic acids. Additionally, the safety feature of this system inhibits activation by the host's RNA. To edit DNA, type VI CRISPR-Cas systems need only the Cas13 protein and crRNA, much as type V systems. There are four forms of VI: VI-A (also known as Cas13a or C2c2), VI-B (also known as Cas13b or C2c6), VI-C (also known as Cas13c or C2c7), and VI-D. (Cas13d). Each of the Cas13 subtypes is a variation that differs primarily in size and the sequence it targets, but all of them have a common trait of two HEPN domains(Onishi et al., 2021).

Chapter 04

4.1 Cancer and its prevalence

Cancer is the second largest disease causing the death all over the world following the cardiovascular diseases. The environment, Genes, the immune system, and other variables all contribute to cancer's varying degrees of lethality. Different parts of the body have unique conditions that might promote the growth of cancer. However, the exact pathways that contribute to cancer formation are unknown. When combined, these characteristics make cancer detection and therapy more difficult. Identification at the earliest possible stage is essential for effective treatment for cancer. Survival rates of previously doomed patients can be increased by early diagnosis (Kim et al., 2021). Cancer can be treated with a variety of techniques, including chemotherapy, radiation treatment, and surgery. However, no individualized treatment plan has been offered for individuals whose cancer has persisted after radiation, chemotherapy, or who are no longer surgical candidates due to the disease's advanced stage. Thanks to recent discoveries in molecular genetics and immunology, individualized cancer treatments may now be developed. However, understanding the mechanisms of cancer resilience that lead to therapy failure is essential. As such, new approaches to treating cancer are needed worldwide. DNA repair genes, Tumor suppressor genes, and proto-oncogenes are the primary targets of genetic manipulation during carcinogenesis and its progression. Unchecked multiplication of otherwise healthy cells occurs when a cascade of mutations simultaneously alters the functions of these three classes of genes. Environment chemicals with carcinogenic properties have a significant immediate or indirect effect on the cell, contributing to alterations of the gene, and hence genetic abnormalities, reinforcing these occurrences(Gong et al., 2021). Many variables, including genetics, the environmental factor, and gene-environment interactions, work together to cause mutations in the human genome. Approximately 7% of all cancer cases can be attributed to environmental risk variables as viruses and radiation bacteria.

Proto- oncogenes are proteins that play a role in maintaining a regular cell cycle but can be turned into oncogenes when exposed to mutagenesis stimuli. At the same time, the mutation-caused loss of function or deactivation of cancer suppressor genes simultaneously initiates an unchecked growth of cells. DNA restoration genes would normally be in charge in translating proteins and enzymes required to fix such damage, but variations cause so much damage that DNA repair is

overwhelmed by the rate of unchecked replication. Mutation can occur in many different ways, corrupting otherwise healthy DNA region(Gu et al., 2021). Chromosomal translocation causes oncogenes and other genetic anomalies, as in the cases of the Bcr and Abl genomes in chronic blood cancer. Point mutations in the Ras gene of colon cancer, deletions in the Erb-B gene of breast cancer, insertion activation in C-myc of acute blood cancer, and amplifying mutations in N-myc of neuroblastoma are all examples of somatic alterations that may occur in cancer. Furthermore, in 60 percent of total of cancer cases, a variation in the gene p53 is discovered to elicit aberrant proteins that, under normal conditions, would have demostrated a crucial role in controlling cell death, cell division, differentiation, senescence, angiogenesis, and DNA metabolism. DNA mutation is just one type of oncogenic change that can occur; Splicing change, DNA methylation, and posttranscriptional/posttranslational changes also play roles in driving aberrant cell proliferation. Engineered hypomethylation in repeating regions leads to a rise in genetic alterations, which in turn causes chromosomal fragility(Porika et al., 2022). Cancer suppressor proteins MASPIN in prostate and breast cancer, SNCG in ovary and breast cancer, S100P in pancreatic cancer, MAGE and DPP6 in melanoma, and so on are all mentioned as instances of tumorigenesis whose expression is inappropriately induced by hypomethylation. Genetic factors in repair (Hmlh1, BBRCA1), sensitivity to vitamins (CRBP1, RARB2), apoptosis (DAPK1, WIF-1), and cell cycle regulation (P16INK4b, P16INK4a) can all have their transcription inhibited by hypermethylation of certain promoter regions. Since this is the case, they can be used as cutting-edge biomarkers in cancer for the detection and monitoring of cancer.

In 2019, the World Health Organization (WHO) predicts that cancer will become the second biggest reason of death worldwide, affecting people under the age of 70 in more than a hundred different nations (Global Health Estimates: Leading Causes of Death, 2020). As time has passed, the percentage of cancer patients who die in the US has remained relatively constant at around 35% . If appropriate treatments are not found and applied quickly, the presence of cancer is a terrifying menace that will continue to wipe out more individuals. Without action, the number of new cases of cancer is expected to rise from its projected 19.3 million in 2020 to a staggering 28.4 million in 2040. The lowering rates of stoke and heart disease, the expanding average lifespan that has led to larger elderly populations, and other risk factors associated to economic and social progress may all be to blame for this trend. In 2020 alone, there were an estimated 19.3 million

new instances of cancer and 10 million deaths from the disease. Female breast cancer was the most common kind, followed by lung cancer in men and prostate cancer(Choi et al., 2019).

4.2 Types of cancer:

4.2.1 Breast Cancer

When it comes to women, breast cancer is by far the most prevalent and deadly. Multiple categories, each with its own unique appearance, have been identified. The Her2-enriched; Triple-negative/basal-like; luminal A; and luminal B are the four primary molecular subtypes based on the activation of estrogen receptor (ER), progesterone receptor (PR), ERBB2 (HER2), p53, and Ki-67. About 70% of breast cancers are ER-positive luminal subgroups, and 30% of individuals with these cancers develop resistance to endocrine treatments. It is especially important to identify novel therapeutic alternatives in the event of a recurrence. Treatment targets in several kinds of breast cancer have recently been identified thanks to investigations mediated by the genome editing tool CRISPR(Moses et al., 2018).

By 2020, there will be approximately 2.3 million new instances of breast cancer globally, making it the most common disease in the worldwide. Female sex hormones, genetic mutations, pregnancy, menopause/menstruation, obesity, ethnicity, breastfeeding, alcohol, age, smoking, physical activity, family history of cancer or cancerous illness of the breast, chemical or radiation exposed, nadequate vitamin supplementation, processed food, drugs, etc. are all risk factors for the development of breast cancer. Breast cancers are categorized according to the presence or absence of three characteristics: the expression of the estrogen receptor (ER), the amplification of the human epidermal growth factor and co factors receptor-2 (HER2), and the presence or absence of mutations in the BRCA1 and BRCA2 genes. Based on this classification system, there are four subtypes: Luminal A, Luminal B, Basal-like, and HER2-amplified. ER-positive tumors can occasionally develop into subtypes such as tubular, invasive cribriform, invasive micropapillary, invasive lobular, and mucinous carcinomas; they are all referred to as Luminal Breast Cancers. In addition, they indicated that ER-positive, PR-negative, and/or HER2+ luminal B cancers are distinguished from ER-positive, PR-positive, HER2-negative Luminal A tumors by their respective proliferative and luminal regulatory mechanisms. In order to inhibit ER actions or lessen the availability of estrogen receptors for activation, endocrine treatments are applied to ER pathways. However, they are always in need of enhancement owing to developed tolerance.

HER2-enriched breast cancers lack ER and PR expression but show high levels of HER2 genetic expression, which is associated with the production of proteins and genes involved in cell proliferation. Another alteration cluster is produced when a change in APOBEC3B leads to a cytosine instability bias(Dehshahri et al., 2021). The lack of ER, PR, and HER2 expressions, as well as the presence of BRCA1 and/or BRCA2 germline mutations, define basal-like or triple-negative breast cancer (TNBC). The research showed that TNBCs may be further classified into several subtypes, including basal-like (BL1 and BL2), mesenchymal stem-like, luminal androgen receptor, immunomodulatory, mesenchymal, and an unclassified category.

4.2.2 Lung Cancer

When it comes to cancer, lung cancer is by far the most lethal form. More than 85% of lung cancers are found in non-small lung cancer (NSCLC) subgroups. Patients with adenocarcinoma, in particular, benefit from knowing the exact genetic abnormalities driving their cancer so that they can receive the most effective treatment possible. Multiple molecular targets, including ALK-EML4, EGFR, BRAF, and cMET, are now in use for the therapy of NSCLC in clinical settings. But the multitude of molecular mechanisms and the emergence of drug resistance have constrained therapy choices. New therapy options and possible therapeutic options for lung cancer have been discovered thanks to CRISPR/Cas9-based in vitro and in vivo investigations in recent years. At an anticipated 2.2 million new cases and 1.8 million deaths in 2020, lung cancer ranks as the second most common disease worldwide. In terms of morphological appearance, lung cancer is typically divided into two subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)(Maier et al., 2019). Non-small cell lung cancer is divided into subtypes called adenocarcinoma, squamous cell carcinoma, and giant cell carcinoma based on the degree of invasion of the lesions. Mutations in the genes that code for receptor-type tyrosine kinase on cellular membrane and their consequent signaling processes are responsible for the overwhelming majority of lung malignancies. After ligands attach to receptors, the receptors can form hetero- or homodimers, which facilitates signaling for cell development and other processes. Independent activation leads to carcinogenesis when a mutation occurs at exon sites 18-21. Lung cancers acquire resistance to EGFR- TK inhibitors (EGFR-TKIs) like gefitinib and erlotinib by amplifying the MET gene via a secondary amplification mechanism. Mesenchymal Epithelial Transition (MET) is yet another tyrosine kinase antibody onto which hepatocyte growth factor (HGF) linkers connect likely to induce invasion, cell proliferation, and metastasis(Azangou-Khyavy et al., 2020).

Moreover, alterations in the PI3K-AKT-mTOR pathway, the RAS-RAF-MEK pathway, the P16-RB pathway, the P14-MDM-2-P53 pathway, and other pathways have also been linked to lung cancer.

4.2.3 Liver Cancer

Liver cancer is more frequent in men and is the third leading cause of cancer-related mortality worldwide. Hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (IHC) are the two most common forms of primary liver cancer (ICC). It's estimated that about 80% of all instances are attributable to HCC, with ICC accounting for the remaining 15%. In the remaining 5% of cases, unusual forms of liver cancer manifest themselves. The prognosis is dismal for people with liver cancer, and incidence rates are common and treatment choices are restricted. Accordingly, CRISPR/Cas9 technology may be an effective means of finding novel treatment targets in this malignancy. Using CRISPR/Cas9, researchers have explored a number of other possible targets in HCC(Mintz et al., 2018). For instance, hepatocellular carcinoma (HCC) was formed in vivo when Pten was knocked out using CRISPR and Nras was overexpressed. Both Pten and Nras expression aberrations are required for liver carcinogenesis, although none is adequate on its own. Accordingly, inhibiting abundantly expressed NRAS in individuals with PTEN loss may reduce tumor development and increase patient survival in a subgroup of HCCs. Numerous cancers are associated with aberrations in the expression of NCOA5, which produces a coregulator for estrogen receptors (ERs). HCC cell viability and migration were considerably slowed when NCOA5 was genetically knocked out. Furthermore, deletion of NCOA5 protein significantly suppressed EMT, a prevalent treatment resistance mechanism in several cancer types. Deleting Nogo-B with CRISPR significantly reduced cell growth in vitro and suppressed tumor development and distant metastasis in vivo (van Essen et al., 2021). Nogo-B is a negative regulator of apoptosis. All of the abovementioned genes may be good candidates for additional in vivo investigations or clinical trials as inhibitors are produced. One of the causes of HCC is infection with hepatitis B virus (HBV). The CRISR/Cas9 system has therapeutic promise for preventing HBV-derived liver cancer by eradicating viral DNA. Upon entering hepatocytes, the rcDNA found in HBV particles is transformed to cccDNA, or covalently closed circular DNA. All HBV transcripts are derived from cccDNA. Therefore, cccDNA removal, such as via cleaving, may be a curative treatment for HBV. Several HBV-specific preclinical studies have revealed encouraging

findings, which might be useful in the fight to eliminate HBV as a significant risk factor for liver cancer.

4.2.4 Colorectal Cancer

In 2020, colorectal cancer was the fourth most common cancer in the globe after breast and lung. Adenocarcinoma, undifferentiated carcinoma, adenosquamous carcinoma, spindle cell carcinoma, squamous cell carcinoma, and neuroendocrine carcinoma are some of its subtypes. Fo fFod, smoking, alcohol intake, hyper- or hypoglycemia, and blood protein component imbalances as environmental risk factors that might accelerate the development of colorectal cancer. CpG island methylator phenotype, microsatellite instability, and chromosomal instability are the three pathogenic processes that lead to colorectal cancer. They claim that mutations in cell proliferation-related genes such as BRAF, cMYC, PTEN, SMAD2, PIK3CA, the RAS gene family, etc., are common in colorectal malignancies. The binding of ligands to receptor tyrosine kinases like EGFR initiates the mitogen-activated protein kinase (MAPK) signaling cascade, which leads to differentiation and proliferation of cells (Waddington et al., 2016). As a receptor tyrosine EGFR plays a major role in initiating the intracellular signals that drive the growth, angiogenesis, differentiation, and metastasis of several cancer cell types. Resistance to anti-EGFR medicines, such as humanized monoclonal antibodies cetuximab and panitumumab, has been linked to alterations in the Gene encoding, which is part of the RAS-RAF-MEK-ERK signaling pathway. This is why RAS genes are often checked for before beginning anti-EGFR therapy. Mutations in the cancer-preventive APC gene are associated with an extremely elevated risk of colorectal cancer. Numerous genes, including LAMB1, CHDH1, HNF4A, SNP rs60-17342, miR-196a2, and the C allele of SNP rs11614913, increase the risk of getting colorectal cancer.

4.2.5 Cervical Cancer

Cervical cancer, the most frequent form of malignancy affecting the reproductive system, poses a serious threat to the well-being of women and is the fifth most prevalent cancer type in women. In 2012, over half a million people were identified with cervical cancers for the first time, and almost a third of a million people lost their lives to the disease. HPV (human papillomavirus) exposure is linked to cervical cancer. HPV's gene mutations are mostly located in its E7 and E6 genes. Cell cycle arrest is caused by the E7 and E6 proteins interacting with the Rb and p53 enzymes, accordingly, and causing inactivation of the Rb protein. Injecting CRISPR/Cas9 into the Si-Ha cell

line (carrying the HPV16 virus) of cervical cancer to modify the E7 and E6 promoter region. Cervical tumor cells were shown to retain p53 and p21 genes, which inhibited cervical cancer cell line Si-Ha growth and tumorigenesis. Second, a mouse model of cervical cancer was developed by injecting tumor cells subcutaneously. Researchers discovered that by targeting the E7 and E6 genes using CRISPR-Cas9, cancer cell proliferation may be slowed and the number of tumor cells present in the body could be reduced (Jia & Patel, 2021). By employing CRISPR/cas9 technique to precisely modify the E6 gene, researchers were able to better understand how tumor cells grow in vivo and in vitro. Patients with cervical cancer may benefit from the high efficiency and selective CRISPR-Cas9 technology that targets E6 expression of genes in high-risk papillomaviruses. The early E6 and E7 genes are essential for the malignant phenotypic of cervical cancer cells to be maintained following HPV infection. For HPV-positive cell lines, the Cas system may be used to zero in on the HPV16-E7 DNA. HPV16 E7 sgRNA-targeted CRISPR/Cas system may lead to death and decrease multiplication of HPV-positive cervical carcinoma cells while having no effect on HPV-negative cervical carcinoma cells (C33A and HEK293)

4.2.6 Skin Cancer

About 1.7% of all cancer cases were caused by melanoma. the entire earth in the year 2020. Among skin malignancies, melanoma has an increased propensity to metastasize to distant organs and tissues. Melanoma is classified into one of five invasiveness levels using the Tumor-Node-Metastasis (TNM) method established by the American Joint Commission on Cancer. Stage 0 refers to intraepithelial cancer, stage 1 to localized cutaneous melanoma less than 2 mm mm thick, stage 2 to tumors more than 2 mm or 1-2 mm with ulcers, stage 3 to local nodes, and stage 4 to distant metastatic dissemination. Proto-oncogene stimulation from BRAF and KIT gene variants, in addition to suppression of tumour suppressor genes, have a significant role in melanoma prognosis. In addition, the activated state of downstream

Excess transcriptions and cell proliferation are the end result of MAPK kinase or ERK enzyme-mediated MEK signal transduction. Vemurafenib and dabrafenib, two antagonists of V600E- and V600K-modified BRAF kinases, were shown to increase melanoma patients' survival in a randomized phase 3 study (BRIM-3). In addition, C-kit, a type 3 transmembrane ligands tyrosine kinase, binds to stem cell factors, which promotes receptor oligomerization, autophosphorylation, and downstream initiation of signal transduction like the mitogen-activated protein kinase

(MAPK) and phosphatidylinositol (PI3K) pathways, which are responsible for gene transcription and cell growth. C-kit gene mutations are seen in 36% of all melanomas, 28% of acral melanomas, and 39% of mucosal melanomas(Elliott et al., 2021).

4.3 Treatment Options for Cancer

4.3.1 Surgery

In surgery, a surgeon uses instruments like knives and scalpels to cut out a tumor or even to debulk (clear sections of) a tumor while the patient is under. The removal of a tumor or a portion of the tumor aids in the reduction of most malignant cells and improves therapeutic compliance from subsequent treatment options. Cryosurgery, also known as cryotherapy, uses the very freezing temperature of liquid nitrogen or argon gas to kill malignant cells or tumors. It is most useful in the treatment of early-stage skin malignancies, retinoblastoma, and first tumor overgrowth on the cervix and skin. Treatment with a laser includes directing a light beam towards the cancer in order to kill or compress the tissue there in(H. Yang & Patel, 2017). Vaginal cancer, b asal cell carcinoma, esophageal cancer, cervical cancer, non-small cell lung cancer, and so on are all examples of cancers commonly treated in this way. Hyperthermia entails subjecting localized regions of tissue to high temperatures in order to destroy cancer cells and make tumors more susceptible to subsequent treatments like radiation and chemotherapy. Drugs used in photodynamic treatment are activated by a specific wavelength of light, killing cancer cells in the process. Treatment of skin cancer, non-small cell lung cancer, and other malignancies diseases like cancer.

4.3.2 Radiation and Chemotherapy

Cancer development and spread can be stymied by chemotherapy, which employs the killing of rapidly proliferating tumor cells and some healthy cells. However, ionizing radiation is used in radiation treatment to destroy cancer cells in a forthright way. Radiation and chemotherapy, the more conventional methods, affect the entire body, including healthy cells, which can lead to unpleasant side effects like pain, diarrhea, cardiotoxicity, nausea, hyperpigmentation, alopecia, and immunosuppression, as well as increased cancer resistance if the treatments fail. Additionally, normal cell types with a faster rate of division (such as bone marrow, hair follicle, digestive system, etc.) do face a larger risk of adverse effects. Depending on the stage and extent of the malignancy,

chemotherapy and radiation treatment may be administered together as either neoadjuvant, adjuvant, or concurrent therapy(Lin et al., 2017). This permits the disease to be targeted from several fronts, leaving it vulnerable enough to be treated with a variety of methods while avoiding resistance. As part of neoadjuvant therapy, the tumor is first reduced in size by surgery, and then various forms of treatment are used to kill off any remaining cancer cells. On the other hand, adjuvant therapy is administered following surgery in cases when the malignant cells or tissues were not eradicated by the previous methods. Hormone receptivity, cancer kind, and lymph node status all have a role in adjuvant therapy. Chemotherapy, radiotherapy, hormone therapy, targeted therapy, immunotherapy, and the other four forms of treatment are the five main categories. Alkylating compounds like topoisomerase inhibitors, cyclophosphamide, like camptothecin, anthracyclines like daunorubicin, plant alkaloids like vinblastine, pyrimidine, and purine antimetabolites like mercaptopurine are all examples of chemotherapy drugs. These anti-cancer drugs work by inhibiting DNA replication or cell division during a certain stage of the cell cycle. This allows us to classify them as either general or epithelial. For instance, alkylating agents are a class of non-cell cycle-specific chemicals that induce apoptosis by the formation of cross links, DNA strand breakage, or caused by mutations in nuclear and mitochondrial DNA. Since they are not cell cycle specific, their increased efficacy versus leukaemia and tumors comes with increased toxicity.

4.3.3 Targeted Therapy

Cancer cells are the exclusive focus of targeted treatment, which has both great efficacy and low toxicity. Imatinib is a tyrosine kinase inhibitor that targets BCR-ABL in myeloid leukemia; it was the first small molecule medication licensed by the US Food and Drug Administration (FDA). There are two main categories of targeted drugs: macromolecules and micromolecules. Because of their diminutive size, small-molecule medicines are able to enter cells and hone in on the key proteins that regulate tumor cell development and metastasis.These macromolecules include such things as polypeptides, monoclonal antibodies nucleic acid, and antibody-drug conjugates.

Kinases are the types of enzymes that transfer a phosphate group from adenosine triphosphate (ATP) to a hydroxyl group on a protein residue. Critical cellular processes including proliferation, differentiation, and survival rely on the actions of protein kinases. Tyrosine kinases, serine/threonine kinases, and tyrosine kinase-like enzymes are all subtypes of protein kinases that

are distinguished by the substrate residues that they catalyze(Jolany Vangah et al., 2020). Protein kinases are the most reliable indicators and targeted therapies for cancer since their dysfunction is linked to a wide variety of disorders. Kinase inhibitors, epigenetic inhibitors BCL-2 inhibitors, , proteasome inhibitors, etc., are only some of the protein kinase inhibitors available.

Anaplastic lymphoma kinase (ALK) proteins, encoded by the ALK gene, facilitate the stimulation of downstream signal transduction and play a critical role in the maturation of the central nervous system. Multiple human malignancies, including anaplastic large cell lymphoma, diffuse large B-cell lymphoma (DLBCL), inflammatory myofibroblastic tumor and non-small cell lung cancer (NSCLC), have been linked to ALK activation due to mutant ALK.

Abelson murine leukemia 1 (ABL1) is a gene on chromosome 9 that encodes a participant of the non-receptor tyrosine kinase family called cAbl, a Bcr-Abl1 inhibitor that has been shown to play a role in the regulation of critical cellular processes like cell cycle and growth, cell differentiation and cell survival. When the ABL1 gene on chromosome 17 and the breakpoint cluster region (BCR) on chromosome 22 fuse together, an abnormal BCR-ABL fusion gene is produced. This fusion gene encodes the oncoprotein p210 Bcr-Abl1, which triggers autophosphorylation and the downstream signaling pathways, resulting in the rapid proliferation of tumor cells. This phenomenon is known as Philadelphia (Ph) chromosome translocation (Miri et al., 2020).

Uncontrolled cell proliferation, brought on by cell cycle aberrations, is well recognized as a hallmark of cancer. CDKs are enzymes that aid cyclin proteins in activating downstream phosphorylation signaling pathways, hence regulating cell cycle growth progression. Palbociclib, ribociclib, and abemaciclib are all CDK inhibitors that work by inhibiting CDK4/6 in particular.

Macromolecular hybridomas are a type of targeted treatment in which B cells are fused into cancer cells (myeloma). These hybridomas inhibit cancer cell activity by complement-dependent cytotoxicity, antibody-dependent cytotoxicity, and interference with protein function and downstream signaling cascades. Antibodies specific to a single target protein that have become deregulated during carcinogenesis are called monoclonal antibodies. Newer monoclonal antibodies acquire humanized fusion cells, making the resultant macromolecules safer and more effective for the human body compared to their earlier mouse equivalent. Adalimumab (brand name Humira) is a monoclonal antibody that has shown great effectiveness in treating autoimmune

diseases. It attaches to cancer necrosis factor-alpha (TNF-alpha), preventing its bioactivity, and promoting the death of TNF-expressing mononuclear cells(Lakshmanan et al., 2021).

Depending on the case, immunotherapy can either boost or dampen the body's natural defenses against illness. Immunotherapy comes in many forms, and one that strengthens the body's T cells to better fight cancer cells is T-cell transfer treatment, also known as adoptive immunotherapy. Tumor-infiltrating lymphocytes (TIL) treatment and Chimeric antigen receptor (CAR) T-cell therapy are two examples of T-cell transfer therapies. Both treatments call for extensive collections of cultured patient immune cells. T cells are extracted from the patient's blood, multiplied in the lab, and injected directly into the patients through an IV. Radiation therapy and Chemotherapy can be administered prior to T cell therapy to boost the efficacy of this immunotherapy by reducing the number of immune cells in the body and clearing the way for the transplanted T cells. Tumor-infiltrating lymphocytes (TIL) are a subset of T cells that invade tumors and serve as unique indicators of tumor cell subtype. These lymphocytes are first identified and then grown to produce bigger amounts for delivery to patients. The high numbers of lymphocytes aid in counteracting the inhibitory signals given out by tumor cells (Tschaharganeh et al., 2016). CAR T-cell treatment is a subset of immunotherapy that, while similar to TIL therapy, necessitates the transformation of T cells into a chimeric antigen receptor (CAR) protein before they can be cultivated and re-administered to the patient. The CAR proteins help the T cells strengthen their anti-cancer capabilities by binding to certain proteins discovered on the surface of cancer cells.

Another target in cancer treatment and relapse prevention is cancer stem cells (CSCs). Surface indicators, signaling pathways, microenvironmental signal interference, efflux pump inhibition, miRNA expression modification, triggering CSC death, and CSC differentiation are some of the key targets or phases tackled in these CSCs (Dragu et al., 2015). 15 Cancer patients can benefit from more targeted and more successful treatment plans by using their tumor's genetic profile to attack the disease at its genetic and epigenetic roots. Epigenetics, refers to the post-translational alterations of gene expression that are independent of direct DNA sequences. DNA methylation and demethylation, post-translational modifications, chromatin structural changes, and other mechanisms are all part of epigenetics. DNA methyltransferases (DNMTs), which help in DNA methylation, and histone deacetylase, which modifies chromatin post-translationally, are two examples of epigenetic targets. Due to quick inactivation by cytidine deaminase enzymes in the

liver, most DNMT inhibitors break down in neutral water solution, creating hazardous analogs, and have thus far shown to be ineffective in clinical trials. When binding proteins for epigenetic proteins are absent, the epigenetic proteins are unable to attach to their intended targets. In turn, this results in ubiquitous, yet non-specific, genetic expression, which can have a wide range of undesirable outcomes. There are many approaches that may be taken to reengineer epigenetic proteins in order to permanently attach to certain targets. The small interfering RNA (siRNA) molecules used in nucleic acid-based gene editing either target the nucleus, where they cause methylation via controlling cognate genes, or the cytoplasm, where they induce posttranscriptional silence of targeted genes by destroying the precursor mRNAs (Granados-Riveron & Aquino-Jarquin, 2018). Proteins make changes to genes primarily by the recognition of particular sites on the gene via the DNA-binding domains present on the protein, which is then fused to a second effector domain on the protein that specifically targets the recognized sites. The zinc finger (ZF) and transcription activator-like effector (TALE) domains are two widely lauded innovations. Since their extremely compressed targets are challenging for protein sequences to access and bind to, the likelihood of these molecular editing features being employed for epigenetic editing remains low. However, when combined with transcription-activating medicines, RNA-guided endonucleases have demonstrated encouraging efficacy.

Chapter 5

5.1 CRISPR/cas9 delivery in cancer cell

5.2 Viral Vector:

The number of research showing the efficacy and promise of CRISPR in combination with virus - based delivery techniques is amazing, given the relatively early stage of development of mammalian gene editing. Adenoviruses, Adeno-associated viruses (AAVs), lentiviruses and retroviruses, and are four of the most often used viral vectors for delivering Cas9 and/or gRNAs thus far.

5.2.1 Adeno-associated Virus

Gene transfer vectors based on adeno-associated viruses are safe, effective, and simple to create. Cas9 and RNA are two essential components of CRISPR, both of which may be delivered by adeno-associated virus (AAV) alone or in combination. Low-molecular-weight Cas proteins The diameter of the envelopeless AAV virus is only 25 nanometers. In studies spanning decades, scientists found that the AAV vectors can fit inside a 5 kilobase (kb) genome, leaving a maximum of 4.7 kb for the transgenic cassette. More effective delivery and a larger selection of possible vector promoters are only two of the benefits of using Cas proteins in AAV vector design. The Staphylococcus aureus Cas9 encoding gene (SauCas9) is 3.2 kb in length, and every given AAV vector may only accommodate two sgRNAs co-transcribed with the Cas9 gene. Recently, a self-linearizing mending templates were found to be included in an AAV8 vector that also houses the SauCas9 gene and two sgRNAs. Mice with a Fah (fumarylacetoacetate hydrolase) mutant that this vector was shown to correct were cured of type 1 hereditary tyrosinemia. Adeno-associated virus has been the most common viral vector used for CRISPR genome editing in cell and animal experiments, although there are many more options (Chen et al., 2022). This indicates that AAV is a fantastic toolkit for CRISPR usage in a wide variety of in vitro and in vivo cell types. Packing constraints frequently necessitate the use of numerous vectors. In several fields, AAVs have found usefulness. Among the several gene-enhancement therapies being tested in human clinical trials, AAVs stand out for their excellent safety profile and wide range of potential therapeutic applications. Furthermore, considerable toxicity is seen in animal studies at larger dosages. While some adeno-associated virus (AAV) DNA may integrate into mitochondrial DNA hotspots or a

specific location on human chromosome 19, the vast majority of transduced AAV DNA remains in the cell's episomes (AAVS1). As of right now, both integration sites are benign and do not stimulate cancer. In addition, AAV is available in a wide range of serotypes, making it possible to deliver targeted genes to specific tissues. Given that certain serotypes have been validated for use in CRISPR-edited tissues. Compared to other viral vectors, the innate immune response triggered by recombinant adeno-associated virus is very mild but nevertheless considerable. An individual's first line of defense against viral infection is their innate immune system. Pathogen-associated molecular patterns (PAMPs) are recognized by pattern recognition receptors (PRRs) on invading viruses or viral nucleic acids. In response to pathogen recognition, these sensors initiate a signal transduction event that limits viral replication and initiates adaptive immune responses. However, the immune system still presents major challenges in terms of reliability and efficiency. Preexisting neutralizing antibodies (NABs) pose a problem when the first vector administering, and immunological responses to a provided vector can result in rapid seroconversion, which tends to make vector re-administration difficult (Sahel et al., 2019). However, temporary immunosuppressive may repress CD8+ T cell responses to the capsid. As our knowledge of AAV biology expands, however, we may one day be able to logically design or chemically change capsids to render them undetectable. Alternatively, variations resistant to neutralizing antibodies may be quickly generated by directed evolution, and such decisions may be combined with evolutionary processes to drive transmission to certain tissues and cells in vivo. Accordingly, the success of Adenovirus vectors may hinge on a diverse and expanding set of tools that are increasingly being used with a wide range of patients and goods.

5.2.2 Adeno Virus

Adenovirus (Ad) vectors are now used to transport genetic material across mammalian cells, and they are quite useful. Given that adenoviruses are small, non-encapsulated dsDNA viruses (their genomes are smaller than 36kb), their viral particles cannot integrate into the host genome and cause oncogenesis or genotoxicity. Because of their ability to catalyse both trying to divide and non-dividing cells at the same time, adenoviruses make in vivo gene transfer possible; however, the wide variety of cell types infected by these viruses necessitates trying to target, especially if the transfected gene is detrimental when voiced in non-targeted tissues. Despite the availability of other vector systems for genetic modification and functional genomics studies, recombinant

adenovirus vectors continue to dominate the field because they are simple to construct, can be produced in huge quantities, and have high transduction efficiency. Adenovirus is a good option for in vivo delivery of breakthrough genome engineering machinery like CRISPR-Cas due to its features and its shown safety profile in clinical research (Zou et al., 2022). Altering the protein capsid of an adenovirus is a potent tool for modulating the virus's behavior in vivo following injection. As a result, vectors have been developed that overcome challenges such 1) viral sequestration in the liver, 2) limited transduction of deficient cells the viral receptor, and 3) preexisting antibody responses to adenovirus.

5.2.3 Retrovirus

Negative-sense RNA viruses, or retroviruses, require the enzyme reverse transcriptase to convert their RNA into DNA so that it may be incorporated into the host chromosome. The canonical genome of a retrovirus includes the pol gene, the env gene, the pro gene, and the gag gene. The reverse transcriptase enzyme gene (RT) is located in the pol gene, while the integrase gene (iH) is located in RNase H. While the gag gene is responsible for making the polyprotein structure, the env gene makes the envelope proteins that help in virus assembly and tropism determination. Retroviral vectors used it to transduce mammalian cells were based mainly on the Moloney murine leukemia virus (MLV), and that they could only transduce dividing cells because the pro gene expresses protease needed for sophistication of the virion by transforming immature polyproteins into functional elements. Correct gene integration is essential for precision focused gene therapy and genome modification, and these vectors have been enhanced to integrate just transgenes of choosing rather than viral genes. Because of this, scientists have designed HIV-derived viral vectors for use in retroviral gene delivery, which have the added benefit of extending viral tropism to non-dividing cells. Since HIV-based vectors may potentially transduce non-dividing cells, the term "lentiviral vector" is widely used to describe them. A number of human illnesses have been affected by vectors based on retroviruses, and as vectors grow safer, their use may increase (Ratan et al., 2018). In treating X-SCID, a self-inactivating (SIN) -retrovirus vector has been shown to be effective, according to preliminary studies. Therefore, "self-inactivating" or "SIN" genomes are commonly seen in modern retro- and lentiviral vectors; in these viruses, the viral long terminal repeat inhibits gene transcription in the transfected cell, reducing worries about the reproduction of live viruses and increasing safety. Infecting immunocompetent animals or humans will always

result in the development of a humoral immune response, mostly directed towards proteins in the viral shell, even if the resulting virus has a completely different genetic makeup than the original.

5.2.4 Lentivirus

Like the adenovirus, the lentivirus (LV) is a spherical virus that replicates in both growing and non-dividing cells due to its single-stranded RNA. Recent lentivirus vector methods separate crucial genes over three plasmids, decreasing the potential for in vivo virus replication. What's more, the lentivirus vector can package up to around 8 kb of DNA. The capacity of lentivirus to be pseudotyped alongside other viral proteins is a major advantage. Because of this, the LV's cellular tropism may be tailored by engineering and modification. Moreover, LV vectors do not include viral genes and therefore do not cause an immune response. As the production of non-integrating lentiviruses is beyond the scope of most laboratories. Fewer adeno-associated virus and adenovirus vectors are used than LV vectors(Bhatkar et al., 2020). Furthermore, illness models are the most common use for LVs. In addition to conventional genetic analysis, LV-delivered CRISPR-Cas9 was used to generate animal models. Animal models were developed by administering CRISPR-Cas9 to them through LV, so the research could go beyond just genetic testing. In order to mimic the genetic complexity of malignancy, researchers have created mice models in which a single hematopoietic stem cell develops acute myeloid leukemia (AML) by modifying up to five genes.

5.3 Non-viral Physical Vectors

Physical transfection is one method for delivering CRISPR-Cas9. Physical transfection creates temporary breaches in the cell membrane using mechanical or electrical pressures, facilitating the entry of target molecules (Das et al., 2022). Additionally, vectors are optional for physical transfection. Thus, in contrast to viral vectors, the size of the cargo is almost infinite, and in contrast to chemical vectors, cell endocytosis is not the rate-limiting mechanism.

5.3.1 Microinjection

Microinjection refers to the practice of delivering genes into cells using extremely small blood vessels. Gene expression can be sped up with the use of microinjection, which enables the direct injection of genetic materials into the nucleus. Microinjection is the best method for single-cell

applications like germline editing. Microinjection of CRISPR plasmids has sped up the creation of model organisms with the necessary traits. Cas9 mRNA and gRNA targeting the myostatin-encoding gene were introduced into the cytoplasmic matrix of the sheep zygote to create animals with inhibited muscular development. Insect vectors, zebrafish, rats, and even pigs have all been successfully injected with CRISPR by scientists thus far. This method is quite successful, but it has low throughput since it requires a skilled technician to inject the cell in a way that keeps it alive (Albitar et al., 2018).

5.3.2 Electroporation

Electroporation is the process of using an electric current to create a cell that is more competent than its predecessor due to increased membrane permeability. RNA, DNA, and targeted proteins can enter target cells thanks to a transient increase in membrane permeability. A technique for introducing CRISPR-Cas9 machinery into cells, electroporation, has emerged in recent years. Studies have investigated the use of electroporation as a means of delivering for genome editing (Dey & Nandy, 2021). Electroporation is used to introduce CRISPR-Cas9 plasmid systems, Cas9 protein-and-sgRNA complexes, Cas9 messenger RNA, and Cas9 single guide RNA.

5.4 Nonviral Chemical Vector

5.4.1 Lipid based Vector

Incorporating lipids into the transfer of genes is one of the first and most reliable methods of gene therapy. The cationic lipid-based vectors liposomes and solid nanomaterials (SLN) are widely used. Due to their improved biochemical properties and low immunogenicity, these liposomes have rapidly gained popularity as a means of transporting genetic material. SLNs are constructed from lipids with a high melting point; these lipids have a solid center and are covered with surfactants to keep them from melting. They are non-viral vectors for systemic delivery of genetic material. Lipid nanoparticles that are solid at room temperature have the potential to be employed as non-viral carriers in a broad variety of settings because of their increased stability during storage, as well as their sterilization and lyophilization. The first non-viral delivery vectors to be employed in human clinical trials were cationic liposomes since they are the most widely used non-viral gene carriers (Batool et al., 2021). Aside from facilitating the delivery of CRISPR/Cas9 elements into cells, lipid nanoparticles can also provide some protection for the payload against

degradation. Commercially available lipid nanoparticle transfection reagents for delivering CRISPR-Cas9 ribonucleoproteins include LipofectamineTM CRISPRMAXTM Cas9 (Thermo Fisher Scientific). Liposome-based gene therapy has been reported to have advanced to clinical trials on many occasions. Human HLA-A2, HLA-B13, and murine H-2K genes were delivered to patients with various malignancies, including leukemia, through DC-Chol/DOPE cationic liposomes as part of phase I study. In particular, in situ gene therapy induced a strong immune response with no serious side effects. Two of eight patients experienced complete clearance of cutaneous lesions after therapy with HLA-A2-DNA liposomes.

5.4.2 Polymer based vector

Polymers have been extensively studied as a non-viral vector for transferring the components of CRISPR/Cas9 due to their biocompatibility, flexibility, and simplicity. Polymeric matrices are an exciting delivery channel for CRISPR/Cas9 components because to their inherent adaptability. Polymeric carriers, in contrast to lipids carriers, can circumvent serum instability concerns with careful molecular design (S. Zibitt et al., 2021). The decreased immunogenicity and simplicity of mass production and functionalization of cationic polymers including polyethylenimine (PEI), poly[2-(dimethylamino) ethyl methacrylate], poly(L-lysine) (PLL), and poly-amidoamine (PAMAM) dendrimers have attracted significant attention in gene therapy.

5.4.3 Chitosan system based vector

An alternative to viral vectors for the safe delivery of genetic components including pDNA, siRNA, and ODNs is chitosan-based vectors (oligodeoxynucleotide). Produced from partly deacetylated chitin, chitosan is a polysaccharide composed of glucosamine and N-acetylglucosamine. Chitosan compounds can be supplied to certain cell types using a variety of cell-targeting ligand conjugates. With properties including low toxicity, low immunogenicity, high biocompatibility, and a strong cationic charge, chitosan is a versatile material with many potential applications. Since chitosan has a net positive charge, it may readily form polyelectrolyte complexes with the negatively charged nucleotides. Substandar specificity and transfection efficiency, however, continue to restrict its clinical use. Clinical studies have begun on a combination of the nonionic poloxamer CRL1005 and the cationic surfactant benzalkonium chloride (Hashemi, 2018). Genetic vaccination with this formulation is being studied as a means

of reducing the risk of cytomegalovirus infection in patients undergoing allogeneic hematopoietic cell transplantation as part of a phase II/III clinical research.

5.5 Extracellular Vesicles as Vector

Generating and self-assembling the viral particles and/or viral basic proteins is required for the production of virally infected extracellular vesicles. Virus-like particles are also the common name for the envelope and structural proteins of viruses (VLPs). The target protein is covalently linked to the Gag polyprotein before being incorporated into a particle. When a virus matures, a Pol enzyme catalyzes the breakdown of the Gag polyprotein, allowing the release of a cargo protein that may be transported to target cells. In contrast to lentiviruses (LVs), which do carry viral DNA, extracellular vesicles (EVs) do not. Therefore, extracellular vesicles are not part of their host genomes and cannot propagate independently. In addition, EVs are a secure method of viral delivery due to their enhanced safety profile (Selle et al., 2020). Off-target alterations and immunogenicity might be caused by CRISPR-Cas9 and the generation of gRNA through viral carriers. Therefore, for therapeutic genome editing, a mechanism of temporary administration is necessary. Chemically driven dimerization of the Cas9 protein facilitates its recruitment into extracellular nanovesicles, where it is followed by the attachment of a viral RNA packing signal and two self-cleaving riboswitches that distribute sgRNA. The potential for off-target effects brought on by sustained expression of Cas9 is, however, greatly reduced by the targeted delivery of CRISPR/Cas via EVs within cells. Additionally, EVs may be produced quickly and cheaply using standard transfection of plasmids into packing cells. Eventually, vesicles will allow for multiplexed gene editing. Vesicles can form without the help of proteases being cleaved through proteins. Thus, it avoids the disadvantages of the VLP-based technique, such as protease-mediated protein denaturation and competition between Gag and Gag-Cas9 proteins during packing (Garcia-Robledo et al., 2020).

Chapter 6

6.1 Applications of CRISPR/Cas System in Cancerous Cell

Using a CRISPR-Cas system like CRISPR/Cas9, it is possible to generate tumor models with several targeted mutations in vivo, providing a more accurate representation of the complicated human illnesses and malignancies that include many stages of carcinogenesis. To better understand the activity of cancer genes in cancer research and the discovery/screening of anti-cancer therapies, mice models are applied to resemble human tumors by remodeling or xenografting. Non-small-cell lung cancer (NSCLC) was mimicked in animal models by inducing chromosomal translocation of Eml4-Alk genes using a lentiviral CRISPR-Cas9 vector; this led to the development of lung cancer in the mice after 8 weeks (Waldt et al., 2021).

By using CRISPR-Cas9 to knock down the Pten and P53 tumor suppressor genes in the liver, we were able to induce tumors in the liver, providing a model system for studying carcinogenesis, tumor invasion, and potential treatment strategies. Techniques like patient-derived xenograft (PDX) have been incredibly useful in oncology research since they allow scientists to develop human tumors in animals. By using CRISPR/Cas9 to knock out Rag1, Rag2, and n Il2 in Sprague Dawley rats, we generated models of rat lymphoid organ development impairment with severe immunodeficiency that were promising prospects for future development into PDX models of lung squamous cell cancer. In the study of cancer, these models are extremely helpful. In addition, the mouse is a popular animal model for the study of several types of cancer, particularly skin cancer, pancreatic cancer, leukemia, breast cancer, glioma, and lung cancer, because of its high reproductive rate, ease of engineering, versatility, and cheap maintenance expenses. Mucosal melanoma zebrafish models lacking the tumor suppressor gene SPRED1 may be generated using CRISPR-Cas systems (S. Li et al., 2021). Therefore, comparable changes occurring in human tumors may be accounted for, allowing for in-depth study of the inhibition of MAPK (mitogen activated protein kinase) resulting from such mutations. Accordingly, cancer models offer a practical means of exploring cancer genes in vitro and in vivo.

6.2 CRISPR/Cas System in Cnacereous viral infection

Since oncoviruses may cause tumors to form by causing changes in the cell cycle of infected cells, they are responsible for a sizable fraction of all cancer occurrences globally. There are currently eight well-known and approved human oncoviruses: hepatitis C virus (HCV), hepatitis B virus (HBV), human papillomavirus (HPV), human T lymphotropic virus 1 (HTLV-1, Epstein–Barr virus (EBV), , human cytomegalovirus (HCMV), herpesvirus associated with Kaposi's sarcoma (KSHV) or human herpesvirus-8 (HHV-8) (HHV-8), Merkel cell polyomavirus (MCPyV), . Using CRISPR-Cas editing technology, researchers have successfully suppressed EBV, HBV, HPV, and HSV-1 in many experiments (Lau, 2018).

Four different combinations of the eight sgRNAs targeting the HBV genotype used in the CRISPR-Cas system were able to decrease HBsAg and HBcAg by 70%. Mice that have their proliferating cell nuclear antigen (PCNA) genes activated using CRISPR-Cas9 develop hepatocarcinogenesis and PCNA increase in the liver. One study found that using CRISPR-Cas9 to introduce sgRNAs targeting genes characteristic of the HBV genotype into HCC cell lines led to lower levels of HBsAg production, slower growth in the lab, and less tumorigenicity in animals. Targeting the HCV-negative RNA region, the CRISPR-Cas9 and Francisella novicida (Fn) bacteria FnCas9 system is able to interfere with viral propagation (Jin et al., 2020). Using sgRNAs to target the EBV genome and control the viral structure, morphological alterations, and latency, CRISPR/Cas9 was used to treat EBV infection in Burkitt's lymphoma patients in a separate research. There was a 50% drop in EBV genome number and a corresponding decrease in viral load in the resultant cell lines. The ability of EBV to transform primary human B cells into immortal lymphoblast cell lines is further supported by the available evidence.

6.3 CRISPR/Cas System in Oncogenic Tumor Diagnostics

Undoubtedly, Cas proteins are the major detecting techniques employed often in cancer diagnosis. Proteins have a number of advantages over other detection methods, including their specificity, sensitivity, speed, low cost, and ability to be employed in multiplex for RNA and DNA quantification and mutation detection. Tumorigenesis and metastasis in colorectal cancer are caused by alterations in a number of signaling pathways and genes, including the WNT signaling system, the EGFP signaling route, the TGFb signaling pathway, the p53 function, and the MYC

and ERBB2 genes. As a result, CRISPR tools and multiplexed detection can help doctors identify cancer at its various phases, when it is most amenable to therapy. Particular forms of cancer, such as lung cancer and brain tumors, have been related to up- or down-regulation of miRNA expression, making miRNA useful biomarkers in the identification and monitoring of these illnesses (Brezgin et al., 2019). CRISPR-based diagnostics have been successful in the detection of microRNAs (miRNAs), as evidenced by the electrochemical detection of miR-19b by the CRISPR/LwaCas13a system in patients with medulloblastoma without requiring preamplification and the testing of RNA isolated from breast cancer cell lines for the presence of miR-17 using the CRISPR/LbuCas13a system. CRISPR diagnostics may also track therapy response indicators like BRAF mutations in cancers like melanoma, which indicates that the tumour is responding to treatment. Preventative precautions are especially important when working with small sample sizes to guarantee the integrity of any genetic material detections. Some research have used CRISPR-mediated targeting of certain alleles for very sensitive mutation detection in tiny mixed nucleic acid samples. One effective method for facilitating the accurate enrichment and identification of low-frequency DNA alterations is the removal of wild-type DNA from mixed samples. CRISPR-SpCas9 was utilized to cleave wild type alleles like the PAM sequence via point mutations; this method is applicable to the detection of oncogenic point mutations in KRAS and GNAQ, despite their low frequency. All remaining mutant alleles were subsequently amplified using PCR. Mutant KRAS DNA is amplified 30-70-fold during the enrichment process. In addition, it was shown that KRAS point mutation amplification was effective in individuals with colon cancer. Similar amplification of GNAQ genes also occurs by CRISPRFnCas12a. SHERLOCK, short for "Specific High Sensitivity Enzymatic Reporter UnLOCKing," is a CRISPR-based diagnostic system that detects sensitive cancer-causing genes, including low-frequency mutations that are difficult to determine using traditional diagnostic approaches, such as sequencing. The diagnostic tool consists of two main parts: the RNA-guided RNase Cas13a, which produces non-specific ssDNA cleavage, and a reporter signal, which marks the completion of an RNA cleavage (Piergentili et al., 2021). This methodology is essential for identifying EGFR L858R and BRAF V600E, two common cancer-causing mutations. The DNA endonucleasetargeted CRISPR trans reporter (DETECTR) is another sensitive diagnostic method for detecting infections in cancer; in this case, Cas12a is involved in cleaving, and recombinase polymerase amplification (RPA) enzymes amplifies the micro-samples being detected. Rapid and

low-cost findings from employing DETECTRs to identify HPV types 16 and 18—the most common types responsible for lung cancer—have been achieved. The DETECTR method, in contrast to the SHERLOCK, does not need the conversion of DNA amplification products into RNA. Non-small cell lung cancer patients with EGFR mutations can be identified using SHERLOCK in conjunction with paper strip lateral flow test; this detection method yields more accurate results when Csm6 is also included. SHERLOCK and DETECTR can be used with the amplification-boosting approach of recombinase polymerase amplification (RPA) to better identify viral components. When used together, SHERLOCK and DETECTR can greatly improve the accuracy with which viruses like HIV are diagnosed, while DETECTR is useful for identifying dsDNA HPV. The HUDSON (for heating unextracted diagnostic samples to annihilate nucleases) technology is another game-changing method for detecting viruses in real time. To identify oncolytic viruses like HPV, HBV, HCV, EBV, etc., this instrument may be indispensable. Combining CRISPR with graphene membrane field effect transistor technology resulted in a new diagnostic method dubbed CRISPR-Chip technology (Mukhopadhyay & Bhutia, 2021). An sgRNA and the recognition protein dCas9 make up the transistor's molecular complex. The dCas9-sgRNA complex detects and attaches to the appropriate target gene upon membrane contact with the sample, prompting the transistor to release an electrical signal. Different from CRISPR-Chip technology, which relies on gene binding to create signals, SHERLOCK and DETECTR rely on gene cleavage. The CRISPR-Chip technique has advantages over other methods since the DNA being examined doesn't need to be amplified.

6.4 Utilizing the CRISPR-Cas System as an Immunotherapeutic Agent for Cancer

6.4.1 Recombinant Antigen Receptor T-Cell Immunotherapy

One kind of adoptive immunotherapy, called chimeric antigen receptor (CAR) T-cell treatment, uses engineered T cells with a chimeric signaling domain on the inside to stimulate the immune system and a single-chain variable segment on the outside to detect a patient's tumor. Due to its very precise targeting and expression of B cells, this kind of immunotherapy has proven to be particularly beneficial in treating hematological malignant tumors, such as leukemia and lymphomas. Autologous (the patient's own T-cells) CAR T-cell production is time consuming, expensive, and labor intensive; therefore, allogeneic universal T-cells chosen to take from healthy persons are the more economic/commercial viable alternatives, albeit needing the withdrawal of

endogenous T-cell receptor (TCR) and human leukocyte antigen (HLA) class I previous to its utilization in treatment to reduce the likelihood of graft-versus-host disease and subsequent rejection in the host immune CAR-T cells with strong anticancer activity may be produced by using CRISPR-Cas9 to damage numerous genomic loci, in addition to causing faulty expressions of TCR and HLA class I (P. Li et al., 2021). Using CRISPR-Cas9, we can knock off Fas (CD95) receptors, that under normal conditions cause T-cell death, therefore enhancing the anti-tumor activity of Tcells and extending the lifespan of tumor-bearing animals. Removing genes linked with co-inhibitory molecules like PD-1 and CTLA-4 using CRISPR-Cas9-sgRNA complexes is another way to boost T-cell functionality. CRISPR-Cas9 systems can also be used to disrupt additional co-inhibitory molecules, such as LAG-3 and TIM-3, to improve the performance of CAR T-cells. Anti-CD19 chimeric antigen receptor T-cell therapies, such as Yescarta and Kymriah, have been approved by the FDA to treat adult dispersed large B cell lymphoma (DLBCL) and pediatric/adolescent B lymphoblastic leukemia, respectively, based on the results of one of the most successful clinical trials currently being conducted (BALL). One study found that knocking down the GM-CSF gene improved CAR-T cell activity and suppressed inflammation and cytokine release syndrome (CRS). Another study used CRISPR/Cas9 technology to knock off the endogenous TGF- receptor II (TGFBR2) in CAR T-cells, which reduced CAR T-cell depletion and improved their solid tumor-killing effectiveness. Similar results were shown in the treatment of T cell acute lymphoblastic leukemia when CD7 and TRAC were removed from CAR T cells (Chinnapaiyan et al., 2020). Many patients' CAR T-cell therapies have failed because of an immunosuppressive tumor microenvironment and/or T cell fatigue. Therefore, methylcytosine dioxygenase TET2 and other genes that make T-cells vulnerable to inhibitory signals from the tumor microenvironment have been silenced utilizing CRISPR-Cas systems, resulting in effective improvement of CAR T-cells efficiency. Transducing CAR genes into T cells typically makes use of lentiviral vectors assisted by CRISPR-Cas9 systems.

6.4.2 Adoptive T Cell Therapy Utilizing T Cell Receptor (TCR) Targets

Insufficient tumor-specific antigens, suppressive mechanisms in the tumor microenvironment, and heterogeneity among tumor antigens all contribute to CAR-T cells' inability to fully fulfill their therapeutic potential. Because of the importance of being able to target a greater range of antigens and thus cure a wider range of cancer types, T-cell therapy based on modified T-Cell Receptors

(TCRs) is a top priority. Each TCR chain in the TCR heterodimer has an extracellular constant area, a changeable antigen binding region, and a membrane region. The TCR/CD3 complex, formed by the constant portions of the main TCR/ chain and the CD3 chains, is responsible for tumor antigen recognition, which is dependent on the major histocompatibility complex. (MHC). Several solid cancers, including sarcoma, melanoma, multiple myeloma, breast cancer, cholangiocarcinoma, and papillomavirus-associated cervical cancer, have responded favorably to TCR T-cell treatment in clinical trials (Al-Sammarraie & Ray, 2021). In addition, natural TCRs have been knocked out using CRISPR-Cas9 to improve the production and functionality of transgenic TCRs. In addition, transduction of stabilized V/V single-chain TCRs (Sc-TCRs) was performed to reduce the likelihood of mispairing. Lentiviral transduction was employed to genetically modify patient-derived autologous T cells such that they express TCRs specific for NY-ESO- 1 and LAGE-1 antigens following isolation of the T cells. After using CRISPR-Cas9 to delete the genes for endogenous TCRs and PD-1, T cells were expanded in vitro and re-administered to the same three patients. Two of the patients were found to have illness states that were stable, while the third patient's condition had worsened (Ghaemi et al., 2021).

6.4.3 Cancer Immunotherapy: Novel Target Screening

A loss-of-function screening platform was constructed by combining sgRNA lentiviral transmission with Cas9 protein electroporation (SLICE) and using a pooled CRISPR delivering single-cell analysis of transcript to discover genes that enhance T-cell activation. This allows for the proper characterization of functional target genes in primary T-cells. Due to inefficient tumor site targeting, lack of effector function, and CAR T-cell restriction of permanent activity, continuous antigen activation and the immunosuppressive tumor microenvironment are two key factors affecting the efficacy of CAR T-cell treatment. Target gene screening and selection for genes responsive to these inhibition can further improve this efficiency (Mathony et al., 2020). Therefore, CRISPR-Cas systems can be widely used to account for the screening component of dealing with these treatments. In order to introduce CAR genes into T-cells, researchers often utilize CRISPR/Cas9 screening, lentivirus, or retrovirus. However, the precision of the findings may be impaired by the efficient screening due to the random variables of CAR genes. Transducing the CAR gene's coding sequence and the editing tools into T-cells at the same time is one strategy for combating this issue. Another research revealed that the danger of ational and off-targeting

might be reduced by expressing CAR genes there under direction of a T-cell genome. One study used a Cas12a-based adeno-associated viral (AAV)-Cpf1 KIKO (knock-in and knock-out) platform to discover genes that control CAR T-cell activity. Human CD4+ T-cells had previously been electroporated to introduce Cpf1 mRNA before being infected with AAV encoding for two crRNAs and a single CAR coding gene (V. W. Xue et al., 2020). CAR gene integration (KI) was guided by the first crRNA, and knockdown of the target gene was achieved by the second (KO). With the KO crRNA swapped out for a crRNA library, the KIKO system as a whole will be able to do a CRISPR screen for the discovery of genes involved in the regulation of CAR-T-cell activity. Furthermore, genome-wide CRISPR screens have not yet comprehensively examined the function of B cells in advancing malignancies and modulating tumor responses to immunotherapy. Cas9-mediated targeting of genes involved in B-cell engagement and transformation in B cells separated from transgenic mouse models led to the development of a highly selective sgRNA design tool. The regulators of the B cell state, its variation, and disease-associated variables were largely characterized using a CRISPR screen (Bahuguna et al., 2021).

6.4.4 Prevention of Immune Checkpoint Signaling

T-cell fatigue occurs when T-cells and tumor cells start to coexist due to persistent exposure to antigen and immunosuppressive substances. This fatigue results in the upregulation of inhibitory receptors on tumor-infiltrating T cells, including PD-1, CTLA-4, lymphocyte activation gene 3 protein, T cell immunoglobulin domain, mucin domain-containing protein 3, and others. Anti-tumor effects in cancer treatment are aided by immune checkpoint inhibitors. Upon antigen identification by the TCR/CD3 complex, CD28 molecules enhance the TCR signals to help in the T-cell activation process. Because it is a homologous receptor of CD28, CTLA-4 may bind to the identical CD28 ligands and provide inhibitory signals to both CD4+ and CD8+ T-cells, inducing opposite effects on each type of cell. By inhibiting CTLA-4, CD28 ligands are released, paving the way for T cell activation (Y. Li et al., 2019). Ipilimumab is an anti-CTLA-4 recombinant IgG1 monoclonal antibody that has been shown to be effective in treating metastatic melanoma and enhancing the immune system's ability to destroy tumor cells. Knocking out or disrupting CTLA-4 genes in cytotoxic T lymphocytes (CTLs) utilizing CRISPR-Cas9 systems has been demonstrated to boost secretions of TNF- and IFN-, hence bolstering the anti-tumor action of CTLs. Like CTLA-4, PD-1 is an immune checkpoint inhibitor that is expressed on immune cells

(such as monocytes, T-cells, dendritic cells, etc.). T-cell exhaustion occurs when the ligands for programmed death 1 (PD-L1) and programmed death 2 (PD-L2), which are found on tumor cells and antigen presenting cells (APCs), interact. Similarly, anti-PD-1 antibodies like pembrolizumab and nivolumab have demonstrated effective anti-tumor action in a variety of tumor forms, including as melanoma, metastatic urothelial carcinoma, non-50 small cell lung cancer, and head and neck squamous cell carcinoma (Uddin et al., 2020).

6.5 Utilization of the CRISPR-Cas System in the Management of Various Cancer

6.5.1 Lung Cancer

Lung cancer treatment options are vast, with surgery and radiation therapy among the most common procedures. Because of their capacity to inhibit EGFR tyrosine kinase activity, selective tyrosine kinase inhibitors (TKIs) like erlotinib and gefitinib are now the drugs of choice. alterations in the EGFR genes are also responsible for the vast majority of lung malignancies. EGFR is a membrane glycoprotein with three distinct regions: a transmembrane region, an extracellular ligand binding region, and an intracellular tyrosine kinase region. When a ligand binds to the extracellular ligand domain, it activates intracellular kinase activities, which in turn promote cell growth, invasion, vascularity, and metastasis while simultaneously suppressing apoptosis and triggering glycolysis (Fellmann et al., 2017). Because of the rise in medication resistance, however, CRISPR-Cas systems are being used in tandem with standard treatments to restore their former effectiveness. Both homologous and non-homologous DNA repair mechanisms are being tested using CRISPR-Cas9 in lung cancer. The first method involves the design of sgRNA supplementary to the EGFR series that can target the specific site for its cleavage by Cas9 proteins, creating a ss- or dsDNA break depending on the particular enzyme used. As a general rule, inhibiting CRISPR-Cas9 raises MHC class I expression, which aids in the detection of tumors by cytotoxic cells and their eventual destruction. The second method includes using CRISPR-Cas systems to improve immune cells, such as T lymphocytes, that have been harvested from patients or donors. For example, CRISPR-Cas9 may silence genes that code for PD-1 and CTLA-4, two inhibitory receptor proteins that normally connect to T-cells and halt their activation and consequent immune response to malignancies (Nidhi et al., 2021). In order to promote caspase activation, which may be used to induce programmed death and apoptosis in tumor cells, it is necessary to inhibit PD-1 protein on immune cells. CRISPR-mediated treatments have been tested

in 13 clinical studies so far, with one research even attempting in-vivo gene editing by the injection of CRISPR-Cas9 editing tools. The remaining papers all focus on in vitro modifications of immune cells utilizing CRISPR-Cas9 systems. The effectiveness of PD-1 knocked-out T cells in treatment metastatic non-small cell lung cancer is being studied in a clinical study. This research uses CRISPR/Cas9 technology to knock off the PD-1 gene ex vivo after isolating autologous T-cells from the peripheral blood. Infusing patients with designed and enlarged PD-1-knockout-out T-cells follows the initial injection of cyclophosphamide.

6.5.2 Breast Cancer

There are a variety of approaches that may be used while attempting to treat breast cancer. One strategy involves exploiting Cas9 proteins and information gleaned through Cas9 whole-genome dropout screens to silence oncogenes that drive cancer cell growth, metastasis, and treatment resistance. Similarly, CRISPR technologies can be used to find TNBC medications that, when used, can counteract the effects of molecular target death. Therapeutic targets for TNBC include medicines that suppress the maternal embryonic leucine zipper kinase (MELK), such as OTS167. Overexpression of the enzyme MELK can stimulate cell growth. Although histone deacetylase (HDAC) inhibition has failed to cure solid tumors and TNBC, reversible DNA methylation and histone acetylation by DNMT and HDAC inhibitors is another strategy to treating breast cancer. In a study of women with metastatic breast cancer, the HDAC inhibitor vorinostat was not beneficial when administered alone (Cheng et al., 2020). It was eventually determined that high levels of JAK1 and BRD4 were responsible for the activation of the LIFR-JAK1 STAT3 signaling pathway, which rendered HDAC inhibition ineffective. The effectiveness of HDAC inhibition in the treatment of TNBC was improved by silencing these two components. When used with paclitaxel, another HDAC inhibitor called romidepsin was efficient in treating aggressive breast tumors. After fusing with regulatory effector domains unique to particular genes, the dCas9 form of the CRISPR-Cas system may be used as an epigenetic regulation tool for a number of genes. LSD1-dCas9 fusion, for instance, has been shown to block certain enhancer genes, hence influencing breast cancer growth. Using gRNAs of varying lengths that are particular to each target locus, these dCas9 fusions have shown that they can activate tumor suppressor proteins and repress oncogenes concurrently, with great specificity and little off-target consequences in vivo. Synthetic biology genetic circuits can be used to further improve these dCas9 modulations. The use of

gRNAs with dCas9 to build genetic circuits is analogous to building logic gates, and it can aid in the targeted modification of breast cancer genes and the precise control of epigenetic states (B. Zhang, 2021). T-cells modified to produce CAR proteins can aid in the identification of antigens unique to breast cancer thanks to the use of Cas9 proteins, which can also be useful in enhancing immunotherapy. Clinical studies of a HER2-targeting CAR T-cell are presently underway. Targeting ER/PR, HER2, BRCA1/BRCA2, and other genes that have been linked to carcinogenesis and progression in breast tumors can be a very successful therapy strategy.

6.5.3 Colon and Rectal Cancer

Treatment options for metastatic colorectal cancer have been improved thanks to the identification and in-depth investigation of key tumor driving pathways as KRAS, TGF, WNT, PI3K, and TP53. Most cases of colorectal cancer (CRC) involve three to six driver mutations, and traditional treatments until recently did not provide the controlled deletion of all of the altered genes at once. However, advances like CRISPR-Cas9 technologies and the creation of organoids (miniature constructions of organ-like tissues) have allowed for more precise therapeutic interventions. For instance, it has been shown that deletion of APC results in WNT-independent organoid development and that alterations in SMAD4, TP53, KRAS, and PIK3CA also result in independent growth in human intestinal cells in vitro. Chromosomal instability, Microsatellite instability, and CpG island methylator phenotype are the three main pathogenic processes that lead to CRC. Mutations in genes such as BRAF, c-MYC, SMAD2, SMAD4, PIK3CA, PTEN, and RAS, as well as the WNT, PI3K, TGF, and TP53 pathways, all contribute to the spread of cell proliferation (C. Li et al., 2021). Furthermore, amplification of EGFR has been strongly linked to malignant progressions of CRC, making these cells a promising target for CRISPR-Cas9 based deletion treatment in CRC patients. Several possible targets in KRAS-mutated CRC have been discovered, including the fructose metabolism and nicotinamide adenine dinucleotide kinase (NADK). Mutations induced by CRISPR-Cas9 systems provide great insight into the identification of novel genetic targets for the diagnosis and treatment of colorectal cancers. CRISPR-Cas9 was used to fix a mutation in the -catenin gene located in the WNT pathway of the HCT116 human CRC cell line, and the results showed an increase in protein phosphorylation and a decrease in CRC cell proliferation in vitro (Mengstie & Wondimu, 2021). Mutations in BRAF, genes involved in DNA mismatch repair, and the microsatellite instability (MSI) gene family are all major contributors to

the development of CRC, which is characterized by the presence of serrated polyps. Serrated polyposis families and sporadic serrated polyps were evaluated for genetic alterations, and it was found that a high frequency of RNF43 mutations influenced by CRISPR-Cas9 reduced the dependence of organoids of bladed adenomas on the R-spondin growth factor, which is necessary for tumorigenesis. oxaliplatin, capecitabine,In addition, CRISPR-Cas systems can enhance the efficacy of a variety of cytotoxic medications, including oxaliplatin, 5-fluorouracil, irinotecan, oxaliplatin, leucovorin, etc., which have been shown to improve mCRC in patients(Huang et al., 2018) .

6.5.4 Melanoma

Several mutations that block tumor suppressor genes and activate proto-oncogenes like BRAF and KIT are responsible for melanoma carcinogenesis. The MAPK or ERK (extracellular signal-regulated kinase) system is controlled by BRAF, a protein kinase involved in growth signal transduction. Most BRAF alterations in metastatic melanomas are located in the gene's V600E region, whereas MAPK/ERK signaling promotes cell proliferation, secretion, and differentiation. An unchecked transcriptional response, unchecked cell proliferation, and an unchecked tumor development are all outcomes of the persistent amplification of the descending MEK/ERK pathway. For the treatment of melanoma, knockout of V600E- and V600K alterred BRAF kinase using CRISPR-Cas systems shows promise (Y. Yang et al., 2021). Phosphatase and tensin homologue (PTEN) genes are a kind of tumor suppressor gene unique to melanoma tumors; they work by blocking the survival, proliferation, and migration of cells. In addition to inhibiting cell survival, cell cycle progression, and the control of transcription, translation, and metabolism, the phosphatidylinositol 3-kinase (PI3K)/AKT pathway is regulated by PTEN through the conversion of PIP3 to PIP2. To put it another way, drug-resistant tumors are aided in their growth by mutations that cause PTEN to become inactive. PTEN levels are controlled in part by genetic alterations, but also by epigenetic changes such as transcriptional and post-transcriptional modifications. From either a panel of melanoma and TNBC, the SK-MEL-28 mammalian cell line was isolated; it was shown to harbor the BRAF V600E mutation and to be resistant to the B-Raf inhibitor dabrafenib. Using the dCas9-VPR transactivation method, we were able to restart PTEN transcription in SK-MEL-28, which led to a higher rate of endogenous gene activation (Sreedurgalakshmi et al., 2021).

By decreasing phosphorylated AKT and blocking cascade oncogenic signaling pathways important for cell proliferation and migration, PTEN activation reduced metastatic behavior and drug resistance. Additionally, when SK-MEL-28 cell responsiveness to B-Raf and PI3K/mTOR antagonists increased, the cells' ability to migrate was greatly diminished. Combining CRISPR-mediated PTEN activation with traditional small molecule inhibitors likeand dactolisib (PI3K/mTOR inhibitoand dabrafenib (B-Raf inhibitor) r) may lead to significantly improved tumor suppression and drug-resistance with minimal adverse effects (Yin et al., 2019).

6.5.5 Cervix Cancer

Molecular epidemiological and clinical investigations have shown that elevated human papillomavirus (HR-HPV) infections, which display strong carcinogenic qualities by prolonging the cell cycle of human keratinocytes, are the leading cause of cervical cancer. Both the host's p53 and RB regulating proteins can be deactivated by these oncogenic viruses' E6 and E7 proteins. Somatic mutations, g enomic instability, and HPV incorporation into the host genome are all exacerbated by latent infection and viral oncogene production, which in turn inactivates p53 and Rb. Despite DNA damage, the E6 protein can prevent cells from undergoing apoptosis and promoting telomerase activity, and it can also prevent the translocation of TP53 and its expression levels in the nucleus (Song et al., 2021). The E7 controls protein stability, which in turn promotes cell growth, cell cycle extension, and transformation, and it degrades RB tumor suppressors through interactions with growth factors. By binding to RB tumor suppressors, E7 proteins can both delay their degradation and extend the keratinocyte cell cycle. In addition to their role in regulating tumor metastasis via the PI3K/AKT signaling pathway, E7 proteins can also increase the production of cyclins A and E, inhibit cyclin-dependent kinase (CDK) activity, and stimulate cell cycle progression. Reactivation of TP53 and pRB is crucial to the CRISPR-Cas9 systems' ability to silence E6 and E7 and trigger apoptosis and cell death. CRISPR/Cas9 may be used to target the sequences of interest in the E6 mRNA, resulting in a drop in the quantity of E6 mRNA and a subsequent rise in TP53 protein levels, which in turn helps to inhibit malignancies. One study found that a decrease in E6 and E7 mRNA level, a boost in TP53 protein, a decline in RB protein, and the induction of apoptosis and inhibition of proliferation in SiHa cervical carcinoma cell lines were all the result of CRISPR/Cas9 targeting the promoters and accessible reading frames of E6 or E7 transcripts (Janik et al., 2020). Tumor growth suppression and apoptosis activation in vivo

were seen after CRISPR/Cas9-mediated targeted of E6/E7 mRNA of HPV16 or HPV18 infections, demonstrating the efficacy of CRISPR/Cas9 as prospective treatments for cervical cancer. In a separate research, CRISPR/Cas9 was crucial in making the cisplatin (CDDP) medication and radiation therapy more effective against cervical cancer caused by the human papillomavirus (HPV). Potential exists for CRISPR/Cas9 to work synergistically with various cytotoxic agents to repair and improve the activities of TP53 and pRB proteins by targeting E6/E7 (X. Zhang et al., 2020).

6.5.6 Osteosarcoma

Osteosarcoma is a kind of bone cancer, the precise cause of which is still largely a mystery. Rothmund-Thomson syndrome, hereditary retinoblastoma, Li-Fraumeni syndrome and Werner syndrome, and genes associated with these conditions, including TP53, RB1, WRN, and RECQL4, have all been linked to the development of this kind of cancer. Treatment for osteosarcoma often consists of neoadjuvant chemotherapy, surgical resection, and further procedures performed after the first resection (Sander & Joung, 2014). The toxicity of chemotherapeutic medications including doxorubicin's cardiotoxicity, cisplatin's carcinogenicity and otorrenal toxicity, methotrexate's bone marrow inhibition, ocular mucosal, liver, and kidney toxicity, and tumor resistance to chemotherapy have led to the search for more cutting-edge options. Knocking down CD11K genes in human osteosarcoma cell lines including KHOS and U2OS using CRISPR/Cas9 technology showed promising results in inhibiting migration, invasion, and cell proliferation, suggesting that this technique may be useful in the treatment of osteosarcoma. Elimination of GLT25D1 and GLT25D2 from human Saos osteosarcoma cells rendered them nonviable in a single research where oncogene knockouts were used (Gunsteren et al., 2013). Other genes that have been knocked out by CRISPR-Cas9 systems that impede growth, metastasis, and mobility include CD44 in human 143B and MNNG/HOS cells, CD81 in murine 143B cell lines, FGF5 in murine MG63 and U2OS cells, and many more. In addition to editing DNA, CRISPR/Cas9 may be utilized to make osteosarcoma cells more vulnerable to chemotherapy. After the mutant TP53 was knocked out in KHOSR2 osteosarcoma cells using the CRISPR-Cas9 method, the cells were more sensitive to Adriamycin. In addition, the doxorubicin and paclitaxel sensitivity of the KHOS and MNNG/HOS cell lines was improved by PD-L1 knockdown. Additionally, suppressor genes may be modified using CRISPR/Cas9 to investigate their biological functions and confirm their potential as

therapeutic targets. Research has shown that apoptosis is reduced in U2OS cell lines when the CNE9, CNE10, or STAG2 genes are knocked out (Tuckerman et al., 2000). In instance, knocking down CNE9 and CNE10 reduced cell growth, demonstrating their anti-cancer functions. Tumor invasiveness, PD-L1 gene expressions, epithelial-mesenchymal transition (EMT), changes in the expression of immune-related genes, and increased resistance to the chemotherapeutic agent cisplatin were all seen in STAG2 deletion cell lines. Thus, it was shown that STAG-2 deficiency in osteosarcoma makes tumors more vulnerable to PD-L1-mediated host immune response, suggesting that STAG2 might be exploited as a biomarker for PD-1- PD-L1 inhibitor treatment in this cancer.

Chapter 7

Future Direction and Limitation

Because of its effectiveness, simplicity, high adaptability, inexpensive, ability to edit numerous genes, and broad application, CRISPR-Cas systems are a promising gene editing technique for treating a wide range of disorders. CRISPR-Cas systems have shown great promise for therapeutic applications, but their ultimate fate is still uncertain because of their many drawbacks and difficulties. When dealing with the difficulties of CRISPR-Cas systems, preventing Cas-sgRNA from attaching to and cleaving off-target homologous sequences is a top priority. Off-target cleavage can result in alterations that can lead to cancer progression, deletion of suppressor genes, chemotherapy resistance, and other undesirable outcomes. To maximize sgRNA/DNA mismatch sensitivity while minimizing off-target impact or collateral cleavage, it is essential that chosen sgRNAs be highly selective and short(Waldt et al., 2021). Another unsolved problem is the improvement of gene knock-in efficiency. Given that HDR repair only occurs during the G2 and S phases of the cell cycle and that the target sites are constantly being re-edited, it is highly challenging to insert a precise alteration through this repairing route in reality. Improving HDR editing efficiency may be as simple as inhibiting DNA ligase or silencing genes encoding proteins involved in the NHEJ process.

Suppression of histone dynamics by Cas9 may also contribute to its ineffectiveness. The inability of Cas9 proteins to cleave can be overcome by extending the duration of the experiment, giving the Cas proteins more time to locate their intended cleavage site. Activating the immune system in humans might be difficult at times as well. Cas proteins, which originate in bacteria, trigger harmful responses in human cells, activating the immune system to produce antibodies against the bacteriophage.

These antibodies can block the therapeutic effects of CRISPR. Two alterations in the epitope-anchoring residues of Cas9 can reduce its immunogenicity. Another difficulty with CRISPR-Cas technology is that each Cas protein needs its own unique PAM sequence in order to target particular places in the genome. The efficacy of CRISPR in identifying and treating various malignancies can also be considerably impacted by the Cas proteins' increased sensitivity to RNA secondary structures and RNA's instability in the presence of RNase. The absence of a safe and

efficient delivery route is a persistent barrier for the expanding number of applications using CRISPR-Cas9-mediated modified T-cells in cancer treatment.

Minor subclone outgrowths are just one problem caused by tumor heterogeneity. Therefore, anticipatory identification of these overgrowths and the use of numerous Cas9/gRNA may be useful in reducing relapse in patients undergoing these treatments. Neurotoxicity, in the form of cytokine release syndrome, is a new problem made more acute by the use of CAR T-cell treatment in tandem with CRISPR-Cas systems. This method is not only time-consuming and costly, but also calls for a sizable number of doctors and other medical staff. These technologies have the potential to completely change the way cancer therapies are developed and administered due to their capacity to control the expression of genes through mechanisms like as silencing, activating, repressing, translocating, duplicating, and inverting. While CRISPR-Cas technology has already helped in certain ways, its potential benefits to oncology and cancer therapy have not been fully realized. However, due to the tumor's heterogeneity, a combination therapy is typically necessary even when diagnosis occurs at a more primitive stage of tumor growth. In addition, this causes resistance to develop and tumor growth due to the effect being targeted on certain genes, a situation that may be improved by the use of CRISPR-Cas systems. One of the few successful trials undertaken by CRISPR-Cas systems has been in non-small cell lung cancer, and recent research have shown that CRISPR-Cas can target several target genes in a heterogeneous tumor mass. CRISPR-Cas based treatment applications have the potential to expand beyond cancer with further study and development. Furthermore, the new methods have the potential to enhance existing therapy methods and, even better, uncover novel methods that eradicate the genomic and epigenomic features of cancer(Jin et al., 2020).

Chapter 8

Conclusion

Positive research outcomes over the past few years have made it obvious that CRISPR-Cas systems have enormous therapeutic promise in treating genome-based disorders like cancer, but effective clinical application of this therapy is strongly dependent on the limitations imposed delivery methods. There is a lot of excitement and anticipation surrounding the early phases of the many ongoing clinical trials using this method. Rising expectations, however, must be met with cutting-edge improvements that permit wide-scale clinical translation while still guaranteeing optimal efficiency, specificity, and safety. Despite the obstacles, the ongoing advancement of CRISPR-Cas systems will undoubtedly lead to significant improvements in the methods now used to treat cancer.

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