A Review on CRISPR-Cas System Based Applications in Oncology

By

Mir Sadia
18146057

A thesis submitted to the School of Pharmacy in partial fulfillment of the requirements for the degree of
Bachelor of Pharmacy

School of Pharmacy
Brac University
February 2022

Declaration

It is hereby declared that

1. The thesis submitted is my 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.
4. I have acknowledged all main sources of help.

Student's Full Name & Signature:

Mir Sadia
18146057

Approval

The thesis titled “A Review On CRISPR-Cas System Based Applications in Oncology” submitted by Mir Sadia (18146057) of Spring 2018 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Bachelor of Pharmacy.

Examining Committee:

Supervisor:
(Member)

Tanisha Tabassum Sayka Khan
Lecturer, School of Pharmacy
Brac University

Program Coordinator:
(Member)

Namara Mariam Chowdhury
Lecturer, School of Pharmacy
Brac University

Deputy Chair:
(Member)

Dr. Hasina Yasmin
Professor, School of Pharmacy
Brac University

Departmental Head:
(Dean)

Dr. Eva Rahman Kabir
Professor, School of Pharmacy
Brac University

Ethics Statement

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

Abstract

Cancer is one of the leading causes of death around the world. The ability to proliferate at a rapid rate, metastasize, and form heterogeneous tumors due to mutagenic occurrences renders cancer as one of the most difficult-to-treat diseases in the world. Owing to its high accuracy and efficiency, the gene editing tool Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems and CRISPR-associated (Cas) enzymes have shown immense potential in the treatment of genome-based diseases, especially cancer. The ability to deliberately activate or repress genes, allows CRISPR-Cas systems to discover novel targets for new potential anti-cancer drugs. In this review, the mechanism of different classes of CRISPR-Cas systems are summarized. Several other applications including diagnostic and therapeutic applications of CRISPR-Cas systems in the field of oncology are also addressed in this review.

Keywords: CRISPR-Cas; cancer treatment; immunotherapy; CRISPR applications; disease models; oncology

Acknowledgement

Foremost, I would like to thank Almighty Allah for blessing me with the knowledge, patience and strength to complete this thesis successfully.

I would like to express my deepest gratitude to my project supervisor, Tanisha Tabassum Sayka Khan (Lecturer, School of Pharmacy, Brac University), whose support and guidance have helped me to complete this project in a proficient manner. I would also like to express gratitude to the School of Pharmacy for giving me the opportunity to complete my thesis and for the wonderful guidance given to me by the faculties over the last four years.

Finally, my utmost gratitude and appreciation go to my parents who have provided me with endless love and support throughout the ordeal. Without them, I wouldn't have been able to make it this far.

Table of Contents

Declaration ii

Approval iii

Ethics Statement iv

Abstract v

Acknowledgement vi

Table of Contents vii

List of Table x

List of Figures xi

List of Acronyms xii

Chapter 1 Introduction 1

1.1 Background 1

1.2 Objectives of the Study 2

1.3 Rationale of the Study 2

Chapter 2 Methodology 4

Chapter 3 Cancer 5

3.1 What is Cancer 5

3.2 Prevalence of Cancer 6

3.3 Types of Cancer 7

3.3.1 Lung cancer 7

3.3.2 Breast cancer 7

3.3.3 Colorectal cancer 8

3.3.4 Melanoma 9

3.4 Treatment Options for Cancer 10

3.4.1 Surgery 10

3.4.2 Chemotherapy and Radiation Therapy 11

3.4.3 Targeted Therapy 12
3.4.3.1 Kinase Inhibitors 12
3.4.3.2 ALK Inhibitors 12
3.4.3.3 Non-Receptor Tyrosine Kinase Inhibitors 12
3.4.3.4 CDK Inhibitors 13
3.4.3.5 Monoclonal Antibodies 13
3.4.4 Immunotherapy 14
3.4.5 Other Treatment Strategies 14
Chapter 4 CRISPR-Cas System 16
4.1 What is the CRISPR-Cas System? 16
4.2 Classification and Mechanism of CRISPR-Cas Systems 22
Type I system 24
Type II (Cas9) system 25
Type III (Cmr/Csm) system 26
Type V system 28
Type VI (Cas13) system 28
Chapter 5 CRISPR-Cas System in Oncology 31
5.1 Targets for Gene Editing 32
5.1.1 Oncogenes 32
5.1.2 Cell Death-related Genes 33
5.1.3 Epigenetic Genes 33
5.1.4 Immune-related Genes 34
5.1.5 Viral Oncogenes 35
5.1.6 Tumor Microenvironment-associated Gene Targets 35
5.1.7 Others 35
5.2 Delivery of CRISPR-Cas System to Tumor Cells 36

5.2.1 Viral Vectors 36
5.2.2 Non-viral Vectors 38
5.2.2.1 Chemical Methods 38
5.2.2.2 Physical Methods 40
5.3 Applications of CRISPR-Cas System in Oncology 41
5.3.1 Application of CRISPR-Cas System in Cancer Modeling 41
5.3.2 Application of CRISPR-Cas System in Combating Oncogenic Virus infection 42
5.3.3 Application of CRISPR-Cas System in Cancer Diagnostics 43
5.3.4 Application of CRISPR-Cas System in Cancer Immunotherapy 46
5.3.4.1 CAR T-Cell Immunotherapy 46
5.3.4.2 T Cell Receptor (TCR)-Based Adoptive T Cell Therapy 47
5.3.4.3 Novel Target Screening for Cancer Immunotherapy 48
5.3.4.4 Inhibition of Immune Checkpoint Signaling Pathway 49
5.3.5 Application of CRISPR-Cas System on Treatment of Different Types of Cancer 50
5.3.5.1 Lung Cancer 50
5.3.5.2 Breast Cancer 51
5.3.5.3 Colorectal Cancer 52
5.3.5.4 Melanoma 53
5.3.5.5 Cervical Cancer 54
5.3.5.6 Osteosarcoma 55
5.3.5.7 Others 57
Chapter 6 Limitations and Future Direction 58
Chapter 7 Conclusion 60
References 61

List of Table

Table 1: Characteristics of the different types of CRISPR-Cas systems 30

List of Figures

Figure 1: Illustration of the adaptation mechanism in the Type I-E system 18

Figure 2: Illustration of the crRNA processing and interference 19

Figure 3: Mechanism of action of the CRISPR-Cas9 system 22

List of Acronyms

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 1

Introduction

1.1 Background

The epidemic of cancer has caught the entire world in a stronghold due to its increasingly sophisticated and evolving tendency to metastasize to heterogeneous tissues through uncontrollable proliferation. The etiological origin underlying such pathological conditions is highly complex, however, environment and genetics are two of the many risk factors perpetuating the condition. According to Hanahan and Weinberg (2000), the causal reasoning driving tumorigenesis and its development are inactivation of tumor growth suppressors, maintaining proliferation signaling, bypassing cell apoptosis, prolonging cell life, aiding angiogenesis, and allowing metastasis. The correct study and characterization of underlying etiology can help researchers and clinicians tremendously in honing the appropriate treatment strategies. In another study, Hanahan and Weinberg (2011) provided an extensive deep dive on their previous characteristics as well as introduced several other hallmarks such as genetic mutation, unstable genome, inflammation, evading immune responses, deregulated cellular energetics, as well as the heterogeneity of tumors in certain cells, such as stromal cells.

Throughout the years, technological advancements have given rise to small molecules and antibodies that target key proteins in the signaling pathways of oncogenes. Although agents like imatinib targeting BCR-ABL in myeloid leukemia or antibodies targeting EGFR in colorectal cancer lower tumorigenesis or tumor development, various other treatment options are still insubstantial when it comes to providing fruitful treatments or tackling resistance mechanisms due to restricted in-depth knowledge in oncology (Zhan et al., 2019). Technological advancements have also brought upon the advent of countless genome-editing molecular tools for the purpose of creating genomic profiles of phenotypes for different diseases. Zinc-finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs) are two of the many molecular tools capable of targeting and modifying genomic sequences. However, these tools were laborious and costly due to the need for individual customization of each DNA target. ZFs also exhibited widespread binding at unnecessary sites which reduced their clinical applications. Clustered, regularly interspaced, short palindromic repeats (CRISPR)/ CRISPR-associated (Cas) protein

systems, another recent breakthrough in medical science, is a system consisting of a programmable RNA molecule and either archaic/bacterial proteins that are capable of targeting sequence-specific genes of various functionalities, such as profiling cancer-causing mutated genes (Song et al., 2021). These systems are highly efficient in treatment strategies for cancer therapy as well as oncology research as they aid in provoking genetic, epigenetic, and transcriptional mutations and evaluating the resulting diseased phenotype (Moses et al., 2018).

1.2 Objectives of the Study

The primary objective of this review is to communicate the significance of CRISPR-Cas systems as an effective diagnostic and therapeutic tool for cancer treatment. To be more specific, the study intends to explore the underlying factors responsible for tumorigenesis, highlight the preexisting treatment options for it, comprehend the working mechanisms of CRISPR-Cas systems in genome editing, underline its applications and the various modes of delivery, highlight the contribution of CRISPR-Cas systems in the treatment of various cancers, and delineate its limitations and future prospects.

1.3 Rationale of the Study

It can be claimed that genome engineering may be the next biggest approach when it comes to the field of oncology. The discovery of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems and Cas enzymes have further revolutionized the way in which cancer is being researched, diagnosed, and treated in patients. Unlike the previous genome editing systems such as the ZFNs and the more recent TALENs, the CRISPR systems are simple and versatile, as well as cost-effective (Song et al., 2021). The many CRISPR-Cas systems enable genomic editing at several target regions on a chromosome, resulting in the therapy of a wide spectrum of hereditary disorders, including cancer. This article provides an overview of the mechanics and uses of various CRISPR-Cas systems. Furthermore, the importance of CRISPR as a diagnostic and therapeutic tool for cancer, as well as alternative CRISPR-Cas delivery tactics employing vectors and other

physical mechanisms, are also discussed. Finally, the article underlines its role in treating various forms of cancer and prospects in oncology.

Chapter 2

Methodology

The information collected in this article were collected mostly from online database resources such as MDPI, PubMed, ResearchGate, Frontiers, Nature, Elsevier, ScienceDirect, and Cell. The information prioritized were mostly from recent years and consisted of research articles, review articles, and reports. The keywords used in searching for relevant information pertaining to the topic included “CRISPR-Cas systems”, “CRISPR application in cancer treatment”, “CRISPR-Cas classifications”, “Types of cancer”, “Cancer treatments”, “oncogenic virus”, “immunotherapy”, “CRISPR-based diagnostics”, CRISPR-Cas delivery systems”, “CAR T-cell therapy”, “Chemotherapy”, “Lung cancer”, “Breast Cancer”, “Colorectal cancer”, “Melanoma”, “Epigenetic editing”, etc. These articles were then reviewed and those with relevant information were included in this review paper. In addition, the papers cited in the reference at the end of this article were also included when appropriate. Duplicate articles were also removed manually from the list before the paper was finalized. The aim of this review paper is to briefly relay the recent findings on the diagnostic and therapeutic applications of CRISPR-Cas systems on different cancer treatments.

Chapter 3

Cancer

3.1 What is Cancer

According to Moses et al. (2018), cancer is a highly complex disease originating in any tissue or organ where under the influence of genetic mutation or environmental carcinogenesis, cells undergo uncontrolled proliferation and metastasize to other organs in the body. The onset of tumorigenesis and its development is brought upon by genetic interventions of three main types of genes: tumor suppressor genes, DNA repair genes, and proto-oncogenes (*What is Cancer?*, 2021). A series of mutations occur concurrently thereby changing the functionalities of these 3 gene types which results in uncontrolled proliferation of normal healthy cells. These phenomena are further reinforced by the influence of environmental chemicals having carcinogenic characteristics that have a profound effect, direct or indirect, on the cell leading to mutations of the gene and thereby genetic disorders. Several risk factors play hand-in-hand in culminating genetic mutations, these are mainly genetic, environmental, and gene-environment interactions. Environmental risk factors include viruses, radiation, and bacteria which account for about 7% of all cancer incidences (Moses et al., 2018).

According to Hassanpour and Dehghani (2020), the normal healthy cell cycle is regulated by proto-oncogenes, however, under mutagenic influences, these genes are activated into oncogenes. Concurrently the lack of or inactivation of tumor suppressor genes also brought upon by mutations, sets off an uncontrollable proliferation of cells. In any normal circumstances, DNA repair genes would take the lead in translating essential proteins and enzymes for repairing such damages, however, due to the damage induced by mutations, DNA repairs are unable to compensate for the uncontrolled proliferation. There are numerous ways in which mutation may happen, affecting the healthy sequences of the genome. Oncogene and its resulting genetic abnormalities occur due to chromosomal translocation which can be seen in the case of genes *Bcr* and *Abl* for chronic blood cancer. Examples of point mutation are seen in the *Ras* gene of colon cancer, deletion is seen in *Erb-B* gene in breast cancer, insert activation is exhibited in *C-myc* of acute blood cancer, and amplification mutation in *N-myc* of neuroblastoma. Moreover, it is the gene *p53* mutation that is

found to elicit abnormal proteins in 60% of cancer cases, whereas in normal circumstances these same healthy *p53* proteins would have played a vital role in regulating cell death, cell division, senescence, differentiation, angiogenesis, and metabolism of DNA.

Apart from DNA mutation, oncogenic alteration is also initiated at other stages in order to bring about abnormal cell proliferation including DNA methylation, splicing alteration, and posttranscriptional or posttranslational modifications. Hassanpour and Dehghani (2017) also stated that hypomethylation induced in repeated sequences results in an increased gene deletion thereby emanating in chromosomal instability. Some of their stated examples of hypomethylation in inducing ectopic expression of oncogenes can be seen in *MASPIN* tumor suppressor genes in prostate and breast cancer, *SNCG* in ovarian and breast cancer, *S100P* in pancreatic cancer, *MAGE,* and *DPP6* in melanoma. On the other hand, hypermethylation of specific promoter regions can inhbit the transcription of genes involved in repairs such as *Hmlh1* and *BBRCA1*, response to vitamins such as *CRBP1* and *RARB2,* apoptosis such as *DAPK1*, and *WIF-1*, and cell cycle control such as *P16INK4b* and *P16INK4a*. As a result, they can work as novel biomarkers in diagnosing cancer in oncology.

3.2 Prevalence of Cancer

According to estimates from the World Health Organization (WHO) in 2019, cancer is now the second leading global cause of death, targeting population groups below the age of 70 in over 100 countries (*Global Health Estimates: Leading Causes of Death*, 2020). Over the years the mortality statistics have only increased with approximately 35% of cancer patients dying in the United States alone by 2014 (Hassanpour & Dehghani, 2017). The existence of cancer is therefore a foreboding threat that will continue to eradicate more people if effective treatment methods are not yet discovered and implemented soon. Without proper intervention, this gradual increase is predicted to result in 28.4 million new cancer cases in 2040 from 19.3 million new cancer cases in 2020. According to Sung et al. (2021), this rising prevalence may be attributed to the declining cases of stroke and cardiovascular diseases, growing life expectancy resulting in increased older population groups, as well as several other risk factors related to socio-economic development. The total number of cancer cases and the death toll in 2020 alone surmounted to approximately 19.3 million

and 10 million worldwide, respectively, with female breast cancer being the highest occurring and lung and prostate cancer being the second and third.

3.3 Types of Cancer

3.3.1 Lung cancer

According to Sung et al. (2021), lung cancer is the second most prevalent cancer in the world with an estimated 2.2 million new cancer incidences and 1.8 million deaths in 2020 alone. Lung cancer is mainly classified into 2 types- small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) based on the appearance of the tumor cells under microscope. Depending on the extent to which lesions are invaded, NSCLC is further categorized into adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Travis, 2011). According to Toyooka et al. (2011), the majority of lung cancers are affected by mutations at the epidermal growth factor receptor (*EGFR*) genes which code for receptor-type tyrosine kinase on cell membranes and their subsequent signaling mechanisms. Ligands, upon binding to receptors, then form hetero- or homodimers, which allows for cell growth signaling as well as other activities. A mutation in exon site 18-21 is what sets off the independent activation which ultimately leads to carcinogenesis. Mesenchymal Epithelial Transition (MET) is another tyrosine kinase receptor onto which hepatocyte growth factor (HGF) ligands bind inducing cell proliferation, invasion, and metastasis, and it is this *MET* gene secondary amplification mechanism lung cancers opt for in acquiring resistance to EGFR-TK inhibitors (EGFR-TKIs), like gefitinib and erlotinib. Furthermore, other genetic mutations associated with lung cancer have also been reported in *PI3K-AKT-mTOR*, *TITF1*, *RAS-RAF-MEK*, *P16-RB*, and *P14-MDM-2-P53*, etc.

3.3.2 Breast cancer

Based on a report by Sung et al. (2021), breast cancer has preceded lung cancer in becoming the most prevalent cancer in the world with over 2.3 million new cases worldwide in 2020 alone. There are multiple risk factors, both modifiable and non-modifiable, instigating the emergence of breast cancer such as female sex hormones, pregnancy, breastfeeding, genetic mutations, obesity,

ethnicity, alcohol, age, smoking, menopause/menstruation, physical activity, family history of cancer or cancerous disease of the breast, chemical or radiation exposure, processed food, drugs, insufficient vitamin supplementation, etc (Lukasiewicz et al., 2021). According to Mintz et al. (2018), classifications of breast cancer are based on three parameters, namely estrogen receptor (ER) expression, amplification of human epidermal growth factor receptor-2 (HER2), and *BRCA1/BRCA2* gene mutation. Accordingly, there are four types- Luminal A, Luminal B, Basal-like, and HER2-amplified.

According to Łukasiewicz et al. (2021), luminal breast cancers are ER-positive tumors that can sometimes differentiate into tubular, invasive lobular, invasive cribriform, invasive micropapillary, and mucinous carcinomas. They also stated that luminal-like tumors A and B are differentiated based on pathways of proliferation and luminal regulation, whereby Luminal A tumors contain/express an ER and/or PR but lack *HER2* expression and Luminal B tumors are ER-positive with PR-negative and/or HER2-positive. According to Chen & Zhang (2018), endocrine therapies are used for ER pathways in order to suppress ER functions or reduce the estrogen ligands for activation. However, due to acquired resistance, they are in constant need of improvement.

Łukasiewicz et al. (2021) also state that HER2-enriched breast cancer exhibits a high expression of the *HER2* gene which includes proliferation-related genes and proteins, without ER and PR expression. Moreover, a mutation occurs in the APOBEC3B resulting in cytosine mutation biases, creating further mutation clusters. Basal-like or triple-negative breast cancer (TNBC) is characterized by an absence of ER, PR, and HER2 expression, as well as accounts for BRCA1 and/or BRCA2 germline mutations. The study also stated that TNBCs can be further subdivided into basal-like (BL1 and BL2), mesenchymal stem-like, immunomodulatory, mesenchymal, and luminal androgen receptor, and an unspecified group.

3.3.3 Colorectal cancer

According to Sung et al. (2021), colorectal cancer garnered around 9.8% of new cases worldwide in 2020 alone, following closely behind breast and lung cancer. It consists of several subtypes namely, adenocarcinoma, adenosquamous, undifferentiated carcinomas, squamous cell, spindle cell, and neuroendocrine (Liu et al., 2019). According to Yoshihara et al. (2007), some of the environmental risk factors catapulting colorectal cancer include diet, smoking, alcohol

consumption, hyper- or hypoglycemia, protein factor imbalances in the blood, etc. Porru et al. (2018) state that, colorectal cancer stems from three different pathogenic mechanisms namely CpG island methylator phenotype, microsatellite instability, and chromosomal instability. According to them, some of the vital genes mutated in colorectal cancers are the *BRAF*, *cMYC*, *PTEN*, *SMAD2*, *PIK3CA*, the family of *RAS* genes, etc. which are involved in cell proliferation. These RAS are components of the mitogen-activated protein kinase (MAPK) signaling pathway which is initiated by ligands binding to receptor tyrosine kinase, like EGFR, and ultimately results in cell growth and differentiation. In general, EGFR is a receptor tyrosine kinase that primarily sets off all the intracellular signals responsible for the proliferation of many different cancer cells, their differentiation, angiogenesis, and metastasis.

Porru et al. (2018) also state that the resistance to anti-EGFR treatments such as humanized monoclonal antibodies- cetuximab and panitumumab were met with *KRAS* gene mutations of the RAS-RAF-MEK-ERK signaling pathway, which is why the *RAS* genes are usually tested for before initiating such treatments. Furthermore, the risk of colorectal cancer is also greatly increased in cases where the *APC* gene which helps in preventing cancer gets defected (Yoshihara et al., 2007). According to new research conducted, CHDH1, HNF4A, LAMB1, miR-196a2, SNP rs60-17342, and C allele of SNP rs11614913 genes, etc. are susceptible to developing colorectal cancer.

3.3.4 Melanoma

According to Sung et al. (2021), melanoma contributed to around 1.7% of new cancer cases worldwide in 2020 alone. Melanoma has a higher tendency among skin cancers in metastasizing to a greater extent throughout the body. According to the American Joint Commission on Cancer Tumor–Node–Metastasis (TNM) system, there are five stages of melanoma of increasing invasiveness. Stage 0 accounts for the intraepithelial disease, stage 1 covers localized cutaneous melanoma that is less than 2 mm thick, stage 2 encompasses tumors that are greater than 2 mm or 1-2 mm with ulceration, stage 3 includes regional nodes, and stage 4 accounts for the distant metastatic spread (Karimkhani et al., 2014).

Karimkhani et al. (2014) also state that melanoma prognosis takes a stronghold due to proto-oncogene activation from mutations on *BRAF* and *KIT* genes, as well as inhibition of tumor

suppressors. Moreover, the mutated BRAF accounts for the continual activation of downstream MEK signaling pathways via MAPK kinase or through ERK enzymes, resulting in excessive transcriptions and cell growth. Inhibitors of V600E- and V600K-mutated BRAF kinases such as vemurafenib and dabrafenib showed improvements in the survival of melanoma patients in a randomized phase 3 trial (BRIM-3). Moreover, the type 3 transmembrane receptor tyrosine kinase, C-kit binds to stem cell factors thereby propagating receptor dimerization, autophosphorylation, and subsequent downstream activation of signaling pathways, including MAPK and PI3K pathways responsible for gene transcriptions and cell growth. These genetic mutations on the C-kit are prevalent in 36 % of chronic, 28 % of acral, and 39 % of mucosal melanomas.

3.4 Treatment Options for Cancer

3.4.1 Surgery

According to the National Cancer Institute, surgery is a technique in which the surgeon removes an entire solid tumor, or debulks (removing parts of a tumor) tumor from the body by the use of sharp tools such as knives or scalpels alongside the administration of anesthesia. Removing a tumor or a part of the tumor helps with reducing majority of the cancerous cells and allows better therapeutic adherence from later treatment strategies. The other forms of non-invasive surgeries include cryosurgery, laser, hyperthermia, photodynamic therapy, etc (*Surgery for Cancer*, 2015). Cryosurgery or cryotherapy employs the extremely cold temperature of liquid nitrogen/argon gas to damage cancerous cells or tumors and its main applications lie in treating skin cancers at an early stage, retinoblastoma, and initial tumor growths on the cervix and skin. Laser treatment involves firing powerful beams of light to cut through tumor tissues, shrinking or destroying them. These are mainly done on the surface of the body or on the internal lining of organs such as basal cell carcinoma, cervical, esophageal, vaginal, non-small cell lung cancer, etc. Hyperthermia involves small areas of tissues to high temperature in order to damage and kill cancerous tumors and deem them sensitive to radiation therapy and chemotherapy drugs in later therapies. Photodynamic therapy calls for administering drugs that activate by reacting to a specific type of light, thereby damaging cancerous tissue. It is used in treating non-small cell lung cancer, skin cancer, etc (*Surgery for Cancer*, 2015).

3.4.2 Chemotherapy and Radiation Therapy

According to Baudino (2015), chemotherapy implements the use of agents that target rapidly dividing tumor cells as well as certain normal cells, thereby inhibiting tumor growth and metastasis. Radiation therapy, on the other hand, utilizes ionizing radiation in killing cancerous cells directly. The more traditional approach of radiation and chemotherapy offer widespread effects on the body, even non-cancerous cells and as a result, these therapies offer greater side effects such as pain, diarrhea, nausea, cardiotoxicity, alopecia, hyperpigmentation, and immunosuppression, as well as a greater resistance in resulting cancer in the event that both therapies fail to work. Moreover, the normal cell types that do experience a greater risk of side effects are cells that have a higher rate of division including those from bone marrow, hair follicle, digestive tract, etc.

Tanvetyanon et al. (2005) state that chemotherapy and radiation therapy may be used adjunctively as neoadjuvant, adjuvant, as well as concomitant therapy depending on the severity/invasiveness of cancer. This allows cancer to be attacked by different approaches, rendering it very weak before alternative treatments may be used whilst preventing resistance. Neoadjuvant therapy calls for decreasing the size of the tumor through surgery before ultimately finishing it off of other treatment approaches. Adjuvant therapy, on the other hand, is used after surgery when other used approaches fall short of removing the cancerous cells/tissues. Adjuvant therapies depend on hormone reception, cancer type and stage, lymph nodes. These therapies are of five types namely chemotherapy, radiation therapy, hormone therapy, targeted therapy, and immunotherapy.

According to Baudino (2015), chemotherapeutic agents include a vast list consisting of alkylating agents such as cyclophosphamide, topoisomerase inhibitors such as camptothecin, anthracyclines such as daunorubicin, plant alkaloids such as vinblastine, pyrimidine, and purine antimetabolites such as mercaptopurine. These agents elicit their anti-cancer effects by interfering with cell division or the synthesis of DNA. on one of the cell cycle phases. As a result, they can be classified as either cell-cycle non-specific or cell-cycle-specific. Alkylating agents, for example, are non-cell cycle-specific compounds that damage nuclear and mitochondrial DNA by adding alkyl groups causing the DNA strands to form cross-links and break or undergo point mutations eventually resulting in apoptosis. Although they have greater effectiveness against leukemia and solid tumors, due to their non-cell cycle specificity, they have greater side effects.

3.4.3 Targeted Therapy

According to Zhong et al. (2021), targeted therapy is specific to cancer cells with high potency and low toxicity. Imatinib, the very first US Food and Drug Administration (FDA) approved small-molecule drug is a tyrosine kinase inhibitor targeting BCR-ABL in myeloid leukemia. Targeted drugs are of two types: small molecules and macromolecules. Small-molecule drugs, like their name suggests, are small enough to invade into the cells where they target vital proteins responsible for controlling the growth of the tumor cells and their metastasis. Macromolecule examples include monoclonal antibodies, antibody-drug conjugates, polypeptides, and nucleic acids.

3.4.3.1 Kinase Inhibitors

Zhong et al. (2021) state that kinases are enzymes that catalyze the γ-phosphate transfer from ATP to protein residues that have hydroxyl groups attached. These protein kinases are vital in performing critical functions such as cell growth, division, and differentiation. Protein kinases are further classified based on the substrate residues that they catalyze, and these include tyrosine kinases, serine/threonine kinases, and tyrosine kinase-like enzymes. Dysfunctional protein kinases have been credited to a myriad of diseases, namely cancer making protein kinases the most accurate biomarkers as well as therapeutic targets for cancer. There are a number of protein kinase inhibitors namely kinase inhibitors, BCL-2 inhibitors, epigenetic inhibitors, proteasome inhibitors, etc.

3.4.3.2 ALK Inhibitors

According to Zhong et al. (2021), the *ALK* gene encoding anaplastic lymphoma kinase (ALK) are proteins that catalyze the activation of downstream signaling pathways and have an important role in nervous system development. Activation of the mutated *ALK* has been associated with countless human cancers such as anaplastic large cell lymphoma, diffuse large B-cell lymphoma (DLBCL), non-small cell lung cancer (NSCLC), and inflammatory myofibroblastic tumor.

3.4.3.3 Non-Receptor Tyrosine Kinase Inhibitors

Zhong et al. (2021) state that as a member of the non-receptor tyrosine kinase Abl family, the c-Abl is a Bcr-Abl1 inhibitor that is encoded by Abelson murine leukemia 1 (*ABL1*) gene on

chromosome 9 and it has been involved in the regulation of vital functions within the cell such as cell differentiation, cell cycle and growth, and cell survival. Philadelphia (Ph) chromosome translocation is a phenomenon wherein the ABL1 and the breakpoint cluster region (BCR) present in chromosome 22 fuse together to form an abnormal BCR-ABL fusion gene which encodes an oncoprotein, p210 Bcr-Abl1 that induces autophosphorylation and the downstream signaling pathways catapulting uncontrolled proliferation of tumor cells.

3.4.3.4 CDK Inhibitors

According to Zhong et al. (2021), cell cycle abnormalities result in uncontrolled cell proliferation and have been considered one of the important hallmarks of cancer. Cyclin-dependent kinases (CDKs) are enzymes controlling cell cycle growth progression with the help of cyclin proteins in the activation of downstream phosphorylation signaling pathways. CDK inhibitors consist of ribociclib, palbociclib, and abemaciclib, whereby they exhibit their functionality by specifically targeting CDK4/6.

3.4.3.5 Monoclonal Antibodies

According to Zhong et al. (2021), another approach of targeted therapy involves macromolecular hybridoma which are fusions of B cells with cancer cells (myeloma). These hybridomas disrupt the functions of cancerous cells by either of three mechanisms: via antibody-dependent cytotoxicity, by interfering with protein function and resulting downstream signaling pathways, or via complement-dependent cytotoxicity. Monoclonal antibodies are created in order to work against the target proteins deregulated during tumorigenesis.

Due to the newer monoclonal antibody acquiring humanized fusion cells, the resulting macromolecules are safer and more efficacious for the human body, compared to their former mouse counterpart. One of the most successful monoclonal antibodies is the adalimumab or Humira tried for the treatment of rheumatoid and psoriatic arthritis, ulcerative colitis, Crohn's disease, etc. and it binds to tumor necrosis factor-alpha (TNF-alpha) and blocks their bioactivity as well as propagates the TNF-expressing mononuclear cells' apoptosis (*HUMIRA® (Adalimumab) | A Biologic Treatment Option*, 2013).

3.4.4 Immunotherapy

Immunotherapy is a biological therapy that amplifies or suppresses the body's own immune system in fighting diseases. T-cell transfer therapy, also called adoptive immunotherapy, is a type of immunotherapy that reinforces the body's T cells in being better capable of attacking cancer cells. T-cell transfer therapy is of two types: Chimeric antigen receptor (CAR) T-cell therapy and tumor-infiltrating lymphocytes (TIL) therapy (*T-Cell Transfer Therapy - Immunotherapy*, 2020). The two therapies require collecting immune cells from a patient and culturing them in large quantities. The patient's own immune cells are collected, cultivated in large numbers, and then these T cells are administered via needle back into the patient through the vein. According to the National Cancer Institute (2020), chemotherapy and radiation therapy can be performed before T-cell therapy in order to increase the effectiveness of this immunotherapy that results from reduced overall immune cells in the body and makes space for the transferred T cells.

There is a variant of T cells invading the tumor in the body referred to as tumor-infiltrating lymphocytes (TIL), which functions as novel biomarkers in determining the type of tumor cells. After identification, these lymphocytes are then cultured into larger quantities before administration to patients. The large quantities of lymphocytes help with overcoming the suppressing signals released by tumor cells (*T-Cell Transfer Therapy - Immunotherapy*, 2020). Another variant of immunotherapy is the CAR T-cell therapy, and although similar to the TIL therapy, the CAR T-cell requires prior modification of T cells into a type of protein called the chimeric antigen receptor (CAR) before they can be cultured and administered back into the patient. The CAR proteins function by aiding the T cells in attaching to specific proteins present on the surface of cancer cells, thus reinforcing their anti-cancer ability (*T-Cell Transfer Therapy - Immunotherapy*, 2020).

3.4.5 Other Treatment Strategies

Cancer stem cells (CSCs) are yet another target in treating cancer as well as preventing their relapse. To be specific, the targets or stages mainly attacked in these CSCs include surface markers, signaling pathways, interfering with microenvironment signals, inhibition of efflux pumps, modifying miRNA expression, inducing CSCs apoptosis and differentiation (Dragu et al., 2015).

Utilizing a patient's cancer genetic profile allows for personalized and more effective treatment strategies targeting both genetic and epigenetic sites. According to Janik et al. (2020), epigenetics is identified with the later modifications made to genetic expressions unrelated to any direct DNA sequences. The processes associated with epigenetics include methylation and demethylation of DNA, posttranslational modifications, modification of chromatin structure, and as well as other various biological processes. According to Sachdeva et al. (2015), examples of epigenetic targets include DNA methyltransferases (DNMTs) aiding in methylation and histone deacetylase in posttranslational modifications. So far, these enzymes have only amounted to clinical failures with a lot of the DNMT inhibitors disintegrating in neutral aqueous solution and forming toxic analogs, wherein their efficacy is considerably reduced due to rapid inactivation by cytidine deaminase enzymes in the liver.

Epigenetic proteins are incapable of binding to their respective targets due to the absence of certain proteins that aid in binding. As a result, they produce non-specific widespread genetic expression, causing a variety of side effects. Sachdeva et al. (2015) stated that reengineering epigenetic proteins in permanently binding to specific targets can be done in a number of ways, either by using nucleic acid or proteins. The nucleic acid-based method of editing implements the use of small interfering RNA (siRNA) molecules that target either the nucleus where they induce methylation via regulating cognate genes or the cytosol where they induce post-transcriptional silencing of target genes by degrading the precursor mRNAs.

Genetic modifications by proteins mainly occur by recognizing specific sites on the gene via the DNA-binding domain present on the protein, which is further fused to a secondary effector domain on the protein that targets the specific sites on the gene. Two well-approved developments are the ZF (zinc finger) and TALE (transcription activator-like effector) domains. Sachdeva et al. (2015) also stated that it is still less likely for these molecular editing tools to be used for epigenetic editing since their highly compacted targets are difficult for protein domains to access and bind to. Even so, endonucleases aided by RNA have shown promising results when used in adjunction with transcription-activating drugs.

Chapter 4

CRISPR-Cas System

4.1 What is the CRISPR-Cas System?

The advent of clustered regularly interspaced short palindromic repeats (CRISPR) from *Escherichia coli* as well as CRISPR associated proteins (CRISPR-Cas) from genes adjacent to CRISPR regions, as an adaptive prokaryotic immune system has revolutionized the way in which healthcare is able to provide specific acquired immunity against abnormal mutagenic genes or genetic elements such as plasmids and viruses, essentially altering the field of molecular biology (Janik et al., 2020). Identified in the year 1987 from the *Escherichia coli* genome, the CRISPRs consist of 29 nucleotide repeat sequences which are separated by 32 sequences of a nucleotide called the spacers. These spacer sequences only function during the emergence of and contact with a phage DNA. Over the years CRISPRs were also found in archaea, and phylogenetically diverse archaeal and bacterial genomes, with around 84% of CRISPRs coming from archaeal genomes and 45% from bacterial genomes (Grissa et al., 2007).

The system by which CRISPR-Cas systems operate in aiding the acquired immune system in prokaryotic cells from incoming genetic elements involves mechanisms or processes resembling that of RNA interference (RNAi) systems in eukaryotes. According to a report made in 2007 by Barrangou et al. (2007), the lactic acid bacteria *Streptococcus thermophilus* struck with a viral infection from a bacteriophage caused it to integrate new spacer sequences from the foreign nucleic acid fragments of viral phage, thus allowing the bacteria to acquire modified resistance phenotype to the phage. Removal or addition of certain spacer sequences corresponding to the invading genetic elements in the CRISPR-Cas system thus allowed resistance specificity to that particular phage. Thus, CRISPRs analogous to such a system provide the same mechanism in acquiring immunity in which a typical CRISPR-Cas system consists of two separate components joined together: an RNA molecule called the guide RNA or gRNA containing the spacer sequences corresponding to the invading genetic elements or healthy normal genetic sequences, and Cas enzymes which are the RNA-protein complexes functioning in dsDNA endonuclease activity (Janik et al., 2020).

Spacer sequences essentially aid in offering a sequence-specific memory for targeted defense from the invasion of exogenous genetic elements. According to Song et al. (2021), the process of immune response involves the CRISPR being transcribed and processed into CRISPR RNA forms called the crRNA. These crRNAs guide the Cas endonuclease in recognizing and cleaving the complementary nucleic acid of invading genetic elements. In case of cancer, these molecular tools show great capability in manipulating the defected genome, epigenome, and transcriptome as well as the immune-related cells by a myriad of approaches. Some of these strategies have been included in engineered T-cells, either allogeneic or autologous, for immunotherapy in several clinical trials. The clinical applicability of the CRISPR/Cas systems in treating cancer is dependent on gene target selection, delivery strategies, and CRISPR/Cas tools enhancement in absence of unnecessary off-target effects.

According to Liu et al. (2019), the prokaryotic CRISPR-Cas system mechanisms are conducted via three stages which include adaptation, pre-CRISPR RNA or crRNA expression or maturation, and interference. The first stage, adaptation, starts off by expressing a complex of Cas proteins from the CRISPR-Cas loci followed by the Cas proteins binding to the target sequences on the DNA, which subsequently ends with two double-strand breaks in the target regions of the DNA. Recognition of Cas proteins onto the target regions is completely determined by 2-4 base short motifs called the Protospacer Adjacent Motif (PAM). After the double strands break, the segment released from the target region, the protospacer, is adjoined between two repeat sequences in the CRISPR assembly, now acting as the new spacer sequence specific to that target DNA. During the maturation or expression stage, the sequences in the CRISPR array get expressed, allowing transcription to form a single long pre-crRNA which then further matures into crRNA with the help of Cas proteins and other accessory factors. Each of the mature crRNA consists of a single spacer specific to the invading genetic material flanked by repeat sequences. In the interference stage, the mature crRNA functions as a guide RNA recognizing similar sequences in the invading genetic RNA, such as the viral RNA, and allowing cleavage and inactivation by Cas proteins, thus allowing protection to the host cells from the invading infection. The expression and interference stages in each of the CRISPR-Cas systems are unique (Liu et al., 2019). The adaptive and heritable defense mechanism of prokaryotes employing Type I-E, II and III CRISPR-Cas systems have been depicted in Figure 1 and 2.

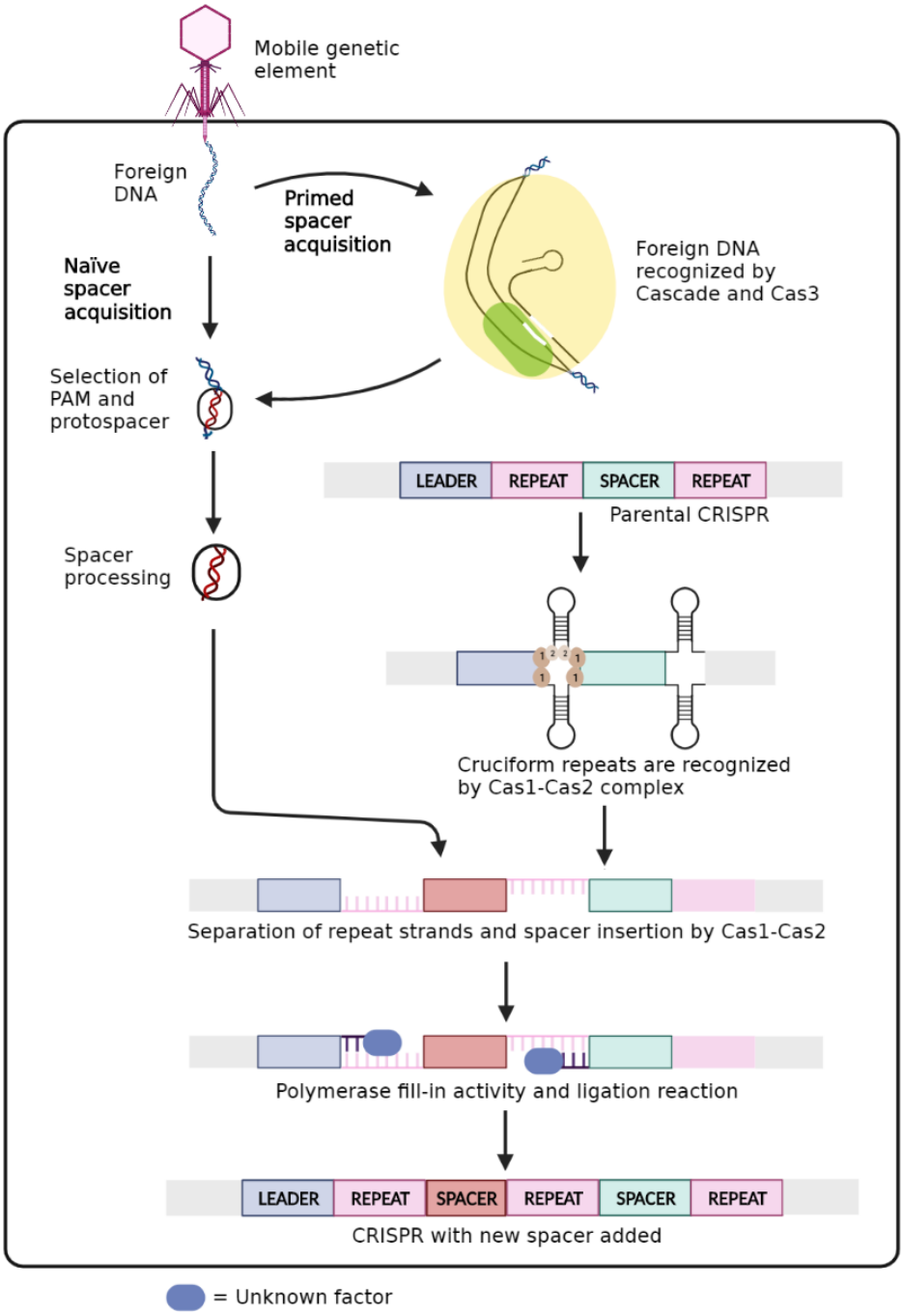

Figure 1: Illustration of the adaptation mechanism in the Type I-E system. There are two types of acquisition phase: primed and naïve, both requiring a PAM (Protospacer Adjacent Motif) and being dependent on Cas1-Cas2 complex. The Cas1-Cas2 complex is responsible for CRISPR array recognition and is known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

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 Rath et al., 2015).

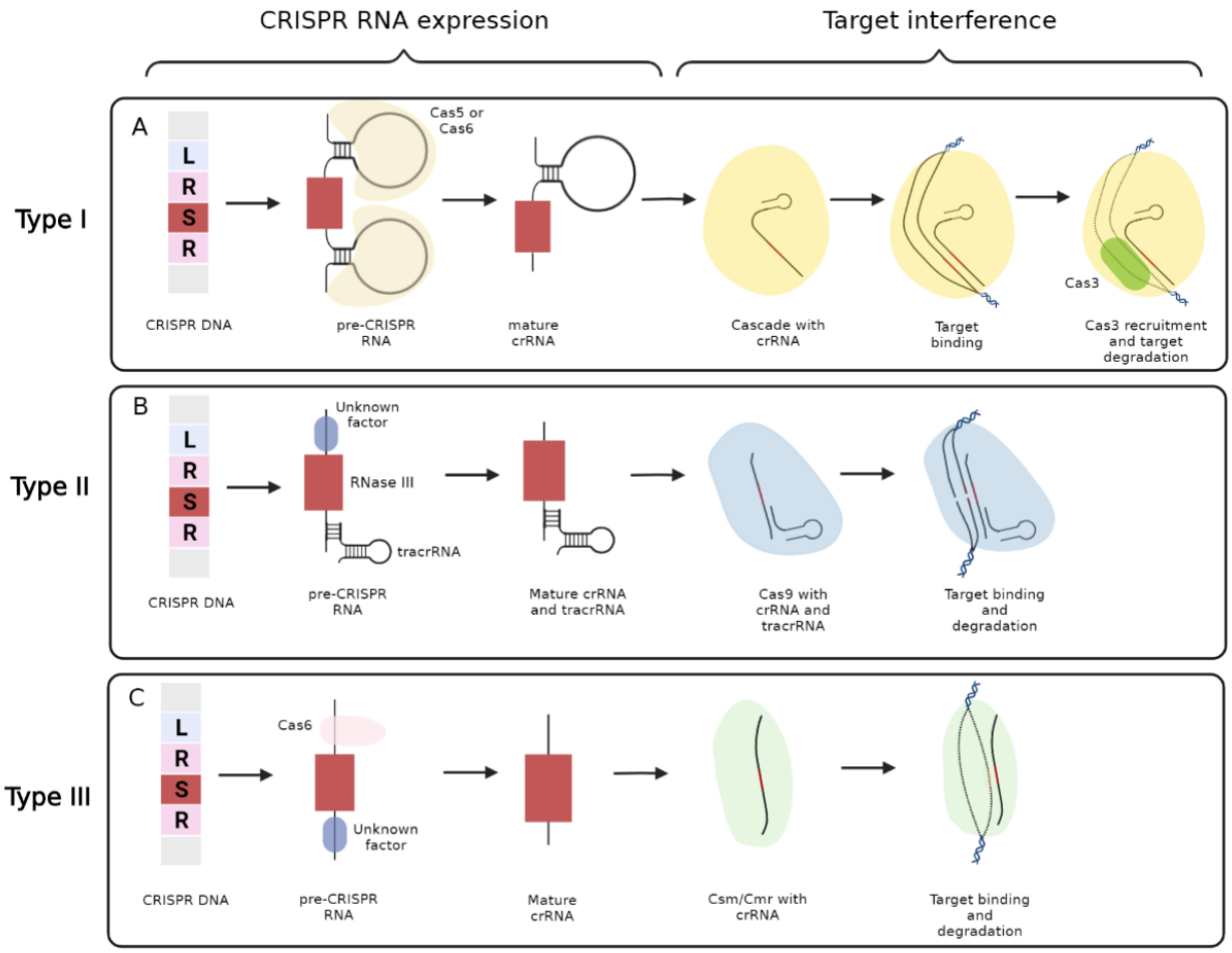

Figure 2: Illustration of the crRNA processing and interference. (A) In the Type I system, Cas5 or Cas6 is used to process the pre-crRNA. Moreover, the target interference utilizes Cas3 proteins including the Cascade and crRNA. (B) In the Type II system, RNase III and tracrRNA are used for processing 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 Rath et al., 2015).

According to Janik et al. (2020), within a specific CRISPR locus, the lengths of the spacer sequences and the repeats are well conserved and specific, although they might differ between other CRISPRs in the same or a different genome. CRISPR repeat sequences range from 23–55 nucleotides, each containing palindromic sequences that form hairpin structures. Likewise, spacers range from 21–72 nucleotides (Grissa et al., 2007). The numerous Cas proteins available bind to nucleic acids, enabling CRISPR systems as a highly adaptive tool in genomic engineering. Unlike the earlier programmable gene-editing molecular tools such as ZFN and TALEN, the CRISPR–Cas tools are easier and faster to use, cheaper, and have a higher targeting efficiency which ultimately leads to fewer side effects (Janik et al., 2020). ZFNs consist of zinc finger protein chains that are fused with nucleases forming systems that are able to make site-specific double-strand breaks (DSBs) by site-specific targeting as they can recognize a 3–4 base pair DNA sequence. The FokI nuclease exists in dimer form which allows for the DSBs. Although sharing structural and dimerization similarities to ZFN, the TALEN provides more specificity in recognizing specific DNA base pairs using the TALE proteins (composed of a C-terminal nuclear localization signal, an N-terminal translocation signal, an activation domain, and an intermediate tandem repeat region). The disadvantage of both ZFNs and TALENs is the induction of mutation at off-target sites (Ates et al., 2020). Moreover, new proteins need to be created each time for targeting a new sequence in the target DNA.

Janik et al. (2020) also stated that, unlike the ZFNs and TALENs, the CRISPR-Cas systems such as the CRISPR-Cas9 containing the Cas9 proteins need modifying only at the short sequence of gRNA for site-specific cleavage. It has greater efficiency due to modifications being made via the direct insertion of RNAs. The system also facilitates multiple gene modifications at a single time since many gRNAs can be introduced into the CRISPR array at the same time. This is due to the target sequences generally situated immediately upstream of the PAM sequence, consisting of short sequences that appear once every eight base pairs, allowing for the design of several gRNAs for one specific target. Moreover, Liu et al. (2019) also state that the CRISPR-Cas9 systems rely on Watson–Crick base pairing which allows for RNA–DNA recognition rather than the protein–DNA-binding mechanism in ZFNs and TALENs.

In vivo or *in vitro* CRISPR genome editing in humans employs several components and two important repair pathways for the introduction or removal of genes into the target site. According to Mengstie and Wondimu (2021), CRISPR-Cas9, being one of the most widely used systems in

treating several human diseases, comprises three major steps in its mechanism: recognition, cleavage, and repair. The designed specific single gRNA (sgRNA), composed of both crRNA and trans-activating crRNA (tracrRNA), recognizes the target site sequence via Watson and Crick base pairing. The Cas9 then gets activated upon recognizing the PAM sequence at 5′-NGG-3′ and cleaves the target site 3 bp upstream to the PAM sequence, creating DNA double-strand breaks (DSBs). The HNH domain of Cas9 cleaves the complementary strand whereas the RuvC domain cleaves the non-complementary strand of the target genome.

According to Xu and Li (2020), the resulting DSBs are repaired by one of the two major mechanisms of the higher eukaryotic host cell: nonhomologous end-joining (NHEJ) and homology-directed repair (HDR). Alongside not requiring an exogenous homologous template, the increased activity of the NHEJ pathway in 90% of the cell cycle allows for the induction of increased genetic mutations, through the introduction of random insertions or deletions (indels) in the target site. These indels can lead to subsequent frameshift mutations or premature stop codon, ultimately resulting in inactivation of the target gene. The potential for inducing loss-of-function mutations makes this pathway suitable for applications involving immortalized cell lines such as cancer treatment, albeit causing permanent loss of gene functions (Li et al., 2020). Mengstie and Wondimu (2021) state that the HDR pathway utilizes an exogenous homologous repair template to introduce highly specific modifications at the target site, such as gene insertions or replacement. This pathway is the most active in late S and G2 phases of a cell cycle. Thus, NHEJ and HDR can induce the desired targeted gene disruption and integrations, respectively. A general mechanism of the CRISPR-Cas9 system has been illustrated in Figure 3.

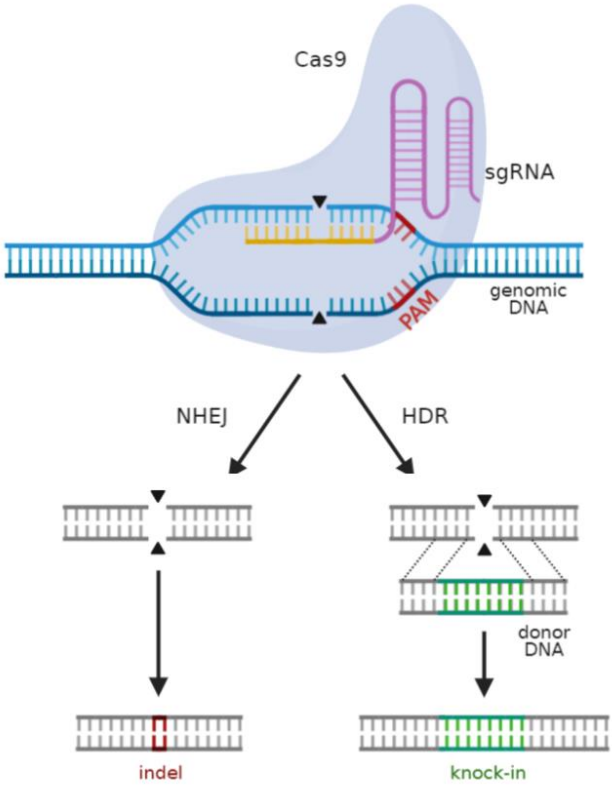

Figure 3: Mechanism of action of the CRISPR-Cas9 system. The sgRNA guides the Cas9 endonuclease to the target site and binds to the complementary DNA region upstream of the PAM sequence by forming a Cas9-sgRNA or RNP complex. The nuclease domain of the Cas9 cleaves the double strands at the target site 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).

4.2 Classification and Mechanism of CRISPR-Cas Systems

There is a large variety of CRISPR-Cas systems in different classes with a distinct set of repeat sequences and genes. The variation mainly lies in the genetically and functionally diverse nucleases that are used to target different regions in a target DNA, some of the most common ones being Cas9, Cas12a, Cas13a, and their respective orthologues (Song et al., 2021).

Effector module structures containing unique Cas proteins account for the different targeted cleavage in each and every system. According to Kumar et al. (2020), CRISPR-Cas systems comprises two classes, class I and class II. The two classes are each divided into three different types, whereby Type I, III, and IV are in class I and type II, V, and VI are in class II. Furthermore, each of these 6 types is again classified into 33 further subtypes (Makarova et al., 2018). These unique Cas proteins consist of unique RNA recognition motifs as well as other functional domains that help with the interaction of different nucleic acids, nuclease motifs, and helicases (Janik et al., 2020). So far, Cas 1 and Cas 2 have been common nucleases in different types of systems whereas Cas3, Cas10, and Cas9 have specifically been found in types I, III, and II, respectively (Makarova et al., 2018). According to Liu et al. (2019), gene sequence, secondary structure, and length of gRNAs are some of the many factors that can influence the specificity and efficiency of these systems. Moreover, target gene locus, accessibility of chromatin, nucleosomes, gRNA sequence as well as other components surrounding the binding sites have an impact on the efficiency of the CRISPR-Cas systems (Chen et al., 2017). The initial 10–12 nucleotides present at the 3' end of gRNA, immediately beside the PAM sequence, also known as the seed sequence, bind to the target gene sequence and confer their specificity. A brief overview of the characteristics of different CRISPR-Cas systems has been highlighted in Table 1.

According to Kumar et al. (2020), in the class I system, the ribonucleoprotein (RNP) complex consists of a crRNA and multiple protein subunits whereas, in a class II system, the RNP employs a single protein subunit alongside a crRNA in targeting the invading viral RNAs. Cas9 proteins belonging to the class II system utilize the tracrRNA and RNase III in processing the pre-crRNA, whereas the Cas13 protein of the type VI system and Cas12 proteins of the type V systems process pre-crRNA single-handedly. The double-stranded target DNA cleavage by Cas12a and Cas12b proteins are based on recognition by the mature crRNA, whereas the Cas13 protein cleaves the target ssRNA. The Cas13 proteins require no PAM in the target RNA, while the Cas12 proteins require PAM in the dsDNA target. Activation of the Cas13 is brought about by complementary binding between the crRNA and the target RNA, subsequently degrading the collateral ssRNA. This property allows for the diagnosis of RNA virus infections. On the other hand, similar activity can also be seen in Cas12 proteins, allowing for the detection of ssDNA viruses.

As stated earlier, the adaptation stage of all CRISPR-Cas systems shares commonalities, albeit the expression and interference stages are unique to each. According to Burmistrz et al. (2020), after

the CRISPR array containing invading spacers is transcribed and matured into crRNA with the aid of Cas proteins and accessory factors, the crRNAs are incorporated into the RNP complexes along with Cas proteins. These RNP complexes recognize and bind to sequences in the nucleic acids complementary to the sequences encoded by crRNA. This recognition activates the interference stage, ultimately degrading the recognized nucleic acid.

Type I system

Type I system is characterized by the Cas3 proteins consisting of both helicase and DNase domains that play a part in degrading the target. According to Rath et al. (2015), there are six subtypes of the Type I system, Type I-A through Type I-F, varying in the number of Cas genes wherein all the systems encode a Cascade-like complex, except for Cas1, Cas2, and Cas3. The Cas3, however, does take part in the cascade complex of the Type I-A system. The cascade facilitates the binding of crRNA and locating of target genetic material after crRNA processing has been done. Moreover, it also enhances spacer acquisition in some of the variants. In type I systems, for most of the variants/sub-types, Cas6-like nucleases are responsible for the primary processing of the pre-crRNA in creating mature crRNAs flanked by a short 50 tag (van der Oost et al., 2014). Type I systems utilize the workings of multiple Cas protein subunits as well as relies on PAM for integrating invading spacers into the host genome for the CRISPR array.

As stated by Makarova et al. (2018), the effector Cas enzyme is an HD nuclease that withholds a Cas3 helicase domain that is essential for target binding. In the Type I system interference phase, cascade nucleolytic activity is steered by the crRNA in binding to the foreign target DNA on a sequence-specific basis and recruits Cas3 for degrading any of the displaced strands via exonucleolytic activity. Unlike other subtypes, type I-B needs Cas4 for adaptation alongside Cas1 and Cas2 whereas type I-F CRISPR-Cas system requires interference machinery to propagate new spacer uptakes. Type I-C systems, on the other hand, do not code for Cas6 enzymes causing the pre-crRNA to be processed by the encoded Cas5d.

Type II (Cas9) system

According to Rath et al. (2015), the Type II system encodes Cas1, Cas2, Cas9, and oftentimes a Csn2 or Cas4 protein. The Type II system is further divided into II-A, II-B, and II-C subtypes. The *CSN2* and *CAS4* genes, both encode for proteins facilitating the adaptation process and are present in Type II-A and Type II-B systems, respectively, whereas a fourth gene is not found in the Type II-C system. Cas9, the signature protein aids in the adaptation process, processing crRNA and cleaving the target DNA with the help of crRNA and tracrRNA. Sachdeva et al. (2015) state that the CRISPR–Cas9 has revolutionized the development of new tools in the field of research and biotechnology. Its simplicity is owed to the requirement of a single Cas protein in genome editing. According to a report by Barrangou et al. (2007), the spacer sequences aid in targeting, wherein the spacer acquisition and phage defense are controlled by Cas9 enzymes. The type II systems consist of at least three parts: the Cas endonucleases, mature crRNA, and tracrRNA. Although there are several Cas protein loci in the different subtypes, only Cas9 is able to demonstrate endonuclease activity (Sachdeva et al., 2015).

As stated earlier by Liu et al. (2019), in the adaptation phase, invading virus or plasmid spacer sequences are integrated into the host chromosome allowing the CRISPR array to be flanked at one end. This is followed by the CRISPR arrays being transcribed into pre-crRNA in the expression phase. According to Sachdeva et al. (2015), in the interference stage, RNase III is utilized in cleaving the hybridized crRNA–tracrRNA and removing the 5′ end of each spacer sequence in order to produce mature crRNAs that are bound to both Cas9 and tracrRNA. The crRNA guides Cas9 in inducing its endonuclease activity and cleaving the target DNA. CRISPR utilizes the Watson and Crick base pairing to bind to the complementary target DNA via the help of PAM in short trinucleotide recognition. Without PAM, the CRISPR array is unable to recognize the target sequence complementary to crRNA, which consequently leads to CRISPR completely ignoring these sequences. Essentially PAM acts as a suitable site in which two strands are able to separate upstream of PAM by allowing R loop formation between the target DNA and the Cas9. In one study, it was discovered that binding of Cas9 to the target alone is insufficient in maximizing the nuclease catalytic activity, rather the extent to which PAM-distal target and 5' crRNA sequence exhibit complementarity is what determines the cleavage activity.

Sachdeva et al. (2015) state that HNH and RuvC/RNase H-like domains are two distinct endonuclease domains characteristic to Cas9 that facilitate the catalyzing of the target DNA strand

cleavage. HNH nuclease domain mainly cleaves the target strand complementary to the crRNA spacer sequence, while the RuvC-like domain cleaves non-target strands allowing the double-stranded DNA cleaving Cas9 to be converted into a nickase. Furthermore, after cleavage of the virus or plasmid DSB, the Cas9 endonuclease repairs the ends by nonhomologous end joining, leading to frameshift mutations including insertions or deletions. Prior to the target DNA cleavage, site recognition is induced by a guide RNA scaffold created from the crRNA-tracrRNA fusion. Therapeutic applications of CRISPR-Cas require site-specific disruption which can be brought about by constructing a system comprised of Cas9 and a guide RNA scaffold in which a 20-nucleotide-long target sequence customized alongside a 5′-NGG-3′ PAM sequence can be integrated. In order to avoid cleavage at potential off-target sites, CRISPR-Cas systems must be designed with the utmost specificity.

Both activation and repression of a target gene can be induced by the CRISPR-Cas9 systems. Kaushik et al. (2019) state that Cas9 bound to target DNA can be exempted from cleaving by inducing point mutations at the spacer sequence of Cas9, which results in nuclease sites such as RuvC and HNH becoming inactivated. The resulting Cas9 is a dead Cas9 protein (dCas9) that has lost its ability to cleave despite being able to bind to the gene sequence. Alongside proteins such as transcriptional activators or repressors, these dCas9 are able to form dCas9 fusion proteins which bind to transcription sites, thereby modulating activator-like activation or repressor-like suppression of the target gene. Moreover, epigenetic engineering for chromatins can also be conducted by Cas9 enzymes upon combining these proteins with epigenetic modifiers like TET1 and p300.

Type III (Cmr/Csm) system

According to Burmistrz et al. (2020), the Type III CRISPR-Cas system which belongs to class I is divided into four subtypes, namely A (Csm), B (Cmr), C, and D. This system is characterized by the presence of a Cas10 protein comprising two domains: the palm domain and the HD-type nuclease domain (Makarova et al., 2018). The type C and D systems, however, lack cyclase activity in the palm domain and lack an HD domain entirely, respectively. The RNP complex in this type of system consists of two parallel filaments, whereby six subunits of Cas7 make up the first one, and the latter is formed from three subunits of a Cas11 homolog, either Csm2 or Cmr5. The crRNA

molecule which encompasses these filaments have their 5' end capped by Cas5 and Cas10 proteins, which accommodates the repeat derived handle. Burmistrz et al. (2020) further state that the maturation or expression stage of this system involves 2 significant steps in the majority of the subtypes. The pre-crRNA transcript gets digested into one spacer sequence by the action of Cas6 enzymes. In the case of a few type III systems, the Cas6 variants play a role in the crRNA processing, whereby the secondary processing leads to the formation of separate varieties of crRNA proteins, each having a different length. A distinctive feature of this system is that it exhibits three separate nuclease activities. The first activity involves cas7 proteins catalyzing the cleavage in sequence-specific sites in which the targeted RNA is placed alongside the crRNA in the RNP prior to being digested. This 'ruler' mechanism is specific to type III systems and brings about 6 nucleotide long intervals of digested RNA molecules. It was demonstrated that nuclease activity was not affected by the lack of complementarity between the target genome and crRNA or the presence of mismatched pairing.

Burmistrz et al. (2020) also state that the second activity of the nucleases involves non-specific ssDNA cleavage which is dependent on the HD domain of Cas10 enzymes, which is a metal-dependent DNase, requiring protospacer sequence transcription. The transcription process starts off with the RNA polymerase exposing the antisense strand of the DNA to the HD domain of the Cas10 proteins in the RNP complex. The initiation of this process is entirely dependent on the complementarity between the target RNA and the crRNA, which ultimately brings about the Cas10 enzyme activation. Any lack in complementarity between the 5' crRNA end and the 3' targeted RNA protospacer region will inactivate the Cas proteins. This safety lock essentially shelters the host's CRISPR array from being targeted and cleaved. The final nuclease activity implements a non-specific RNA degradation. As the Cas10 proteins cleave the target region, the palm domain induces conversion of ATP into cyclic oligoadenylate which functions in activating Csm6 or Csx2 proteins. Like the ssDNA cleavage, the non-specific RNA degradation is activated by the RNP complex binding to the target RNA with non-complementarity between the target RNA and crRNA. Moreover, the Csm6 or Csx2 integrated into RNP complexes, consist of two domains: N-terminal CRISPR-associated Rossman fold (CARF) which detects the Cas10-produced cyclic oligoadenylate, and the C-terminal higher eukaryotes and prokaryotes nucleotide-binding (HEPN) which cleaves the RNA. An advantage of this multi nuclease activity is hypothesized to be preventing the mutants who avoid recognition from escaping.

Type V system

The Type V system is mainly known for the presence of Cas12a proteins also known as Cpf1. The system consists of several subtypes: A (Cpf1), B (C2c1), C (C2c3), D (Cas Y), and E (Cas X) (Makarova et al., 2018). Unlike the blunt ends created by the Cas9, the Cas12a as a single cRNA-guided enzyme generates a staggered end when cleaving the target gene as well as the PAM sequence during DNA acquisition. According to Song et al. (2021), by targeting unique PAM sequences, the Cas12a protein variants are able to target a wide range of locus on the target DNA and process the pre-crRNA on its own without the aid of any tracrRNAs. This shows great potential in cancer therapy due to the heterogeneity of tumors. Moreover, the non-specific cleavage activity of ssDNA allows for the CRISPR/Cas12a systems to demonstrate detection applications for viral DNA. According to Mintz et al. (2018), Cpf1 utilizes a T-rich PAM and RuvC-like domain for cleavage. Inhibiting the Ruv-C-like domain via mutation removes its catalytic activity entirely. The main differences between the Cpf1 and Cas9 are crRNA maturation without the need for tracrRNA, staggered DNA DSB, and target DNA cleavage adjacent to a short T-rich PAM.

Type VI (Cas13) system

Cas13a, or C2c2, is the primary Cas protein of the type VI system which targets the invading RNA. According to Song et al. (2021), upon target RNA binding, Cas13a activates the collateral cleavage at the off-target RNAs apart from the usual target RNAs. The RNA-targeting aspect of this system has been demonstrated in biomedical fields including detection of specific viral RNA sequences and tumor RNA in patients by its ability to control the various RNA molecules, both coding, and non-coding. Furthermore, after the mature crRNA recognize and bind to the complementary target sequences near the adjacent PAM sequences with the help of Cas proteins, a few of the Cas proteins get converted into non-specific ssRNase or ssDNase enzymes, cleaving any ssRNA or ssDNA alongside target nucleic acids. Additionally, the binding between crRNA and targeted RNA also triggers the conformational change of the RNP complex. This further induces both the HEPN domains to come closer together and create a single catalytic site (Liu et al., 2017). Burmistrz et al. (2020) also state that the presence of this catalytic site near the crRNA-targeted RNA or DNA adjunction propagates the cleavage of ssRNA and even ssDNA, alongside target nucleic acids. This non-specificity conferring off-target cleavage is referred to as collateral cleavage and can be utilized for the detection of nucleic acids in vitro. Moreover, the safety-lock mechanism of this

system prevents the RNA of the host from activating it. Similar to type V, the VI CRISPR-Cas system requires just the Cas13 protein and crRNA for genome editing. Type VI comprises four subtypes: VI-A (Cas13a or C2c2), VI-B (Cas13b or C2c6), VI-C (Cas13c or C2c7), and VI-D (Cas13d). Each of the subtypes is a variant of the Cas13, sharing a common feature of two HEPN domains and only differing in size and the sequence targeted.

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

CRISPR-Cas systems		Subtypes	Effector protein	Nuclease domains	Target	PAM Requirement	tracrRNA
Class I	Type I	A, B, C, D, E, F	Cas3	HD	DNA	—	Not needed
	Type III	A (Csm), B (Cmr), C	Csm3, Cmr4	Autocatalytic	RNA	—	Not needed
			Csm6, Csx1	HEPN	—		
		D	Cas10	HD	DNA		
	Type IV	A, B	Csf1	—	DNA?	—	Not needed
Class II	Type II	A, B, C	Cas9	RuvC, HNH	DNA (RNA for B)	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 VI	A (Cas13a), B, C, D	Cas13	HEPN	RNA	Low	Not needed

Chapter 5

CRISPR-Cas System in Oncology

Identifying new targets is essential for uncovering new drugs. This is the essence of drug discovery and development, whereby enhancing or repressing biological targets such as receptors and genes, will culminate in therapeutic effects for a particular disease after being approved in relevant preclinical and clinical studies. In oncology, drug discovery and development measures aim to identify molecules that can target genetic abnormalities in genes such as tumor suppressor genes and oncogenes that cause tumor progression. According to Martinez-Lage et al. (2018), some groundbreaking drug discoveries include vemurafenib targeting the *BRAF* V600E mutations in the case of melanoma; imatinib targeting the *BCR–ABL*1 fusions in chronic myeloid leukemia; or Osimertinib in treating mutated *EGFR* in non-small cell lung cancer. Genome engineering is of great benefit in identifying target genes propagating a certain tumor. However, unlike previous ZFNs and TALENs, the CRISPR-Cas system is quicker and less laborious, allowing rapid development of disease models that are far more efficient and precise (Ahmad & Amiji, 2018).

Cancer is caused by mechanisms that are impacted by underlying genetic factors. Understanding a disease's molecular genetics is critical in figuring out disease mechanisms. According to Tian et al. (2019), cell lines and animal models are significant in understanding the link between genotype, chemotherapeutic effects, and immunological milieu. Using lentivirus-directed Cas9-sgRNA, several inactivated genes such as *Runx1*, *Tet2*, *Dnmt3a*, *Ezh2*, *Nf1*, and *Smc3* were targeted in primary hematopoietic stem and progenitor cells (HSPCs) in order to create leukemia models. Moreover, the CRISPR/Cas technology also played a part in generating multiple cancer models, such as the colon cancer model whereby mutations were introduced in *APC*, *TP53*, *SMAD4*, *TP53*, *PIK3CA*, and *KRAS* genes (Tian et al., 2019). Furthermore, the CRISPR-Cas also identifies the interactions between different genes in inducing tumorigenesis which could be blocked for bringing about therapeutic effects.

Tian et al. (2019) stated that combinatorial gene screening and synthetic lethal drug target identification have been demonstrated by combined systems of CRISPR-based double knockout (CDKO) and a double sgRNA library system in K562 leukemia cells. Moreover, follow-up functional genome screening after initial CRISPR-Cas-related treatments could reveal changes in

gene expression and allow for resistant-gene identifications for targeted drugs. CRISPR libraries comprising the screening of genetic profiles of billions of functional variants provide the utmost precision when it comes to cancer medicine, even showing great potential for developing personalized genotype-based therapies from genome-specific targets (Tian et al., 2019).

5.1 Targets for Gene Editing

The selection of appropriate gene targets is essential for CRISPR-based anticancer therapy in order to maximize the efficacy whilst lessening the toxicity, at the same time accounting for interactions between tumor, host, and environment which influence the CRISPR treatments received (Jiang et al., 2019). The ability of CRISPR-Cas-based systems to precisely recognize target sequences makes it an effective tool in recognizing overexpressed or overactivated genes and regulating them to a molecular level (Jiang et al., 2019). Knockout of mutated, overactivated, or overexpressed target genes offer therapeutic end results when treating cancer.

5.1.1 Oncogenes

The onset of tumorigenesis is a result of multiple factors, such as overexpression or mutation of the oncogenes. In normal circumstances, malfunctioning or nonfunctional cells undergo programmed cell death or apoptosis. Activation of oncogenes causes uncontrolled cell proliferation and increases survival capability of the cancer cells. Knock-out approach for these specific mutated genes such as *EGFR, NESTIN, FAK, CTNND2, RSF1*, and *IGF1R* can be studied in lung cancer treatment, whereby the mutation of these genes has resulted in the lung cell's increased ability to proliferate and metastasize (Jiang et al., 2019). Some of the oncogenic proteins such as *Kras* and *Myc* have demonstrated increased functionalities in inhibiting tumor growth and regression of tumors *in vivo* upon genomic editing of mutant *Kras* and *Myc* genes by CRISPR-Cas systems, respectively (Song et al., 2021). These oncogenes are therefore vital for developing target specific cancer treatments.

5.1.2 Cell Death-related Genes

Targeting the cell death of tumor cells has proven to be another crucial approach for treating cancer. Genes related to apoptosis show an increased rate of cell death upon increasing their expression on cancer cells, thus presenting themselves as potential therapeutic targets in cancer treatment (Song et al., 2021). Successful cases of such implementation can be seen in the case of ovarian tumors dying upon apoptosis-related genes such as *MTH1* being disrupted using CRISPR/Cas9 plasmid systems, subsequently regressing tumor growth as well as preventing metastasis. Moreover, targeting other cell death-related events such as autophagy and necroptosis also confer potential therapeutic strategies for CRISPR-Cas systems in treating cancer.

5.1.3 Epigenetic Genes

Epigenetics involve heritable changes in the phenotype without any alteration in the genotype and it is essential for mammalian development. During this period, the epigenetic mechanical framework enables spatial and temporal control of genetic activity. Anomalous changes to the epigenetic processes in normal cells may, however, risk the actuation of malignant tendencies (Song et al., 2021). Genes expressing proteins regulating epigenetic processes such as DNA methyltransferase, histone-modifying enzymes, etc. are some of the potential therapeutic targets for CRISPR-Cas systems in treating cancer. Increased activation of histone-modifying proteins can either activate oncogenes or suppress tumor suppressor genes (TSGs). According to Song et al. (2021), CRISPR-Cas9 based disruption of an epigenetic-related gene such as DNA (cytosine-5) methyltransferase 1 (DNMT1) gene, which is responsible for the methylation of DNA after DNA replication during cell division, can significantly reduce expression of DNMT1, forcing the inhibition of paclitaxel resistance-acquired tumor growth *in vivo* (Song et al., 2021). DNA methylation is also important in silencing the expression of several oncogenes, however, non-specific interference with the activity of DNMT1 may lead to global loss of DNA methylation resulting in activation of the cancer promoting genes and anomalies in cellular functional integrity. Therefore, the DNMT1 knockout by CRISPR-Cas9 system should be cancer cell specific. In addition to DNA methyltransferases, histone modifying enzymes, whose abnormal production can either repress tumor suppressor genes (TSGs) or activate oncogenes are also potential therapeutic targets of CRISPR-Cas9 system (Song et al., 2021).

5.1.4 Immune-related Genes

Cancer cells require evasion of the host immunity for the tumor to grow and progress as well as acquire resistance against conventional immunotherapy (Song et al., 2021). Immune-related genes are preferred targets for CRISPR/Cas systems in genome editing for ensuring the proper immune responses are elevated to combat this resistance and allow cancer regression. Some examples include the disruption of PD-1 (programmed cell death 1) proteins via *PD-1* genes in engineered CAR-T cells for enhancing its anti-tumor capacity. Disruption of CD47 proteins via *CD47* genes exhibit immunotherapeutic potential for small cell lung cancer.

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), a membrane glycoprotein present on activated effector T-cells (Teffs), facilitates the repression of T-cell proliferation, cytokine production, and cell cycle progression (Zhao et al., 2018). Single nucleotide polymorphisms in the *CTLA-4* gene promoter region regulate CTLA-4 protein expression and are linked to different types of malignancies including colorectal cancer, cervical cancer, breast cancer, melanoma, renal cell carcinoma, etc., whereas a lack of these proteins is linked to esophageal cancer and breast cancer in certain ethnicities (Zhao et al., 2018).

According to Zhang et al. (2019), the use of targeted antibodies such as ipilimumab and tremelimumab in CTLA-4 can help block the inhibitory effects of CTLA-4 in T-cells in a variety of malignant cancer cases, including melanoma, non-small cell lung cancer, breast cancer, pancreatic cancer, prostate cancer, etc. Immune checkpoint proteins such as PD-1 and CTLA-4 activation on T-cells can result in exhaustion of T-cells and subsequent tumor progression. In a study conducted by Zhang et al. (2019), dysregulated checkpoint molecules in peripheral blood T-cells were disrupted by CRISPR-Cas9 in order to improve cytotoxic T-cell anti-tumor efficacy in bladder cancer. Using an anti-CTLA-4 antibody to block CTLA-4 and disrupting CTLA-4 by CRISPR-Cas9 allowed cytotoxic T lymphocytes to exhibit enhanced cellular immune response as well as conferred cytotoxicity to the CD80/CD86-positive bladder cancer cells (Zhang et al., 2019).

5.1.5 Viral Oncogenes

According to Song et al. (2021), most virus-associated malignancies result from the oncogenic characteristics of high-risk human viruses. The methodology by which critical sequences of viral oncogenes are targeted has shown potential in treating virus-induced cancers. CRISPR/Cas9 system-based delivery into HeLa cells, targeting either E6 or E7 (HPV oncogenes), leads to cell death.

5.1.6 Tumor Microenvironment-associated Gene Targets

The tumor microenvironment (TME) plays a vital role in progressing tumorigenesis. Components of the TME such as VEGF, which plays a pivotal role in multiple functions constituting angiogenesis of tumor, can function as effective targets for cancer treatment (Song et al., 2021). The antibody bevacizumab, alongside CRISPR-Cas9, has been shown to target the angiogenic factor VEGF in breast cancer patients and inhibit tumor growth. According to Song et al. (2021), the growth of orthotopic osteosarcoma and angiogenesis was also reduced immensely due to the utilization of CRISPR/Cas9 plasmid targeting the vascular endothelial growth factor A (VEGF-A) in treating osteosarcoma. Moreover, anti-angiogenic combined with other therapeutic approaches like checkpoint inhibitors or chemotherapy have offered effective therapeutic results in cancer treatment.

5.1.7 Others

According to Jiang et al. (2019), one particular study concerning lung adenocarcinoma in mice models showed an overactivation of Nrf2 and increased tumor survivability and growth in mice upon knockout of the *Keap1* gene using the CRISPR/Cas9 system. Another study demonstrated that the knockout approach on tumor-suppressor genes such as Mito fusion 2 (MFN2) in lung cancer increased cell survival, cell viability, cell growth, invasion, and metastasis upon upregulation of the mTORC2/Akt signaling pathway. Moreover, the genetic knockout of miR-1304 increases cell survivability and growth via increased heme oxygenase-1 (HO-1) expression. Chemotherapy resistance by tumor cells compromises the efficacy of chemotherapeutic agents used in treating the uncontrollable proliferation of malignant tumors. CRISPR/Cas9 systems may

be used to study sensitized and resistant genes to lung cancer chemotherapeutics such as carboplatin, cisplatin, and paclitaxel, and edit these genes in order to increase the sensitivity of tumor cells to chemotherapeutics (Jiang et al., 2019). According to a study, paclitaxel-resistant genes such as *RSF1* genes in H460 and H1299 cells were knocked out using CRISPR-Cas systems and administered alongside paclitaxel, resulting in cell cycle arrest, inhibition of cell proliferation and induced apoptosis. Moreover, the knockout of *ERCC1* by CRISPR/Cas9 also increases the sensitivity of lung cancer cells to cisplatin.

5.2 Delivery of CRISPR-Cas System to Tumor Cells

The greater molecular weight and negative charge of the RNP complex of Cas9 proteins prevent the Cas9 RNP molecule from crossing the cell membrane and targeting the genes for editing. Even inside the cells, the Cas9 proteins are forced to evade the harsh conditions of intracellular degradation enzymes. It is therefore essential that CRISPR/Cas9 system delivery is prioritized in order to confer safe and efficient gene editing. Delivery strategies for CRISPR/Cas9 are mainly classified into viral and nonviral approaches whereby the non-viral methods include various physical and chemical delivery systems.

5.2.1 Viral Vectors

Adeno-associated viruses (AAVs)

According to Yip (2020), AAVs are common viral vectors employed for the delivery of genes, standing out from other vectors due to their distinctive characteristics such as defective replication, lower immunogenicity in humans, and non-integration into the host genome. Upon transduction, these vector genomes reside episomally in the nucleus and eventually get diluted by cell division, providing safe transient genome expression (Yip, 2020).

CRISPR/Cas9 process AAVs for delivery in one of four ways. According to Lino et al. (2018), the first mechanism involves *S. pyogenes* Cas9 proteins encoding gene (SpCas9) and sgRNA packaged directly into a DNA plasmid, after which this plasmid is then delivered alongside one AAV particle. Certain enhancers/promoters or routes of administration may be used to facilitate the increased size a lot of these vectors may face in delivering gene-editing tools as well as

accommodating for other elements required for editing such as fluorescent tags, multiple sgRNAs, reporters, DNA templates in homology-directed repair, etc. Some examples include the use of mini-cytomegalovirus enhancer/promoter with SpCas9 to enhance correction of DMD-causing mutations in mice groups and the delivery of AAVs intramuscularly, intraperitoneally in enhancing muscle functions to differing extents (Lino et al., 2018).

The second approach involves AAVs delivering sgRNAs into cells previously expressing Cas9 after engineering. For example, in a cardiovascular research mouse model, Carroll et al. (2016) used AAVs to deliver sgRNAs into mouse cells after utilizing microinjection to transfect the mouse embryos so that Cas9 was expressed in cardiomyocytes for the rapid introduction of insertions/deletions in heart tissue. In the third approach, SpCas9 and sgRNA were packaged into two separate AAV particles in order to use them for co-infection. Moreover, Lino et al. (2018) state that the last approach involves S. aureus Cas9 (SaCas9) which allows for the usage of a single vector and also accommodates more space within the particle for multiple tags and markers. These have been used in these SpCas9 in AAV vector systems for targeting the *PCSK9* gene in regulating cholesterol.

Lentiviruses (LVs)

According to Yip (2020), LVs are another type of viral vector encompassing a greater cloning capacity than AAV vectors, enabling them to clone both sgRNA and Cas9 into a single LV vector. Apart from being less laborious, the LV transduction process enables a greater efficiency when it comes to performing functions in both dividing and nondividing cells, allowing for both in vivo and in vitro delivery at the cost of off-target integration in host cell genomes. The safety of such vectors may be promised by the developing integration-defective lentiviruses where plasmids express mutant integrase (Yip, 2020).

Adenoviruses (AVs)

Yip (2020) states that AVs are another group of vectors commonly used in clinical trials for the delivery of genes, transducing both dividing and nondividing cells without integrating them into host cell genomes. However, they do trigger a greater innate immune response which leads to tissue inflammation filled by AV vectors removal. For both LVs and AVs, the Cas9- and sgRNA-containing viral particles, used to infect the target cells, are created using the transformation of

HEK 293 T-cells, following a delivery mechanism similar to that of AAVs (Lino et al., 2018). In a recent study, AV was used to inactivate genes situated in normal human bronchial epithelial cells and lung fibroblasts.

Lino et al. (2018) also state that a distinctive lentiviral CRISPR/Cas9 system was synthesized via Golden Gate synthesis, whereby one Cas9 and four unique sgRNAs, controlled by a different promoter, were expressed to induce the genome editing of multiple unique cell types. CRISPR/Cas9 delivery by LV can be witnessed in the modification of five genes where a single LV delivers DNA plasmid encoding for Cas9, fluorescent marker, and sgRNA in creating a mouse model with acute myeloid leukemia. CRISPR/Cas9 delivery by LV targets *PCSK9* mutation which has lost its function in mouse liver (Lino et al., 2018).

Owing to its high safety profile, the AAV is used in many clinical trials. In one clinical trial, Cas9 plasmid was combined with AAV-sgE6 in treating human cervical cancer cell lines in vitro. As a result, expression of E6 was reduced, leading to improved p53 expression, increased apoptosis, as well as inhibition of growth (Yoshiba et al., 2018). In another human cervical cancer, CRISPR/ Cas9 system directed by lentivirus was used alongside sgRNA-721 (LV-H721) in order to knock out the *HIF-1α* for treatment (Song et al., 2021). This resulted in a reduced expression of *HIF-1α* in the cervical tumor tissues.

5.2.2 Non-viral Vectors

5.2.2.1 Chemical Methods

Non-viral vectors are also efficient in delivering CRISPR/Cas systems for *in vitro* experiments. Song et al. (2021) state that the delivery of CRISPR/dCas9-VP64 by flexible dendrimers has been demonstrated in human breast cancer MCF-7 cells where tumor suppressor *MASPIN* genes are upregulated. In another specific case, X-tremeGENE HP DNA transfection reagent was used to transduce Cas9 and SgRNA-E7 plasmid into human cervical cancer Siha cells in order to accentuate the apoptosis and inhibit the cell viability by destroying the *E7* gene. Moreover, novel nanocarriers were developed for delivering CRISPR/Cas9 complexes which are based on graphene oxide (GO)-polyethylene glycol (PEG)-polyethyleneimine (PEI) in human gastric cancer AGS cells, where *GFP* was expressed with an efficiency of 39% (Song et al., 2021).

Lipid-based nanoparticles (LNPs) are arguably the most common nucleic acid delivery systems from the nanoparticles. Liposomes, consisting of spherical bilayers similar to biological membranes, make for a suitable system due to their structural advantage in fusing these complexes across cell membranes and into cells. According to Yip (2020), CRISPR/Cas9 system delivery can be facilitated in one of three ways: DNA plasmid, mRNA which comprises both Cas9 and sgRNA, or proteins such as RNP. Since the system relies on the endosomal pathway, for the most part, its efficiency is low when compared to electroporation or viral transduction methods.

Cell-penetrating peptides (CPPs) are short peptides characterized by their ability to translocate across biological membranes. Yip (2020) states that apart from facilitating the delivery of a wide range of elements such as CPP-Cas9, CPP-sgRNAs, and Cpp-Cas9-sgRNAs conjugates, the CPP provides safer deliveries due to their ability to evade insertional mutagenesis and random genome integration, albeit providing a lower efficiency. Gold nanoparticles (AuNPs) deliver Cas9 RNPs in an efficient manner with the added advantage of the absence of immunogenicity due to its chemical inertness. Moreover, Yip (2020) also states that donor ssDNA is hybridized to 50 thiol modified ssDNA sequences which are then conjugated to AuNPs, followed by Cas9 RNPs being loaded into the donor DNA, and finally using silicate particles and polymer PAsp in order to coat the system. CRISPR-Gold is efficient in inducing HDR in cell lines and primary cells in vitro. Implementation of these systems has been demonstrated in the induction of HDR by CRISPR-Gold via intramuscular injection in correcting dystrophin gene point mutations in mice.

According to Huang et al. (2018), polypeptides are also one of the primary nanoparticles alongside lipid nanoparticles employed in developing RNA-based treatment strategies. Unlike AAVs, CRISPR-Cas systems delivered by these nanoparticles are able to contain more nucleic acid elements for editing whilst evading genomic integration and overexpression. Cas9-sgRNA fused RNP complexes delivered via cationic lipids into mouse inner ear cells are able to alleviate hearing loss, surmounting to the capability of these systems in being able to treat skin cancers such as melanoma.

Huang et al. (2018) also state that successful deliveries of the nanoparticle-delivered Cas9- sgRNA RNP complexes have been demonstrated in the U2OS xenograft of the human osteosarcoma cell lines. Co-delivering small peptides or changing the protein's electrostatic charge can help to accentuate the overall delivery across the cell membrane. Examples of such cases can be demonstrated in the delivery of multiple SV40 nuclear localization sequencers alongside Cas9

RNP complexes in the brain of a mouse, thus providing potential in deliveries in treating brain tumors and neurodegenerative disease.

5.2.2.2 Physical Methods

Electroporation

As one of the most common physical methods employed in delivering editing tools into the cell, the electroporation method utilizes an electric field in disturbing lipid bilayers in order to enhance the permeability of biological membranes in the electro transfer of CRISPR/Cas systems into tumor cells. Song et al. (2021) state that this method is highly efficient in vitro, however, cell death of the experimented tumor cells prevents this method from producing effective results in in vitro investigations despite several precautions taken to enhance the technique. The membrane deformation allows for easier delivery of CRISPR/Cas9 system into different types of tumor cells with greater cell viability and efficiency, such as in the case of NUAK2 gene locus in HCC HeLa cells via Cas9 and sgRNA-NUAK2 coding plasmid introduction, in SU-DHL-1 cells of the anaplastic large cell lymphoma in human, etc., whereby the EGFP knockout efficiency in the latter case increased more than 70%.

Song et al. (2021) also state that the CRISPR/Cas9 plasmid was also successfully transduced in U2OS cells of human osteosarcoma via electroporation which resulted in the suppression of CDK11 expression at subsequently inhibiting proliferation, migration and invasion of tumor. Moreover, the CRISPR/Cas9 plasmid was also demonstrated to targeting *ASXL1* genes and the ssDNA repair template was inserted into the KBM5 cells of human myeloid leukemia via electroporation, allowing for *ASXL1* gene expression, the same results being also exemplified in the case of human leukemia K562 cells.

Hydrodynamic delivery (HD)

According to Ates et al. (2020), hydrodynamic delivery technique involves the rapid injection of large volumes of gene solution into the blood circulation, creating high pressure that causes the permeabilization of the biological membrane so that intracellular gene transfer can be facilitated, allowing for in vivo applications. Song et al. (2021) state that the technique has been applied

alongside CRISPR-Cas9 system in the vein of M-TgHBV mouse model tail where two open reading frames (ORFs) in the hepatitis B virus (HBV) transcription template were deleted in order to reduce liver HBcAg and serum HBsAg, and thereby providing potential in the treatment of human HBV-induced hepatocellular carcinoma (HCC). Despite its simplicity and cost-effectiveness (Ates et al., 2020), the HD technique has low transfection rates and a high risk of liver damage due to the injection of a large volume of gene solution (Song et al., 2021). According to Lino et al. (2018), potential physiological complications are also reported including elevated blood pressure, cardiac dysfunction, and potential accidental mortality.

Microinjection

Yip (2020) states that Microinjection involves physically injecting Cas9-sgRNAs complexes into cells directly using a microscope and a needle which pierces through the biological membrane, delivering its content into the nucleus, thus allowing larger editing systems an efficient delivery into the cell. Physical injection also allows for the doses administered to be controlled, even though the process is laborious and technically challenging. Microinjections are mostly employed in applications involving animal zygotes in order to create transgenic animal models via germline modifications. According to Lino et al. (2018), the sgRNA is usually inserted into the nucleus directly whereas the Cas9 component should be delivered into the cytoplasm, however, due to microinjections being technically challenging, both components are delivered into the cytoplasm, allowing for quicker translation of the mRNAs. Moreover, the sgRNA after being bound to the Cas9 can then be taken into the nucleus, wherein they can undergo modification of the target DNA.

5.3 Applications of CRISPR-Cas System in Oncology

5.3.1 Application of CRISPR-Cas System in Cancer Modeling

According to an article written by Zhang et al. (2021), CRISPR-Cas systems such as the CRISPR/Cas9 are able to construct tumor models having several specific mutations *in vivo* in order to work as an overall better model for studying complex human diseases and cancers with multistep carcinogenesis. The applications of mouse models in mimicking human cancers via remodeling or xenografting is to better understand functionality of cancer genes in oncology research and

discovery/screening of anti-cancer drugs. The chromosomal translocation of *Eml4-Alk* genes were induced in mouse models by employing a lentiviral CRISPR-Cas9 vector to mimic the non-small-cell lung cancer (NSCLC), thus resulting in the development of lung cancer in the mice after 8 weeks of the procedure.

Zhang et al. (2021) further state that the disruption of *Pten* and *P53* tumor suppressor genes in mouse liver via CRISPR-Cas9 generated liver tumors, thus allowing for in-depth understanding on the mechanisms behind tumorigenesis and its invasion as well as discovery of effective therapeutic approaches. Oncology research is greatly benefitted by the advent of techniques such as patient-derived xenograft (PDX), wherein the resulting animal models created from said technique induces human tumor exogenous growths. CRISPR/Cas9 employed in the knockout of *Rag1*, *Rag2*, and *n Il2* in rats having Sprague Dawley, created lymphoid organ development-impaired rat models having severe immunodeficiency which were appropriate candidates in developing into further PDX models with lung squamous cell carcinoma. These models offer a great understanding in cancer research.

Moreover, Zhang et al. (2021) emphasizes that zebrafish, due to its genomic similarity with humans, high rate of reproduction, easy engineering, flexibility, and low costs in maintenance have become arguably one of the most commonly used animal model in studying a variety of cancers, including skin cancer, pancreatic cancer, leukemia, breast cancer, glioma, and lung cancer. CRISPR-Cas systems can be used to create zebrafish models having tumor suppressor gene *SPRED1*-deficient mucosal melanoma. As a result, the inhibition of MAPK (mitogen activated protein kinase) occurring from such mutations can be studied in great detail, accounting for similar mutations happening in human cancers. Thus, cancer modeling provides an efficient approach into investigating cancer genes both in vitro and *in vivo*.

5.3.2 Application of CRISPR-Cas System in Combating Oncogenic Virus infection

Oncoviruses have contributed to a significant number of cancer cases worldwide due to their ability to alter cell cycle of the infected cells, subsequently inducing tumor growth. According to de Almeida et al. (2021), there are currently eight well-known and approved human oncoviruses: hepatitis B virus (HBV), hepatitis C virus (HCV), human papillomavirus (HPV), Epstein–Barr

virus (EBV), human T-lymphotropic virus 1 (HTLV-1), human cytomegalovirus (HCMV), Merkel cell polyomavirus (MCPyV), herpesvirus associated with Kaposi's sarcoma (KSHV) or human herpesvirus-8 (HHV-8). Several studies have demonstrated the inhibition of EBV, HBV, HPV, and HSV-1 via CRISPR-Cas editing technologies.

de Almeida et al. (2021) stated that the implementation of CRISPR-Cas system using eight sgRNAs specific to HBV genotype resulted in four of the combinations inhibiting HBsAg and HBcAg, with subsequent suppression by 70%. Long-coding RNA (lncRNA) proliferating cell nuclear antigen pseudogene 1 (*PCNAP1*) genes are capable of inducing HBV replication once activated by CRISPR-Cas9 and expressed, which ultimately results in hepatocarcinogenesis followed by subsequent PCNA elevation in the liver of experimented mice. In one study, sgRNAs specific to genes of the HBV genotype in the HCC cell lines by CRISPR-Cas9 resulted in a decreased HBsAg expression, attenuated *in vitro* HCC proliferation, and subsequently reduced tumorigenicity *in vivo*.

de Almeida et al. (2021) further state that FnCas9, a system of CRISPR-Cas9 and a *Francisella novicida* (Fn) bacterium, having characteristic endonucleolytic cleavage is able to interfere with the viral replication upon targeting the HCV-negative RNA sequence. In another study, CRISPR/Cas9 was applied in treating EBV virus infection in Burkitt's lymphoma patients by using sgRNAs to target EBV genome regulating the viral structure, morphological changes, and latency. The resulting cell lines had a reduced number of EBV genome by 50% as well as a decreased viral load. Moreover, EBV has also been demonstrated in successfully forming immortal lymphoblast cell lines from primary human B cells.

5.3.3 Application of CRISPR-Cas System in Cancer Diagnostics

The primary detection tools used commonly in cancer diagnostics are undoubtedly Cas proteins. According to Huang et al. (2018), these tools are ahead of several other detection techniques due to proteins being highly specific, sensitive, rapid, cost-effective, and withholding multiplex capabilities in being used to quantify both RNA and DNA quantification, as well as detect multiplexed mutations. Colorectal cancer advances through several different stages in its cycle, driven by several factors catapulting these events such as WNT signaling pathway, EGFP signaling activation, inactivation of TGFb signaling, p53 function loss, and up- or downregulation of *MYC*

and *ERBB2* genes which eventually lead to tumorigenesis and metastasis. Multiplexed detection alongside CRISPR tools can therefore aid in distinguishing between the different stages of cancer formation, thus allowing appropriate treatment measures to be taken for the specific cancer type.

According to Kaminski et al. (2021), up- or downregulation of miRNA expression have been linked to particular cancer types such as lung cancer or tumors in the brain, thus rendering miRNA as effective biomarkers in the detection and monitoring of these diseases. The detection of microRNAs or miRNAs have been successful due to CRISPR-based diagnostics, as can be seen in the case of miR-19b electrochemical detection by CRISPR/LwaCas13a system in patients with medulloblastoma without relying on preamplification, as well as testing of RNA taken from breast cancer cell lines for the presence of miR-17 using CRISPR/LbuCas13a system. CRISPR diagnostic methods can also monitor for genetic markers that are characteristic to treatment response, such as *BRAF* mutations indicative to the treatment of melanoma skin cancer.

In the presence of minimal samples, it is essential that appropriate preemptive measures are taken to ensure accurate and precise genetic material detections are carried out. Several studies have implemented CRISPR-mediated targeting of specific alleles for highly sensitive mutations in detecting small mixed nucleic acid samples. According to Lee et al. (2020), one efficient approach is to erase wild type DNA from mixed samples to ensure the accurate enrichment and detection of low-frequency DNA mutations can be done. Oncogenic point mutations in *KRAS* and *GNAQ* having low frequencies, can be detected in a similar way, where CRISPR-SpCas9 was used to cleave wild type alleles such as PAM sequence via point mutations. PCR was then utilized to amplify the remaining mutant alleles. The enrichment process undergoes successive rounds of increasing amplification if needed, wherein the mutant *KRAS* DNA increases 30-70 fold. Moreover, it was demonstrated that the amplification of *KRAS* point mutations was successful in colon cancer patients. *GNAQ* genes are also amplified in the same way via the use of CRISPR-FnCas12a.

Tian et al. (2019) stated that Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) is a CRISPR-based diagnostic system which detects sensitive cancer-causing genes, including mutations of low frequency not easily determined by other diagnostic methods such as sequencing. Two essential elements make up the diagnostic tool: RNA-guided RNase Cas13a which induces non-specific ssDNA cleavage, and a reporter signal which signals the end

of an RNA cleavage. These methods are crucial for the detection of two particular cancer mutants, such as the EGFR L858R and BRAF V600E.

Tian et al. (2019) further state that another sensitive diagnostic tool is the DNA endonuclease-targeted CRISPR trans reporter (DETECTR), whereby Cas12a present is involved in cleaving whereas recombinase polymerase amplification (RPA) enzymes amplifies the micro-samples being detected and screens for infections in cancer. DETECTRs have been successful in detecting type 16 and 18 of HPV causing lung carcinomas, producing rapid and cost-effective results. Unlike the SHERLOCK, the DETECTR system does not require the transcription of amplified DNA into RNA products.

According to Gootenberg et al. (2018), SHERLOCK used with paper strip lateral flow assay as an adjunctive detection technique enables *EGFR* mutations in the non-small cell lung cancer to be detected, with a stronger signal reading if Csm6 is also combined. Bhattacharyya et al. (2018) state that recombinase polymerase amplification (RPA) is a technique that can be coupled with both SHERLOCK and DETECTR to enhance amplification when detecting viral components. This combination with SHERLOCK can play a vital role in optimizing the diagnosis of viruses such as HIV, whereas DETECTR plays a role in detecting dsDNA HPV.

Another revolutionary technology that detects real-time detection of viruses is the HUDSON (for heating unextracted diagnostic samples to obliterate nucleases) technology. This tool could be critical in detecting oncolytic viruses such as HPV, HBV, HCV, EBV, etc. According to Zhang et al. (2021), another diagnostic approach called CRISPR-Chip technology was generated by combining CRISPR with graphene membrane field effect transistor technology. The transistor comprises a complex of dCas9 for recognition and a sgRNA. When the membrane comes in contact with the sample, the dCas9-sgRNA complex recognizes and binds to the specific target gene and an electrical signal is sent out by the transistor.

The cleaving of genes is what generates the signals in both SHERLOCK and DETECTR whereas binding of genes generates signals in CRISPR-Chip technology. Despite these differences the CRISPR-Chip technology is advantageous in terms of the DNA being tested not requiring any amplification (Zhang et al., 2021).

5.3.4 Application of CRISPR-Cas System in Cancer Immunotherapy

5.3.4.1 CAR T-Cell Immunotherapy

Xia et al. (2018) state that chimeric antigen receptor (CAR) T-cell therapy is a type of adoptive immunotherapy employing an intracellular chimeric signaling domain and an extracellular single-chain variable fragment that has the ability to activate T cells and recognise specific tumor antigens, respectively. This type of immunotherapy has proved to be highly effective in treating hematological malignant cancer, including leukemia and lymphomas due to highly specific targeting and expressing B cells. Due to the time consuming, expensive, and laborious efforts required in autologous (patient's own T-cells) CAR T-cell production, allogeneic universal T-cells taken from healthy donors are the more economic/commercial potential substitutes, albeit requiring the removal of endogenous T-cell receptor (TCR) and human leukocyte antigen (HLA) class I prior to its usage in therapy to reduce the risk of graft-versus-host disease and subsequent rejection in the host immune system.

Furthermore, Xia et al. (2018) also state that utilizing CRISPR-Cas9 to disrupt multiple genome locus alongside defective expressions of TCR and HLA class I generates CAR-T cells that exhibit potent antitumour activity. Knockout of Fas (CD95) receptors, which under normal circumstances induces T-cell apoptosis, via CRISPR-Cas9 can help to strengthen the anti-tumor activity of T-cells in tumor-bearing mice, thus prolonging their life span. CRISPR-Cas9-sgRNA complexes can also be used in removing co-inhibitory molecules such as PD-1 and CTLA-4 associated genes to improve T-cell functional efficiency. Moreover, other co-inhibitory molecules such as LAG-3 and TIM-3 can also be disrupted via CRISPR-Cas9 systems to enhance CAR T-cells function.

Ou et al. (2021) state that applying anti-CD19 CAR T-cells in treating B-cell malignancy has been demonstrated as one of the most successful clinical trials conducted today, hence why anti-CD19 CAR T-cells such as Yescarta and Kymriah have been approved by the FDA in treating adult diffuse large B cell lymphoma (DLBCL) and pediatric/adolescent B lymphoblastic leukemia (B-ALL). In one study, granulocyte-macrophage colony-stimulating factor (*GM-CSF*) gene was knocked out to enhance CAR-T cells function alongside reducing inflammation and cytokine release syndrome (CRS). Another study employed the use of CRISPR/Cas9 technology in knocking out the CAR T-cell endogenous TGF-β receptor II (TGFBR2) to reduce the exhaustion of CAR T-cells as well as enhance the solid tumor-killing efficacy. In a similar fashion, eliminating

CD7 and *TRAC* in CAR T cells led to an increased efficacy in treating T cell acute lymphoblastic leukemia (Ou et al., 2021).

According to Liu et al. (2020), immunosuppressive tumor microenvironment and T cell exhaustion are two critical factors that have been attributed to the failure of CAR T-cell therapy in many patients. As a result, using CRISPR-Cas systems to silence genes that render T-cells susceptible to tumor microenvironment inhibitory signals, such as methylcytosine dioxygenase *TET2*, have resulted in successful enhancement of CAR T-cells efficiency. Lentiviral vectors are primarily utilized in transducing CAR genes into T cells with the help of CRISPR-Cas9 systems to aid in its delivery.

5.3.4.2 T Cell Receptor (TCR)-Based Adoptive T Cell Therapy

Ou et al. (2021) stated that CAR-T cells are functionally limited due to the lack of tumor specific antigens, tumor microenvironment suppressive factors, and heterogeneity of tumor antigens. Thus, engineered T-Cell Receptor (TCR) based T-cell therapy is prioritized in many aspects in targeting a wider variety of antigens and subsequent treatment of varied cancer types. The TCR heterodimer comprises TCR α and TCR β chains whereby each chain contains an extracellular constant region, variable antigen binding region, and a transmembrane region. Complex formation between the constant regions of the main TCRα/β chain with the CD3 chains produces TCR/CD3 complex which is responsible for tumor antigens recognition which is reliant on the major histocompatibility complex (MHC). Melanoma, sarcoma, multiple myeloma, cholangiocarcinoma, breast cancer, and papillomavirus-associated cervical cancer are some of the solid tumors that have shown successful clinical response to TCR T-cell therapy.

Furthermore, Ou et al. (2021) also state that CRISPR-Cas9 has been employed in knocking out endogenous TCRs in order to enhance transgenic TCR expression and functions. Moreover, the transduction of a stabilized Vα/Vβ single-chain TCRs (Sc-TCRs) was carried out to diminish any mispairing risks. After isolation of autologous T cells from patients, lentiviral transduction was used to engineer the T-cells into expressing TCRs specific for NY-ESO- 1 and LAGE-1 antigens. CRISPR-Cas9 was then used to disrupt the endogenous TCRs and PD-1 genes, after which the newly modified T cells were grown in vitro before being re-administered into three patients. It was

demonstrated that two of the patients faced stable disease conditions, on the other hand, the third patient had a progressed disease state.

5.3.4.3 Novel Target Screening for Cancer Immunotherapy

According to Liu et al. (2020), for the genome scale screening of primary T-cells of human, a loss-of-function screening platform was generated by combining sgRNA lentiviral infection with Cas9 protein electroporation (SLICE) as well as utilized a pooled CRISPR delivery single-cell analysis of transcriptome for identification of genes that facilitate T-cell activation to be modified. As a result, functional target genes are allowed to be appropriately characterized in primary T-cells. Continuous antigen stimulation and the immunosuppressive tumor environment are two primary factors limiting the efficacy of CAR T-cell therapy due to the upregulation of inhibitory receptor upregulation, tumor site targeting inefficiency, loss of effector function, and CAR T-cell limitation of persistent functionality. This efficacy can be reinforced by screening and targeting target genes that silence genes susceptible to these inhibitory signals. CRISPR-Cas systems can therefore be prevalent in accounting for the screening aspect of when dealing with these types of therapy.

Liu et al. (2020) state that in CRISPR/Cas9 screening, lentivirus or retrovirus are generally used to transduce CAR genes into T-cells. However, due to the random integration of CAR genes the accuracy of the results may be compromised when it comes to efficient screening. To combat this problem, both coding sequence of CAR gene and the editing tools are transduced simultaneously into the T-cells. Moreover, according to a study, it was confirmed that expressing CAR genes under the control of a T-cell genome could prevent the risk of random integration and off-targeting. In one particular research, an AAV-Cpf1 KIKO (knock-in and knock-out) system was developed, whereby Cas12a was utilized in order to identify genes that regulate the CAR T-cell activities. After the delivery of Cpf1 mRNA into human CD4+ T-cells was achieved by electroporation, the T-cells were then infected with AAV, carrying two crRNAs and a single CAR coding gene. The first crRNA directed the integration of CAR gene (KI) whereas the second one knocked out the desired gene (KO). Assuming that the KO crRNA is replaced with a crRNA library, the overall KIKO system will then be capable of conducting CRISPR screen identification of genes that are involved in CAR-T-cell activity regulation (Liu et al., 2020).

Moreover, the role of B cells in progressing malignancies and regulating tumor responses to immunotherapy have not yet been extensively studied by genome-wide CRISPR screens. Liu et al. (2020) further state that a highly specific sgRNA design tool was developed for targeting genes responsible for B-cell activation and differentiation in B cells isolated from transgenic mice models by Cas9. The CRISPR screen was essentially used in characterizing regulators that control the B cell state, its variation, and disease-associated factors.

5.3.4.4 Inhibition of Immune Checkpoint Signaling Pathway

According to Ou et al. (2021), T-cell exhaustion is a phenomenon brought about by the continuous exposure of antigen and immunosuppressive factors that culminates in the T-cells and tumor cells coexisting together. This exhaustion causes tumor-infiltrating T lymphocytes to induce high expression of inhibitory receptors such as CTLA-4, PD-1, T cell immunoglobulin domain, lymphocyte activation gene 3 protein, and mucin domain-containing protein 3, etc. These immune checkpoint inhibitors facilitate the anti-tumor activity in cancer treatment.

Ou et al. (2021) stated that upon recognition of antigens by TCR/CD3 complex, the CD28 molecules aid in the activation of T-cells by amplifying the TCR signals. CTLA-4, being a homologous receptor of CD28, acts as an antagonist and induces opposing functions on both CD4+ and CD8+ T-cells by binding to the same CD28 ligands and delivering inhibitory signals. Blocking CTLA-4 enables CD28 ligands to become available and allow subsequent T cell activation. Ipilimumab, a recombinant IgG1 monoclonal antibody is able to block CTLA-4 and strengthen patents' immunity in killing tumor cells as well as improving metastatic melanoma.

Furthermore, Ou et al. (2021) also state that using CRISPR-Cas9 systems to knockout or disrupt *CTLA-4* genes in cytotoxic T lymphocytes (CTLs) have been shown to successfully increase secretions of TNF-α and IFN-γ which aid in enhancing anti-tumor activity of CTLs. PD-1 are another examples of immune checkpoint inhibitors, usually expressed on immune cells such as monocytes, T-cells, dendritic cells etc. that provide the same effects as CTLA-4. Ligands of PD-1 (PD-L1) and PD-2 (PD-L2) which are present on tumor cells and antigen presenting cells (APCs), interact with one another and suppress activation of T-cells and their functions, ultimately resulting in T cell exhaustion. Likewise, anti-PD-1 antibody such as pembrolizumab and nivolumab have shown successful anti-tumor activity in a wide range of tumor types, including melanoma, non-

small cell lung cancer, metastatic urothelial carcinoma, and head and neck squamous cell carcinoma (Ou et al., 2021).

5.3.5 Application of CRISPR-Cas System on Treatment of Different Types of Cancer

5.3.5.1 Lung Cancer

A myriad of different treatment options are available for lung cancer, surgery and radiation being one of the more widely used choices. According to Tiruneh et al. (2021), selective TKIs such as erlotinib and gefitinib are currently more opted for due to their ability to block the tyrosine kinase activity of EGFRs. Moreover, the majority of lung cancers are affected by mutations at the EGFR genes. The EGFR is a membrane glycoprotein composed of three domains namely transmembrane, extracellular ligand binding, and intracellular tyrosine kinase domains. Upon binding of ligand onto the extracellular ligand domain, intracellular kinase activities are activated which brings about cellular proliferation, invasion, neovascularization, and metastasis, followed by a reduction in apoptosis and glycolysis activation. However, due to the increased resistance to these drugs, CRISPR-Cas systems are now being implemented alongside popular treatment strategies to reinforce their efficacy.

Tiruneh et al. (2021) further state that, CRISPR-Cas9 in lung cancer is being employed in one of two ways, the first being designing sgRNA complementary to the EGFR sequence that can target the specific site allowing for its cleavage by Cas9 proteins creating a ss- or dsDNA break based on the particular enzyme used, followed by a homologous or non-homologous DNA repair mechanism. CRISPR-Cas9 inhibition essentially enhances the MHC class I expression which subsequently improves the recognition of cytotoxic lymphocytes and tumor lysis. The other strategy involves enhancing immune cells like T lymphocytes taken from patients or donors by CRISPR-Cas systems. CRISPR-Cas9 are able to knock out genes encoding for inhibitory receptor proteins such as PD-1 and CTLA-4, that in normal circumstances would bind to T-cells and prevent their activation and subsequent immune response to tumors. Knocking out PD-1 protein on immune cells is essential for facilitating caspase activation which can then be utilized for programmed cell death and apoptosis in tumor cells.

According to Nair et al. (2020), thirteen clinical trials are documented for CRISPR-mediated treatment all over the world, with one study conducting gene editing in vivo by the direct administration of CRISPR-Cas9 editing tools into the human body. The rest of the studies involve using CRISPR-Cas9 systems for modification of immune cells in vitro. One particular trial is concerned for the treatment of lung cancer in which the safety of T-cells undergoing PD-1 knockout is evaluated in treating metastatic non-small cell lung cancer. The study involves isolating autologous T-cells from the peripheral blood and utilizing CRISPR/Cas9 systems to knock out PD-1 gene ex vivo. After an initial administration of cyclophosphamide, the engineered and expanded PD-1-knockout-out T-cells are then infused into the patients.

5.3.5.2 Breast Cancer

Treatment strategies for breast cancers may be conducted in several ways. The first approach is using Cas9 proteins to knock out oncogenes responsible for cancer cell proliferation, metastasis, and drug resistance with the help of Cas9 whole-genome dropout screens that contains essential information on these essential genes. According to Mintz et al. (2018), CRISPR systems can also be utilized in discovering TNBC drugs whose utilization can overcome molecular target deaths. Moreover, drugs that inhibit the maternal embryonic leucine zipper kinase (MELK) such as OTS167 can prove to be effective therapeutic targets for TNBC. MELK is an enzyme that upon overexpression of *MELK* can lead to an increased cell proliferation.

According to Mintz et al. (2018), another approach involves the reversible DNA methylation and histone acetylation by DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors to treat breast cancer, despite HDAC inhibition falling short of treating solid tumors and TNBC. In a metastatic breast cancer research trial Vorinostat, an HDAC inhibitor, used proved to be insufficient in working as an effective monotherapy. This ineffectiveness of HDAC inhibition was later attributed to the LIFR-JAK1-STAT3 signaling pathway which was put into effect due to an elevated JAK1 and BRD4. Inhibition of these two components by siRNA resulted in an increased efficacy of the HDAC inhibition when treating TNBC. Romidepsin, another HDAC inhibitor, was successful in treating inflammatory breast cancers when used in combination with paclitaxel.

Mintz et al. (2018) further state that the dCas9 variant of CRISPR-Cas system can also be utilized as an epigenetic modulation tool for several genes after fusion with regulatory effector domains specific to those genes. For example, LSD1-dCas9 fusion has proven to be effective in modulating breast cancer progression by suppressing specific enhancer genes. These dCas9 fusions have demonstrated the ability to simultaneously activate tumor suppressor genes and suppress oncogenes by employing gRNAs of different lengths unique for each specific target locus, offering high specificity and little-to-no off-target effects in vivo. These dCas9 modulations can be further enhanced by using synthetic biology genetic circuits. Genetic circuits are essentially logic gates constructed from gRNAs and dCas9 that can facilitate in highly specific epigenetic modulations and gene editing of breast cancer treatment.

Furthermore, Mintz et al. (2018) also state that Cas9 proteins can prove to be beneficial in improving immunotherapy, whereby T-cells engineered to express CAR proteins can facilitate in the recognition of antigens specific to breast cancer. CAR T-cell targeting HER2 antigen for the treatment of breast cancer is currently undergoing clinical trials. All in all, these treatment strategies can be highly effective when targeting *ER/PR, HER2, BRCA1/BRCA2* genes, as they are highly accredited to the tumorigenesis and its progression when it comes to breast cancers.

5.3.5.3 Colorectal Cancer

According to Jefremow et al. (2021), discovering critical tumor driving pathways such as KRAS, TGFβ, WNT, PI3K, amd TP53 and extensively studying them have allowed treatment strategies specific to metastatic colorectal cancer to be enhanced. Generally, CRC is propagated by 3-6 driver mutations, and earlier conventional therapies did not allow for simultaneous controlled deletion of multiple mutated genes. However, the development of organoids, small constructs of organ-like tissues along with CRISPR-Cas9 systems have resulted in better controlled treatment strategies. For example, in human intestinal organoids with *APC, SMAD4, TP53* and *SMAD4* knockouts as well as *KRAS* and *PIC3CA* knock-ins, it was demonstrated that deleting *APC* resulted in WNT-independent organoid growth and mutations in *SMAD4*, *TP53*, *KRAS*, and *PIK3CA* also resulted in independent growth in organoids.

Porru et al. (2018) state that there are three different pathogenic mechanisms responsible for CRC, including microsatellite instability, chromosomal instability, and CpG island methylator

phenotype. Cell proliferation is propagated mainly by the WNT, PI3K, TGFβ, TP53 pathways as well as mutations of *BRAF, cMYC, SMAD2, SMAD4, PIK3CA, PTEN*, and *RAS* genes. In addition, overexpression of *EGFR* has been heavily associated with malignant progressions of CRC which can be potential targets for CRISPR-Cas9 based knockout therapy in CRC patients. The fructose metabolism, and nicotinamide adenine dinucleotide kinase (NADK) are also some of the potential targets identified in *KRAS*-mutated CRC.

Jefremow et al. (2021) further stated that the induction of mutations by CRISPR-Cas9 systems offers valuable insight into discovering new potential genetic targets for screening and treating CRCs. In one study, the correction of gene β-catenin mutation by CRISPR-Cas9 in the WNT pathway of human CRC cell line HCT116 resulted in an increase in the protein phosphorylation followed by a decrease in proliferation of CRC cells in vitro. Moreover, serrated polyps are also one of the main causes of CRC, which are brought about by *BRAF* mutations, DNA mismatch repair genes, and MSI.

Evaluation of genetic alterations of serrated polyposis families and sporadic serrated polyps in a study revealed that a high frequency of *RNF43* mutations induced by CRISPR-Cas9 showed a reduced dependency in organoids of serrated adenomas on the R-spondin growth factor which is required for tumorigenesis (Jefremow et al., 2021). Moreover, according to Porru et al. (2018), the administration of a combination of different cytotoxic drugs such as 5-fluorouracil, irinotecan, oxaliplatin, capecitabine, oxaliplatin, leucovorin, etc. have been successful in demonstrating the amelioration of mCRC in patients and has a greater potential if used alongside CRISPR-Cas systems.

5.3.5.4 Melanoma

According to Karimkhani et al. (2014), melanoma tumorigenesis can be attributed to several mutations activating the proto-oncogenes, such as *BRAF* and *KIT*, and inhibiting tumor suppressor genes. BRAF is a growth signal transduction protein kinase which regulates the MAPK (mitogen-activated protein kinase) or the ERK (extracellular signal-regulated kinase) pathway. The MAPK/ERK signaling facilitates cell division, secretion, and differentiation, whereas a mutation at the V600E site in BRAF accounts for the majority of the *BRAF* mutations in metastatic melanomas. The continuous propagation of the downstream MEK/ERK pathway results in an

uncontrolled transcription, cell growth, and tumor progression. Knockout of *V600E*- and *V600K*-mutated BRAF kinase by CRISPR-Cas systems has potential in treating melanoma.

Moses et al. (2019) state that the tumor suppressor genes specific to the melanoma cancers include phosphatase and tensin homolog (*PTEN*) genes that inhibit various cellular processes for survival, cell cycle progression, and migration. The PTEN is also responsible for converting phosphatidylinositol-trisphosphate (PIP3) to phosphatidylinositol-disphosphate (PIP2) in the phosphoinositide 3-kinase (PI3K)/AKT pathway, thereby diminishing cell survival, cell cycle progression, as well as regulation of transcription, translation, and metabolism. Mutations leading to the loss of PTEN activity contributes to the subsequent development of drug-resistant malignancies. Besides genetic mutations, transcriptional, post-transcriptional and other epigenetic modifications are responsible for regulating PTEN levels.

Moses et al. (2019) also state that in a study, a SK-MEL-28 human cell line was taken from a panel of melanoma and TNBC, whereby the SK-MEL-28 consisted of a BRAF *V600E* mutation as well as exhibited resistance to B-Raf inhibitor dabrafenib. By utilizing dCas9-VPR transactivation system, the *PTEN* transcription in SK-MEL-28 was reactivated increasing the level of endogenous gene activation. As a result, the *PTEN* activation led to a decreased metastatic behavior and therapy resistance due to significant reduction of phosphorylated AKT and inhibition of the downstream oncogenic signaling pathways responsible for cell proliferation and migration. Moreover, the migratory potential of the SK-MEL-28 cells was also significantly reduced with an increase in the sensitivity to B-Raf and PI3K/mTOR inhibitors. A combination of *PTEN* activation by CRISPR alongside conventional small molecule inhibitors such as dabrafenib (B-Raf inhibitor) and dactolisib (PI3K/mTOR inhibitor) could be successful in exhibiting a far more effective tumor inhibition and drug-resistance, with little-to-no off-target effects.

5.3.5.5 Cervical Cancer

Owing to molecular epidemiological and clinical studies, the majority of cervical cancers stem from high-risk human papillomavirus (HR-HPV) infections that exhibit potent carcinogenic properties by elongating the cell cycle of human keratinocytes. According to Zhen and Li (2017), these oncogenic viruses express E6 and E7 proteins responsible for the inactivation of *p53* and *RB* regulatory proteins of the host, respectively. Persistent infections and viral oncogenes expression

lead to the constant inactivation of *p53* and *Rb*, and subsequent increase in genomic instability, somatic mutations, and HPV integration into the host genome. The E6 protein is able to bring about cellular changes such as prolongation of cellular life cycle by blocking apoptosis and increasing the telomerase activity, as well as block TP53 translocation and their gene expression in the nucleus, resulting in continuous cell division in spite of the damage dealt to the DNA. The E7 is responsible for regulating stable protein folding and facilitating cellular proliferation, cell cycle prolongation, and transformation, as well as degrade *RB* tumor suppressors via growth factor interactions. The E7 proteins are also able to prolong keratinocyte cell cycle and degrade *RB* tumor suppressors upon binding to them. Furthermore, E7 proteins are also able to upregulate cyclins A and E expression, inactivate cyclin-dependent kinase (CDK), and induce cell cycle progression, as well as regulate tumor metastasis via PI3K/AKT signaling pathway.

Zhen and Li (2017) also state that the E6 and E7 silencing by CRISPR-Cas9 systems in order to induce apoptosis and cell death is primarily dependent on TP53 and pRB reactivation. Targeting sequences specific to the E6 mRNA by utilizing CRISPR/Cas9 decreases the amount of E6 mRNA which then leads to an increase in TP53 protein levels, facilitating the suppression of tumors. In one study, targeting the promoters and open reading frames of E6 or E7 transcripts by CRISPR/Cas9 led to a decrease in the E6 and E7 mRNA level, an increase in TP53 protein, a decrease in *RB* protein, thus inducing apoptosis and inhibition of growth in SiHa cervical carcinoma cell lines. CRISPR/Cas9-mediated targeting of E6/E7 mRNA of the HPV16 or HPV18 infections induced tumor growth inhibition and apoptosis activation in vivo, proving the effectiveness of CRISPR/Cas9 as potential therapeutics for cervical cancer. In another study, CRISPR/Cas9 also played a major role in targeting and sensitizing HPV E6 and E7 oncogenes cisplatin (CDDP) drug and radiation therapy in treating cervical carcinoma. The combination of CRISPR/Cas9 alongside other cytotoxic agents carries potential in targeting E6/E7 in a synergistic manner in order to restore and enhance the functions of TP53 and pRB proteins.

5.3.5.6 Osteosarcoma

Responsible for approximately 1% of adult tumors, osteosarcoma is a type of bone cancer whose exact etiological explanation remains largely unknown. According to Liu et al. (2021), it has been widely speculated that hereditary diseases such as Rothmund-Thomson syndrome, Li-Fraumeni

syndrome, Werner syndrome, hereditary retinoblastoma, and their related genes such as *TP53*, *RB1*, *WRN*, and *RECQL4* have been accredited to the pathogenesis of this cancer type. Currently, the treatment plan for osteosarcoma includes a combination of neoadjuvant chemotherapy, surgery, and postoperative surgery. However, due to the toxicity of chemotherapeutic drugs such as cardiotoxicity of doxorubicin, carcinogenicity and otorrenal toxicity of cisplatin, bone marrow suppression, ocular mucosal, liver, and kidney toxicity of methotrexate, as well as resistance of tumors to chemotherapy, more advanced alternatives are sought.

Liu et al. (2021) state that CRISPR/Cas9 technology have shown potential effectiveness in treating osteosarcoma when it was utilized in knocking out *CD11K* genes in human osteosarcoma cell lines such as KHOS and U2OS, exhibiting profound inhibition in migration, invasion, and cell proliferation. In one study, it has also been applied in oncogene knockouts, where removal of *GLT25D1* and *GLT25D2* made the human Saos osteosarcoma cells nonviable. Other knockout genes bringing about inhibition of growth, metastasis, and mobility via CRISPR-Cas9 systems include *CD44* genes in human 143B and MNNG/HOS cells, *CD81* genes in murine 143B cell lines, *FGF5* gene in murine MG63 and U2OS cells, etc. CRISPR/Cas9 can also be used in sensitizing chemotherapeutic drugs to osteosarcoma cells. KHOSR2, an osteosarcoma cell line with a multidrug-resistant accumulation, displayed an increased sensitivity to Adriamycin after the knockout of mutant *TP53* by CRISPR-Cas9 system. Moreover, the knockout of *PD-L1* rendered KHOS and MNNG/HOS cell lines more sensitive to doxorubicin and paclitaxel treatment.

In addition, Liu et al. (2021) also state that CRISPR/Cas9 may also be utilized in modifying suppressor genes to study and validate their respective biological roles in order to discover and develop potential therapeutic targets. According to one study, the knockout of *CNE9*, *CNE10* or *STAG2* genes in the U2OS cell lines resulted in decreased apoptosis. The knockout of *CNE9* and *CNE10* in particular inhibited cell proliferation which proved the cancer suppressing roles of these genes. The *STAG2* knockout cell lines exhibited tumor invasiveness, *PD-L1* gene expressions, epithelial-mesenchymal transition (EMT), as well as demonstrated modifications in the expression of immune-related genes and an enhanced resistance to chemotherapeutic drug cisplatin. Thus, the absence of *STAG-2* safeguarding tumors from *PD-L1*-mediated host immune response was demonstrated which is indicative of the potential of *STAG2* being used as biomarkers for PD-1-PD-L1 inhibitor therapy in osteosarcoma, where the *STAG2* is deficient.

5.3.5.7 Others

In recent years, CRISPR-Cas systems have been applied in various other trials for studying its effectiveness in treating cancers of different types. According to Sharma et al. (2020), in a phase 1 in-human clinical trial employing CRISPR/Cas9 system was performed in three patients affected by advanced-stage refractory cancer. It was shown that the knockout of *TRAC* and *TRBC* genes encoding endogenous TCR and PDCD1 chains in T-cells increased anti-tumor immunity. Moreover, the introduction of transgene *NY-ESO-1* in T-cells enabled efficient tumor recognition. Eyquem et al. (2017) state that CARs are synthetic receptors that have been successful in redirecting and reprogramming T-cells to facilitate tumor rejection. CAR T-cell therapy has been successful in treating many cancers. In one particular human cell line, CD19-specific CAR integrated into T-cell receptor α constant (TRAC) exhibited uniform expression of *CAR* genes in peripheral blood T-cells and potentiated T-cells functions. Targeting the TRAC locus also prevents tonic *CAR* signaling and enables proper internalization and re-expression of *CAR* genes, thus slowing down the differentiation and exhaustion of effector T-cells.

In another clinical trial, Sharma et al. (2020) state that CAR T-cells were administered in patients with refractory hematological malignancies having *CD19* negative tumor cells. Two CAR genes were integrated into the TRAC locus of T-cells taken from patients in order to recognize *CD19* negative cells. Moreover, gene-disrupted allogeneic universal CD19-specific CAR-T cells were also employed in treating patients with refractory CD19+ leukemia and lymphoma in another clinical trial. Electroporation methods were used alongside CRISPR RNA in disrupting the endogenous *TCR* and *B2M* genes. This has potential in allowing for the reduction of immunogenicity by bypassing graft-versus-host-disease, although the results have yet to be published. Recently, the FDA approved the usage of CTX130 in a clinical trial, whereby the allogeneic CRISPR/Cas9-modified T Cell targets CD70 for the treatment of renal cell carcinoma and hematologic malignancies.

Chapter 6

Limitations and Future Direction

CRISPR-Cas systems are a highly effective gene editing tool that show great potential in treating a myriad of diseases due to its high efficiency, simplicity, high pliability, low cost, capability to edit multiple genes and wide applicability. However, due to its many limitations and challenges, the future of CRISPR-Cas systems in being implemented in therapy is still largely undecided. According to Mollanoori et al. (2018) preventing Cas-sgRNA in binding to and cleaving non-target homologous sequences, or off-targets, takes precedence when it comes to tackling the challenges of CRISPR-Cas systems. The resulting mutations from off-target cleaving may give rise to oncogene activation, inactivation of tumor suppressor genes, resistance to chemotherapeutics, etc. It is therefore crucial that selected sgRNAs are highly selective and truncated to minimize off-target effect or collateral cleavage and enhance the sgRNA/DNA mismatch sensitivity. According to Jubair and McMillan (2017), efficient gene knock-ins are yet another challenge yet to be tackled. In practice, insertion of a precise modification through the HDR repairing pathway is extremely difficult, due to the HDR repair only occurring during the G2 and S phases of the cell cycle and the continuous re-editing in the target sites. Inhibiting DNA ligase and gene silencing of the NHEJ pathway proteins can be effective when it comes to improving the HDR editing efficiency.

According to Kozovska et al. (2021), the lack of efficiency can further be attributed to the inhibition of histone dynamics in Cas9. This failure of cleavage by Cas9 proteins can be combated by prolonging the experimental time in order to allow Cas proteins to find the appropriate target site. Moreover, the possible immune reaction activation in humans can also prove to be challenging a lot of the time. The prokaryotic nature of Cas proteins is what initiates the toxic reactions in human cells, thus producing Cas protein specific antibodies via immune reaction activation. Kumar et al. (2020) state that these antibodies are capable of interfering with CRISPR therapy. Incorporating two mutations in Cas9 epitope anchor residues can help to ameliorate this immunogenicity. Furthermore, the requirement of individual PAM sequences specific to each Cas protein for targeting specific genome sites is yet another challenging aspect of CRISPR-Cas technology. In addition, heightened sensitivity of Cas proteins to RNA secondary structures and

RNA instability to RNase can also greatly affect CRISPR efficiency in diagnosing and treating different cancers.

The increasing number of applications utilizing CRISPR-Cas9-mediated engineered T-cells in cancer therapy is constantly being challenged by the lack of a safe and effective delivery method. According to Liu et al. (2019), the heterogeneity of tumors brings about issues such as minor subclone outgrowths. Thus, identifying these overgrowths beforehand and employing multiple Cas9/gRNA can prove to be effective in minimizing relapse in patients getting these therapies. Zhang et al. (2021) state that the combination of CAR T-cell therapy alongside CRISPR-Cas systems also introduces the issue of neurotoxicity, causing cytokine release syndrome. Apart from being highly laborious and expensive, this technique also requires medical equipment and personnel in large quantities.

Due to its ability to inhibit, activate, repress, translocate, duplicate, or invert genes, these technologies have potential in revolutionizing cancer therapeutics. According to Sachdeva et al. (2015), despite intercepting a lot of the limitations addressed above, CRISPR-Cas technology has yet to benefit oncology and cancer treatment in a profound way. Even though diagnosis may happen at an earlier stage of tumor development, due to the heterogeneity of the tumor a combination therapy is usually required for the treatment to be effective. Moreover, this also results in development of resistance and tumor progression due to targeted effect on specific genes which can be ameliorated by the use of CRISPR-Cas systems. Sachdeva et al. (2015) also state that recent studies have demonstrated CRISPR-Cas's ability of targeting multiple target genes in a heterogeneous tumor mass in non-small cell lung cancer, resulting in one of the only few successful trials to ever be conducted by CRISPR-Cas systems. With more research and development in this field, the CRISPR-Cas based therapeutic applications can tread into a wider variety of diseases apart from cancer. Moreover, the new approaches could improve current treatment strategies and better yet discover newer strategies that erase the disease-associated genomics and epigenomic aspects of cancer.

Chapter 7

Conclusion

The immense therapeutic potential of CRISPR-Cas systems in treating genome-based diseases like cancers, is clearly evident from the vast amount of positive research results over the past few years, although effective clinical implementation of this therapy is heavily reliant on the shortcomings of current delivery methods. The various clinical trials employing this system are still at the earliest stages and are being monitored with great expectations all around the world. However, the rising anticipations are to be met with up-to-date optimizations in order to allow for widespread clinical translation to ensure the highest efficacy, specificity, and safety, all while maintaining a cost-effective approach. Despite the challenges, this continuous development of CRISPR-Cas systems is sure to make great contributions in improving the current cancer treatment strategies.

References

Ahmad, G., & Amiji, M. (2018). Use of CRISPR/Cas9 gene-editing tools for developing models in drug discovery. *Drug Discovery Today, 23*(3), 519–533. https://doi.org/10.1016/j.drudis.2018.01.014

Ates, I., Rathbone, T., Stuart, C., Bridges, P. H., & Cottle, R. N. (2020). Delivery Approaches for Therapeutic Genome Editing and Challenges. *Genes, 11*(10), 1113. https://doi.org/10.3390/genes11101113

Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., Romero, D. A., & Horvath, P. (2007). CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science, 315*(5819), 1709–1712. https://doi.org/10.1126/science.1138140

Baudino, T. (2015). Targeted Cancer Therapy: The Next Generation of Cancer Treatment. *Current Drug Discovery Technologies, 12*(1), 3–20. https://doi.org/10.2174/1570163812666150602144310

Bhattacharyya, R. P., Thakku, S. G., & Hung, D. T. (2018). Harnessing CRISPR Effectors for Infectious Disease Diagnostics. *ACS Infectious Diseases, 4*(9), 1278–1282. https://doi.org/10.1021/acsinfecdis.8b00170

Burmistrz, M., Krakowski, K., & Krawczyk-balska, A. (2020). RNA-Targeting CRISPR – Cas Systems and Their Applications. *International journal of molecular sciences, 21*(3), 1122. https://doi.org/10.3390/ijms21031122

Carroll, K. J., Makarewich, C. A., McAnally, J., Anderson, D. M., Zentilin, L., Liu, N., Giacca, M., Bassel-Duby, R., & Olson, E. N. (2015). A mouse model for adult cardiac-specific gene deletion with CRISPR/Cas9. *Proceedings of the National Academy of Sciences, 113*(2), 338–343. https://doi.org/10.1073/pnas.1523918113

Chen, X., Liu, J., Janssen, J. M., & Gonçalves, M. A. (2017). The Chromatin Structure Differentially Impacts High-Specificity CRISPR-Cas9 Nuclease Strategies. *Molecular Therapy - Nucleic Acids, 8*, 558–563. https://doi.org/10.1016/j.omtn.2017.08.005

Chen, Y., & Zhang, Y. (2018). Application of the CRISPR/Cas9 System to Drug Resistance in Breast Cancer. *Advanced Science, 5*(6). https://doi.org/10.1002/advs.201700964

de Almeida, N. A. A., Ribeiro, C. R. D. A., Raposo, J. V., & de Paula, V. S. (2021). Immunotherapy and Gene Therapy for Oncoviruses Infections: A Review. *Viruses, 13*(5), 822. https://doi.org/10.3390/v13050822

Dragu, D. L., Necula, L. G., Bleotu, C., Diaconu, C. C., & Chivu-Economescu, M. (2015). Therapies targeting cancer stem cells: Current trends and future challenges. *World Journal of Stem Cells, 7*(9), 1185–1201. https://doi.org/10.4252/wjsc.v7.i9.1185

Eyquem, J., Mansilla-Soto, J., Giavridis, T., Van Der Stegen, S. J. C., Hamieh, M., Cunanan, K. M., Odak, A., Gönen, M., & Sadelain, M. (2017). Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature, 543*(7643), 113–117. https://doi.org/10.1038/nature21405

Global health estimates: Leading causes of death. (2020). World Health Organization. https://www.who.int/data/gho/data/themes/mortality-and-global-health-estimates/ghe-leading-causes-of-death

Gootenberg, J. S., Abudayyeh, O. O., Kellner, M. J., Joung, J., Collins, J. J., & Zhang, F. (2018). Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science, 360*(6387), 439–444. https://doi.org/10.1126/science.aaq0179

Grissa, I., Vergnaud, G., & Pourcel, C. (2007). CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. *Nucleic Acids Research, 35*(Web Server), W52–W57. https://doi.org/10.1093/nar/gkm360

Hanahan, D., & Weinberg, R. A. (2000). The Hallmarks of Cancer. *Cell, 100*(1), 57–70. https://doi.org/10.1016/s0092-8674(00)81683-9

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell, 144*(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Hassanpour, S. H., & Dehghani, M. (2017). Review of cancer from perspective of molecular. *Journal of Cancer Research and Practice, 4*(4), 127–129. https://doi.org/10.1016/j.jcrpr.2017.07.001

Huang, C. H., Lee, K. C., & Doudna, J. A. (2018). Applications of CRISPR-Cas Enzymes in Cancer Therapeutics and Detection. *Trends in Cancer, 4*(7), 499–512. https://doi.org/10.1016/j.trecan.2018.05.006

HUMIRA® (adalimumab) | A Biologic Treatment Option. (2013). HUMIRA® adalimumab. https://www.humira.com/

Janik, E., Niemcewicz, M., Ceremuga, M., Krzowski, L., Saluk-Bijak, J., & Bijak, M. (2020). Various aspects of a gene editing system—crispr–cas9. *International Journal of Molecular Sciences, 21*(24), 1–20. https://doi.org/10.3390/ijms21249604

Jefremow, A., Neurath, M. F., & Waldner, M. J. (2021). CRISPR/Cas9 in Gastrointestinal Malignancies. *Frontiers in Cell and Developmental Biology, 9*. https://doi.org/10.3389/fcell.2021.727217

Jiang, C., Lin, X., & Zhao, Z. (2019). Applications of CRISPR/Cas9 Technology in the Treatment of Lung Cancer. *Trends in Molecular Medicine, 25*(11), 1039–1049. https://doi.org/10.1016/j.molmed.2019.07.007

Jubair, L., & McMillan, N. A. J. (2017). The Therapeutic Potential of CRISPR/Cas9 Systems in Oncogene-Addicted Cancer Types: Virally Driven Cancers as a Model System. *Molecular Therapy - Nucleic Acids, 8*(September), 56–63. https://doi.org/10.1016/j.omtn.2017.06.006

Kaminski, M. M., Abudayyeh, O. O., Gootenberg, J. S., Zhang, F., & Collins, J. J. (2021). CRISPR-based diagnostics. *Nature Biomedical Engineering, 5*(7), 643–656. https://doi.org/10.1038/s41551-021-00760-7

Karimkhani, C., Gonzalez, R., & Dellavalle, R. P. (2014). A review of novel therapies for melanoma. *American Journal of Clinical Dermatology, 15*(4), 323–337. https://doi.org/10.1007/s40257-014-0083-7

Kaushik, I., Ramachandran, S., & Srivastava, S. K. (2019). CRISPR-Cas9: A multifaceted therapeutic strategy for cancer treatment. *Seminars in Cell and Developmental Biology, 96*(January), 4–12. https://doi.org/10.1016/j.semcdb.2019.04.018

Kozovska, Z., Rajcaniova, S., Munteanu, P., Dzacovska, S., & Demkova, L. (2021). CRISPR: History and perspectives to the future. *Biomedicine and Pharmacotherapy, 141*(April), 111917. https://doi.org/10.1016/j.biopha.2021.111917

Kumar, P., Malik, Y. S., Ganesh, B., Rahangdale, S., Saurabh, S., Natesan, S., Srivastava, A., Sharun, K., Yatoo, M. I., Tiwari, R., Singh, R. K., & Dhama, K. (2020). CRISPR-Cas System: An Approach With Potentials for COVID-19 Diagnosis and Therapeutics. *Frontiers in Cellular and Infection Microbiology, 10*(November), 1–13. https://doi.org/10.3389/fcimb.2020.576875

Lee, S. H., Park, Y. H., Jin, Y. B., Kim, S. U., & Hur, J. K. (2020). CRISPR Diagnosis and Therapeutics with Single Base Pair Precision. *Trends in Molecular Medicine, 26*(3), 337–350. https://doi.org/10.1016/j.molmed.2019.09.008

Li, H., Yang, Y., Hong, W., Huang, M., Wu, M., & Zhao, X. (2020). Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. *Signal Transduction and Targeted Therapy*, *5*(1). https://doi.org/10.1038/s41392-019-0089-y

Lino, C. A., Harper, J. C., Carney, J. P., & Timlin, J. A. (2018). Delivering CRISPR: a review of the challenges and approaches. *Drug Delivery*, *25*(1), 1234–1257. https://doi.org/10.1080/10717544.2018.1474964

Liu, B., Saber, A., & Haisma, H. J. (2019). CRISPR/Cas9: a powerful tool for identification of new targets for cancer treatment. *Drug Discovery Today*, *24*(4), 955–970. https://doi.org/10.1016/j.drudis.2019.02.011

Liu, D., Zhao, X., Tang, A., Xu, X., Liu, S., Zha, L., Ma, W., Zheng, J., & Shi, M. (2020). CRISPR screen in mechanism and target discovery for cancer immunotherapy. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, *1874*(1), 188378. https://doi.org/10.1016/j.bbcan.2020.188378

Liu, W., Wang, S., Lin, B., Zhang, W., & Ji, G. (2021). Applications of CRISPR/Cas9 in the research of malignant musculoskeletal tumors. *BMC Musculoskeletal Disorders*, *22*(1), 1–12. https://doi.org/10.1186/s12891-021-04020-2

Makarova, K. S., Wolf, Y. I., & Koonin, E. V. (2018). Classification and Nomenclature of CRISPR-Cas Systems: Where from Here? *The CRISPR Journal*, *1*(5), 325–336. https://doi.org/10.1089/crispr.2018.0033

Martinez-Lage, M., Puig-Serra, P., Menendez, P., Torres-Ruiz, R., & Rodriguez-Perales, S. (2018). CRISPR/Cas9 for cancer therapy: Hopes and challenges. *Biomedicines*, *6*(4), 1–13. https://doi.org/10.3390/biomedicines6040105

Mengstie, M. A., & Wondimu, B. Z. (2021). Mechanism and Applications of CRISPR/Cas-9-Mediated Genome Editing. *Biologics: Targets and Therapy*, *Volume 15*, 353–361. https://doi.org/10.2147/btt.s326422

Mintz, R. L., Gao, M. A., Lo, K., Lao, Y. H., Li, M., & Leong, K. W. (2018). CRISPR Technology for Breast Cancer: Diagnostics, Modeling, and Therapy. *Advanced Biosystems*, *2*(11), 1–24. https://doi.org/10.1002/adbi.201800132

Mollanoori, H., Shahraki, H., Rahmati, Y., & Teimourian, S. (2018). CRISPR/Cas9 and CAR-T cell, collaboration of two revolutionary technologies in cancer immunotherapy, an

instruction for successful cancer treatment. *Human Immunology*, *79*(12), 876–882. https://doi.org/10.1016/j.humimm.2018.09.007

Moses, C., Garcia-Bloj, B., Harvey, A. R., & Blancafort, P. (2018). Hallmarks of cancer: The CRISPR generation. *European Journal of Cancer*, *93*, 10–18. https://doi.org/10.1016/j.ejca.2018.01.002

Moses, C., Nugent, F., Waryah, C. B., Garcia-Bloj, B., Harvey, A. R., & Blancafort, P. (2019). Activating PTEN Tumor Suppressor Expression with the CRISPR/dCas9 System. *Molecular Therapy - Nucleic Acids*, *14*(March), 287–300. https://doi.org/10.1016/j.omtn.2018.12.003

Nair, J., Nair, A., Veerappan, S., & Sen, D. (2020). Translatable gene therapy for lung cancer using Crispr CAS9—an exploratory review. *Cancer Gene Therapy*, *27*(3–4), 116–124. https://doi.org/10.1038/s41417-019-0116-8

Ou, X., Ma, Q., Yin, W., Ma, X., & He, Z. (2021). CRISPR/Cas9 Gene-Editing in Cancer Immunotherapy: Promoting the Present Revolution in Cancer Therapy and Exploring More. *Frontiers in Cell and Developmental Biology*, *9*(May), 1–12. https://doi.org/10.3389/fcell.2021.674467

Porru, M., Pompili, L., Caruso, C., Biroccio, A., & Leonetti, C. (2018). Targeting kras in metastatic colorectal cancer: Current strategies and emerging opportunities. *Journal of Experimental and Clinical Cancer Research*, *37*(1), 1–10. https://doi.org/10.1186/s13046-018-0719-1

Rath, D., Amlinger, L., Rath, A., & Lundgren, M. (2015). The CRISPR-Cas immune system: Biology, mechanisms and applications. *Biochimie*, *117*, 119–128. https://doi.org/10.1016/j.biochi.2015.03.025

Sachdeva, M., Sachdeva, N., Pal, M., Gupta, N., Khan, I. A., Majumdar, M., & Tiwari, A. (2015). CRISPR/Cas9: Molecular tool for gene therapy to target genome and epigenome in the treatment of lung cancer. *Cancer Gene Therapy*, *22*(11), 509–517. https://doi.org/10.1038/cgt.2015.54

Sharma, G., Sharma, A. R., Bhattacharya, M., Lee, S. S., & Chakraborty, C. (2021). CRISPR-Cas9: A Preclinical and Clinical Perspective for the Treatment of Human Diseases. *Molecular Therapy*, *29*(2), 571–586. https://doi.org/10.1016/j.ymthe.2020.09.028

Song, X., Liu, C., Wang, N., Huang, H., He, S., Gong, C., & Wei, Y. (2021). Delivery of CRISPR/Cas systems for cancer gene therapy and immunotherapy. *Advanced Drug Delivery Reviews, 168*, 158–180. https://doi.org/10.1016/j.addr.2020.04.010

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians, 71*(3), 209–249. https://doi.org/10.3322/caac.21660

Surgery for Cancer. (2015, April 29). National Cancer Institute. https://www.cancer.gov/about-cancer/treatment/types/surgery

T-cell Transfer Therapy - Immunotherapy. (2020, August 25). National Cancer Institute. https://www.cancer.gov/about-cancer/treatment/types/immunotherapy/t-cell-transfer-therapy

Tanvetyanon, T., Clark, J. I., Campbell, S. C., & Lo, S. S. (2005). Neoadjuvant Therapy: An Emerging Concept in Oncology. *Southern Medical Journal, 98*(3), 338–344. https://doi.org/10.1097/01.smj.0000145313.92610.12

Tian, X., Gu, T., Patel, S., Bode, A. M., Lee, M.-H., & Dong, Z. (2019). CRISPR/Cas9 – An evolving biological tool kit for cancer biology and oncology. *Npj Precision Oncology, 3*(1). https://doi.org/10.1038/s41698-019-0080-7

Tiruneh G/Medhin, M., Abebe, E. C., Sisay, T., Berhane, N., Snr, T. B., & Dejenie, T. A. (2021). Current applications and future perspectives of CRISPR-Cas9 for the treatment of lung cancer. *Biologics: Targets and Therapy, 15*, 199–204. https://doi.org/10.2147/BTT.S310312

Toyooka, S., Mitsudomi, T., Soh, J., Aokage, K., Yamane, M., Oto, T., Kiura, K., & Miyoshi, S. (2011). Molecular oncology of lung cancer. *General Thoracic and Cardiovascular Surgery, 59*(8), 527–537. https://doi.org/10.1007/s11748-010-0743-3

Travis, W. D. (2011). Pathology of Lung Cancer. *Clinics in Chest Medicine, 32*(4), 669–692. https://doi.org/10.1016/j.ccm.2011.08.005

van der Oost, J., Westra, E. R., Jackson, R. N., & Wiedenheft, B. (2014). Unravelling the structural and mechanistic basis of CRISPR–Cas systems. *Nature Reviews Microbiology, 12*(7), 479–492. https://doi.org/10.1038/nrmicro3279

What Is Cancer? (2021, May 5). National Cancer Institute. https://www.cancer.gov/about-cancer/understanding/what-is-cancer

Xia, A. L., He, Q. F., Wang, J. C., Zhu, J., Sha, Y. Q., Sun, B., & Lu, X. J. (2018). Applications and advances of CRISPR-Cas9 in cancer immunotherapy. *Journal of Medical Genetics*, *56*(1), 4–9. https://doi.org/10.1136/jmedgenet-2018-105422

Xu, Y., & Li, Z. (2020). CRISPR-Cas systems: Overview, innovations and applications in human disease research and gene therapy. *Computational and Structural Biotechnology Journal*, *18*, 2401–2415. https://doi.org/10.1016/j.csbj.2020.08.031

Yip, B. H. (2020). Recent advances in CRISPR/Cas9 delivery strategies. *Biomolecules*, *10*(6). https://doi.org/10.3390/biom10060839

Yoshiba, T., Saga, Y., Urabe, M., Uchibor, R., Matsubara, S., Fujiwara, H., & Mizukami, H. (2018). CRISPR/Cas9-mediated cervical cancer treatment targeting human papillomavirus E6. *Oncology Letters*. https://doi.org/10.3892/ol.2018.9815

Yoshihara, M., Hiyama, T., & Tanaka, S. (2007). Epidemiology of colorectal cancer. Nihon Naika Gakkai Zasshi. *The Journal of the Japanese Society of Internal Medicine*, *96*(2), 200–206. https://doi.org/10.2169/naika.96.200

Zhan, T., Rindtorff, N., Betge, J., Ebert, M. P., & Boutros, M. (2019). CRISPR/Cas9 for cancer research and therapy. *Seminars in Cancer Biology*, *55*(March 2018), 106–119. https://doi.org/10.1016/j.semcancer.2018.04.001

Zhang, H., Qin, C., An, C., Zheng, X., Wen, S., Chen, W., Liu, X., Lv, Z., Yang, P., Xu, W., Gao, W., & Wu, Y. (2021). Application of the CRISPR/Cas9-based gene editing technique in basic research, diagnosis, and therapy of cancer. *Molecular Cancer*, *20*(1). https://doi.org/10.1186/s12943-021-01431-6

Zhang, M., Eshraghian, E. A., Jammal, O. Al, Zhang, Z., & Zhu, X. (2021). CRISPR technology: The engine that drives cancer therapy. *Biomedicine and Pharmacotherapy*, *133*(October 2020), 111007. https://doi.org/10.1016/j.biopha.2020.111007

Zhao, Y., Yang, W., Huang, Y., Cui, R., Li, X., & Li, B. (2018). Evolving Roles for Targeting CTLA-4 in Cancer Immunotherapy. *Cellular Physiology and Biochemistry*, *47*(2), 721–734. https://doi.org/10.1159/000490025

Zhen, S., & Li, X. (2018). Oncogenic Human Papillomavirus: Application of CRISPR/Cas9 Therapeutic Strategies for Cervical Cancer. *Cellular Physiology and Biochemistry*, *44*(6), 2455–2466. https://doi.org/10.1159/000486168

Zhong, L., Li, Y., Xiong, L., Wang, W., Wu, M., Yuan, T., Yang, W., Tian, C., Miao, Z., Wang, T., & Yang, S. (2021). Small molecules in targeted cancer therapy: advances, challenges, and future perspectives. *Signal Transduction and Targeted Therapy*, *6*(1). https://doi.org/10.1038/s41392-021-00572-w

Łukasiewicz, S., Czeczelewski, M., Forma, A., Baj, J., Sitarz, R., & Stanisławek, A. (2021). Breast Cancer—Epidemiology, Risk Factors, Classification, Prognostic Markers, and Current Treatment Strategies—An Updated Review. *Cancers*, *13*(17), 4287. https://doi.org/10.3390/cancers13174287