A Review on RNAi Technology and its Therapeutic Potential in Cancer

By

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing my 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 that 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.

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Ethics Statement

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

Abstract

Cancer is still considered as the major global cause of death. RNAi technology serves as one of the tools to treat cancer with its strategies, designs and delivery methods. This gene silencing technique causes knockdown of genes that encodes for diseased proteins or prevents overexpression of genes. Cancer development and progression occurs as a result of overactivation of different signaling pathways involved in cell proliferation, cell differentiation and cell survival. There are several proteins acting as signaling molecules which can induce cancer from their overproduction. Mutation can also lead to activation of oncogenes that codes for cancer causing proteins. To inhibit the production of the responsible protein in cancer, RNAi gene silencing technique is utilized with siRNA, shRNA and mRNA via degradation of the target mRNA responsible for the disease. This review paper provides an insight on the concept of RNAi technology and its therapeutic potential in different types of cancer.

Keywords: RNAi technology, gene silencing, siRNA, miRNA, shRNA, nanoparticles, cancer

Dedication

This work is dedicated to my parents, who have always supported me and given me the best

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List of Acronyms

siRNA: Small interfering RNA

shRNA: Short hairpin RNA

miRNA: microRNA

mRNA: Messenger RNA

NHEJ: Non-homologous end joining

DsRNA: Double-stranded RNA

SNA: Spherical nucleic acid

PEG: poly(ethylene glycol)

PLGA: poly lactic-co-glycolic acid

PEI Polyethyleneimine

DISE: death induced by survival gene elimination

RT: Radiation Therapy

EBRT: External beam Radiation therapy

BSA: Body Surface Area

MRD: Minimal residual disease

MHT: Menopausal hormone therapy

HRT: Hormone replacement therapy

sgRNA: Single guide RNA

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

DSBs: Double-strand breaks

HDR: Homology-directed repair

SNA: Spherical Nucleic Acid

PBAVE: Poly butyl amino vinyl ether

RISC: RNA-induced silencing complex

HRNP: Hypoxia-responsive nanoparticle

Chapter 1

Introduction

1.1 Background

Cancer is still considered as a significant public health concern worldwide. Cancer is defined as a condition where cells divide uncontrollably and often invade neighboring tissues. The main cause of cancer is changes in DNA sequence resulting in the expression of proteins that either trigger abnormal cell proliferation or silence the expression of tumor suppressor genes. In a study published in Nature Reviews Clinical Oncology, the prevalence of a number of early-onset cancers such as tumors of the breast, colon, esophagus, kidney, liver and pancreas, has gradually increased globally since 1990.

The lack of target specificity of conventional drugs used for cancer treatment such as chemotherapy renders a number of side effects deteriorating the patients' quality of life and makes them vulnerable to infections. Moreover, a significant obstacle to the successful treatment of cancer is drug resistance. Drug resistance continues to be a major barrier to the curative treatment of different cancers. Drug target alteration, drug inactivation by enzymes, overexpression of drug efflux pumps, increased DNA repair capacity, gene mutations, and epigenetic modifications are some of the most prevalent drug resistance mechanisms in cancer chemotherapy. Researchers are developing strategies and technologies that could treat cancer effectively and improve the quality of life of cancer patients. RNA interference (RNAi) technology is one of the fields for cancer therapy. RNAi is a cancer therapeutic that is effective and has the potential to be used as a target specific treatment. RNAi is a method which involves sequence-specific post-transcriptional gene silencing in animals stimulated by the formation of a double-stranded RNA (dsRNA). It represents the most important recent advancement in the field of cell biology. RNAi is used to increase the precision, effectiveness, and stability of medical procedures, especially genetic therapies (Uchino et al., 2013).

A group of molecules that is most significant in RNAi technology is small interfering RNA (siRNA) and microRNA (miRNA). siRNA and miRNA trigger enzymatic degradation of target

mRNA and hence, inhibits the production of corresponding proteins. The discovery that the delivery of siRNAs or miRNAs to mammalian cells cause sequence-specific gene silencing by RNAi indicated that RNAi could potentially be applied as a specific means of inhibiting the expression of any gene of interest in mammalian cells and will possess relatively fewer side effects compared to chemotherapy. Researchers in several studies demonstrated that RNAi-mediated gene silencing significantly inhibits the growth of tumor cells and has few side effects because of its target specific property and high gene silencing efficiency (D. Li et al., 2021). A key gene-modification method in cancer therapy is that the RNAi therapy can have synergistic effects in cancer cells when used with anticancer medications. Pre-clinical investigations in animal models induced with human disease supports the use of RNAi for practical application, despite the fact that clinical applications of RNAi-based therapeutics are not yet fully realized (Uchino et al., 2013). The major purpose for using this kind of therapy is to treat cancer due to its capability to specifically inhibit a broad range of genes related to the disease without the draggability of the protein products produced by those genes. As a result, RNAi therapy may be used to treat cancers that are currently incurable (Mansoori et al., 2014).

The development of RNAi technology over the past ten years has been phenomenal and it is hoped that further research will lead to the wider adoption of RNAi therapies as a cancer treatment. For cancer treatment, RNAi technology is a systemic delivery method. However, to minimize RNA degradation, off-targeting, toxic effects, and recognition by the immune system as a foreign agent, the development of safer, more dependable and more efficient drug delivery systems is of highest concern for RNAi treatment techniques (Uchino et al., 2013). As a result, features of various nanocarriers are compiled for efficient tumor delivery of RNAi therapies (D. Li et al., 2021).

1.2 Objectives of the Study

The objectives of this study are-

- to provide an overview on the concept of RNAi technology
- to investigate the therapeutic potential of RNAi technology in cancer

1.3 Rationale of the Study

Traditional medicines for cancer treatment, such as chemotherapy, have shown several side effects in patients including compromised immune system and death of healthy neighboring cells due to its target non-specificity. Radiotherapy is also not very effective in cancers that have undergone metastasis. Moreover, surgical removal of the malignant cells sometimes does not prevent relapsing of cancer. To overcome the side effects associated with conventional therapies of cancer, it has become very necessary to develop treatment strategies that are target specific. A number of targeted therapies have been developed in the last few decades, some of which are already FDA approved and some are undergoing preclinical and clinical trials. The targeted therapies include small molecule inhibitors, immunotherapy and gene therapy. In this review paper, a comprehensive insight will be provided on one of the gene therapy techniques known as RNAi technology and its therapeutic potential in cancer will be discussed. RNAi technology targets the mRNAs responsible for translating into proteins that contribute to the development and progression of cancer.

Chapter 2

Methodology

This study reviews the therapeutic potential of RNAi technology in cancer. For this extensive review, initially the outline was made and then all the information and data were collected from databases like PubMed, Scopus, ResearchGate, Elsevier, ScienceDirect, Springer. The articles were prioritized from the last few years. The keywords searched for relevant information are “RNAi technology”, “RNAi technology in cancer”, “chemotherapy and radiotherapy”, “gene splicing”, “siRNA induced gene”, “shRNA induced gene”, “miRNA induced gene”, “treatments of cancer”, “type of cancer”, “RNAi on each type of cancer” etc. The information was extracted from the selected articles and cited properly.

Chapter 3

Cancer

3.1 What is Cancer?

Cancer is a genetic disease caused by mutations in the genes that regulate cells' functions, particularly how they grow and divide. Cancer is a condition when some of the body's cells grow out of control and spread to other bodily regions. In the millions of cells that make up the human body, cancer can appear basically anywhere. Human cells typically grow and divide to create new cells as the body requires them. New cells replace old ones when they die as a result of aging or damage. Cancerous tumors can move to remote regions of the body to generate new tumors. They can also invade and destroy surrounding tissues. This mechanism, known as metastasis, is a major factor in cancer-related fatalities. This systematic mechanism is disrupted in cancer, causing damaged or aberrant cells to proliferate against their normal process. Cancer cells do not respond to signals to stop dividing or to let go and die, in contrast to regular cells. It is uncontrollably growing and unable to identify its own natural limits. Genes in cancer are the essential building blocks of inheritance that can change in manner. Chromosomes are long, tightly packed DNA strands where genes are organized. A heuristic method for reducing the enormous complexities of cancer phenotypes and genotypes to a tentative set of guiding principles is the hallmark of cancer conceptualization. Other aspects of the disease have surfaced as potential improvements of the mechanisms behind cancer have grown. The complexities of cancer, which spans genetics, cell and tissue biology, pathology, and therapeutic response, is intimidating. At this period, the eight hallmarks include: capabilities for sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing/accessing vasculature, activating invasion and metastasis, reprogramming cellular metabolism, and avoiding immune destruction (Hanahan, 2022).

Cancer is a kind of pain that has a significant negative impact on quality of life and is related to a range of psychosocial reactions. Patients report what type of discomfort makes it difficult for them to focus on or think clearly and that it makes it difficult to carry out regular daily tasks. More than one-third of patients say that their cancer's associated pain is upsetting or perhaps intolerable (van den Beuken-Van Everdingen et al., 2016).

3.2 Treatment for Cancer

There are several types of cancer treatment options. The type of cancer and their stages will determine the kind of treatment the patients will get. Some cancer patients will only receive one treatment. However, the majority of patients have a combination of therapies including surgery along with chemotherapy and radiation therapy and other treatment options as required.

3.2.1 Chemotherapy

Chemotherapy is a cancer treatment which uses chemical agents, most commonly cytotoxic agents, to kill the cancer cells. During cancer treatment, one or more than one anti-cancer drugs may be used. Chemotherapy is provided with the goal of curing the disease, extending life and reducing symptoms. It is the first-line treatment for small cell lung, non-small cell lung, breast, colon, ovarian, pancreatic, bladder, hematological, and high-risk cervical cancers in addition to surgery and radiation therapy. There are different types of chemotherapeutic which differ in the mechanism to destroy the cancer cells. The agents can be classified as alkylating agents, antimetabolites, antitumor antibiotics, topoisomerase inhibitors, and mitotic inhibitors. The therapeutic window for cytotoxic chemotherapy is considered to be relatively short; if exposure levels are beyond the ideal range, there is a risk of toxicity or ineffectiveness. When there are toxicity symptoms, chemotherapy doses and dosing regimens are frequently changed. The considerable adverse effect that toxicities have on patient quality of life shows the need of avoiding unnecessary toxicity. Moreover, chemotherapeutic agents destroy healthy cells along with the diseased ones due to its target non-specificity which can deteriorate the patients' quality of life and make them immunocompromised. As a result, a key element in raising the chance of a positive outcome is the design of a therapeutic strategy that has been meticulously tailored for the specific patient and is specific to the cancer cells (Knezevic & Clarke, 2020). Combination chemotherapy is more successful than single drug treatment in most of the cancers for which chemotherapy is effective. Cytotoxic agents with qualitatively different toxicities and with different molecular sites and mechanisms of action, are usually combined at full doses. This results in higher response rates due to additive and potentiated cytotoxic effects and nonoverlapping host toxicities.

3.2.2 Neoadjuvant and Adjuvant Therapies

Neoadjuvant and adjuvant therapies involve multiple treatment modalities like chemotherapy, hormone therapy, radiation therapy, chemotherapy and immunotherapy which have been utilized for decades to increase the cure for many solid tumors. Neoadjuvant therapy is administered to shrink the tumor size prior to surgery. The main goal of this type of treatment is to reduce the scope and increase the effectiveness of the primary treatment by downstaging cancer and eliminating circulated cancer cells. Adjuvant therapy is administered after the primary treatment with the goal of reducing the chance of cancer relapse by destroying any remaining cancer cells thereby, increasing the cure rate. It potentially prevents postoperative problems and extends recovery from delaying systemic treatment in certain patients. According to the choice of treatment, neoadjuvant immunotherapy offers more benefits over adjuvant immunotherapy that aims to increase survival and cure chances. Neoadjuvant therapy is more effective when patients are not immunosuppressed, it can induce more immune response against antigens, better interaction between immune cells and the tumor microenvironment because of the intact structure of the lymphatic system (Bilusic, 2022).

3.2.3 Radiation Therapy

The multidisciplinary area of radiation oncology crosses the borders of physics, chemistry, biology and medicine. External beam RT (EBRT), which uses high-energy photons (6–25 MV) to deposit energy deeply in the tissue while protecting the skin from radiation exposure accounts for the majority of clinical RT for cancer treatment. Radiation is administered for curative therapy over a period of 6 to 8 weeks in a series of daily fractions of 1.8 to 2 Gy. To increase the therapeutic ratio for such fractionated radiotherapy, it takes use of the disparities between the tumor and normal tissues' capacity for healing. Conventional fractionated EBRT has been the "mainstay" of clinical radiation therapy. A growing body of research has shown the therapeutic advantages of other RT modalities such as modified fractionation (hypofractionation and hyper fractionation), internally applied RT (brachytherapy) and particle therapy (proton, carbon). The physical and radiobiological characteristics of each form of RT vary which has an impact on the therapeutic applications and results. The principles of various forms of RT are outlined and the justifications and clinical outlook were examined in some research. Chemoradiation, which involves giving chemotherapy along with RT, is one of the methods for overcoming the

drawbacks of single modality RT. Chemo-RT uses interactions at the cellular or molecular levels to sensitize cells to radiation, giving it distinct advantages over either modality alone (Allen et al., 2017).

3.2.4 Surgery

Solid tumors are typically treated with surgical excision. There is substantial evidence that tumor removal may actually have a negative impact on MRD's (Minimal residual disease) natural path. There have been several reported processes by which surgery could affect MRD's ensuing growth. After the main tumor has been removed, these can be roughly categorized as mechanisms that disseminate, facilitate or speed up the expansion of tumor cells. First, in tumor modification there is spread of tumor cells like circulating tumor cells. These could increase the overall tumor size and cause MRD. Second, the effects of surgery will produce a time period wherein cancer is more likely to occur in the patient. Postoperative immunosuppression may enhance MRD expansion easier during this stage of wound healing by encouraging immune escape. Thirdly, removing the tumor may change the biological characteristics of cancerous cells, increasing cellular proliferation and decreasing cell death. These conditions will raise the potential for tumorigenicity and expedite the growth of the MRD. The possibility that some of these mechanisms are at action is the fourth theory in surgery. For instance, changes resulting from genetic instability may make circulating tumor cells dispersed following surgery more tumorigenic. The important underlying mechanisms include the spread of tumor cells throughout the body after the surgery. The creation of an environment that is favorable for tumor growth and the direct modification of neoplastic characteristics results in faster tumor growth. For tumor removal, post operative period is the window opportunity to protect against cancer (*Personal View Excisional Surgery*, n.d.).r.

3.2.5 Molecular Targeted Therapy

Molecular targeted therapies interfere with particular molecules to stop the growth, progression, and metastasis of cancer. The Food and Drug Administration (FDA) has approved different molecular targeted therapies that have achieved clinical success in the treatment of a variety of cancer types, including breast, leukemia, colorectal, lung, and ovarian cancers. The idea of a "magic bullet," which was first put forth by Paul Rich in the late 1800s, served as the foundation

for the concept of targeted therapy. For molecular targeted therapies for cancer to be developed successfully is to find the ideal targets. A genetic profile change that results in changes in proteins and receptors that support cell survival and proliferation is one of the causes of cancer in molecular targeted therapy. The development of molecularly targeted therapies can use these particular genetic alterations to identify cancer cells from healthy cells. The physiology and properties of particular molecular targets in cancer can be understood by researchers in order to identify new molecular methods to block the growth and spread of tumors. Genome sequencing enables the comparison of expression of genes and proteins in normal and malignant cells and then discover changes in those expressions can be used to find cancer markers. Cancer treatments that use molecularly targeted therapeutics can have a variety of characteristics and functions. It influences cell surface antigens, growth factors, receptors, or signaling pathways, which control cell cycle progression, cell death, metastasis, and angiogenesis according to the molecular targets. Small molecules, monoclonal antibodies, immunotherapeutic cancer vaccines and gene therapy are the different classes of substances employed in molecular targeted therapy (National Cancer Institute, 2017; Padma, 2015). In order to destroy cancer cells, drugs employed in molecular targeted therapy can disrupt signals that encourage cancer cell development and interfere with the control of the cell cycle or trigger cell death. Moreover, these medications work to stimulate the immune system by targeting both cancer cells and elements in the tumor microenvironment (Lee et al., 2018).

3.2.6 Immunotherapy

Immunotherapy is a kind of treatment used for cancer patients to boost the immune response of the body against cancer cells. Cancer immunotherapy has revolutionized the manner that cancer is treated well because it aims to increase antitumor immune responses while having fewer unintended side effects than chemotherapies and other treatments that kill cancer cells directly. Agents are employed in cancer immunotherapy to stimulate or enhance the immune system's ability to kill cancer cells naturally. With various drugs in clinical and preclinical development, a number of immunotherapy approvals is on the rise (Riley et al., 2019). Immunotherapy includes-

i. Immune Checkpoint Inhibitors

Immune checkpoints physiologically regulate normal immunological responses and defend healthy organs from immune damage by checkpoint inhibitors. T cells express PD-1 when they are activated in response to inflammation, which allows them to identify aberrant and malignant cells. Tumor cells express PD-L1 that binds to PD-1 on T cells to make those cells inactive. This helps the cells to avoid detection and eradication by T cells. Therefore, by preventing this interaction using monoclonal antibodies (mAbs) that specifically target PD-1 or PD-L1, T cell-mediated tumor cell killing is now made possible. The co-inhibitory molecule CTLA4, another immunological checkpoint, controls the degree of T cell activation. T cell function is inhibited by interactions between CTLA4 and its ligands (CD80 and CD86) which accelerates the growth of tumors. T cells continue to be active and recognize and kill tumor cells by preventing the interaction between CTLA4 and these ligands.

ii. Cytokines

Cytokines were the first group of immunotherapies that was taken into the medical context with the authorization of recombinant IFNα (interferons) treatments in 1986. In contrast to checkpoint blocking strategies, this approach directly stimulates immune cell development and activity through injection of cytokines. Interleukins, interferons, and granulocyte-macrophage colony stimulating factor are the three primary cytokine types that have been pursued for immunotherapy (GM-CSF).

iii. Cancer Vaccines

Tumor cell lysate, dendritic cells, nucleic acids (like mRNA) and neo-antigens are examples of cancer vaccines. The class of cell-based cancer vaccines that is most frequently explored is dendritic cell vaccines. Dendritic cells extracted from patients and modified to express tumor-associated antigens which in turn triggers T lymphocytes to target cancer cells (Riley et al., 2019).

iv. Hormone Therapy

Tumors may develop in hormone dependent tissues e.g., breast, prostate gland, uterus which are marked as hormone dependent due to presence of hormone receptors. In certain cases, the elimination of a hormonal stimulation can result in tumor regression. Hormone therapy plays an essential role in the therapeutic management of sex hormone-dependent cancers because it slows down tumor growth and reduces cancer symptoms. Hormone therapy can decrease growth by using hormone antagonists which prevent hormones from having their intended effect. Agents that prevent hormone production and hormone agonists can desensitize or produce negative feedback (Deli et al., 2020).

v. Gene Editing Techniques

Cancer is a genetic disease built upon with repeated genetic and epigenetic abnormalities. Current cancer therapies are limited due to the complexity of its mechanisms. The ability to precisely manipulate almost any genomic sequence is now possible because of advances in technology like CRISPR-Cas9-mediated genome editing technology. Researchers from all across the world are interested in the genome editing technology known as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9. Single guide RNA (sgRNA) and DNA endonuclease Cas9 make up the CRISPR-Cas9 system. The latter leads to particular DNA sequences to cleave double-stranded DNA site-specifically. Currently, sgRNA for site-specific recognition is widely used in CRISPR-mediated genome editing to specifically change one or more target genes in a cell type or organism. After Cas9 binds to and cleaves the target DNA sequences, double-strand breaks (DSBs), which are introduced in the genome sequence of interest at about -3 nucleotides before the protospacer adjacent motif (PAM) sequence. After introducing the genome through DNA repair machinery and through less common homology-directed repair (HDR) or the predominant non-homologous end joining (NHEJ) are then initiated. While NHEJ typically causes genomic insertions or deletions for gene disruption with high efficiency. HDR uses donor DNA template to accurately repair DSBs for gene alteration with low efficiency. CRISPR-Cas9 system can readily make multiple double-strand breaks (DSBs) and retarget new DNA regions, enabling more complex gene editing through the simultaneous expression of several alternative sgRNAs and basic changes to the sgRNA

sequence, respectively. Additionally, the target gene's transcription is either activated or blocked by fusing the inactive Cas9 mutant with different effector domains, known as CRISPRa and CRISPRi. More recently, it has been discovered that some members of the Cas9 family can target RNA in addition to DNA. Several RNA-targeting Cas9 (RCas9) systems have been developed, setting new applications like RNA virus infection prevention, intracellular transcript imaging and post-transcriptional gene silencing (Chen et al., 2019).

RNAi technology is another gene editing technology that is being widely investigated for its effectiveness against different types of cancer. A comprehensive insight on this gene silencing strategy will be provided in this study.

Chapter 4 RNAi Technology

RNA interference (RNAi) technology is referred to as a gene silencing method that inhibits the production of particular proteins involved in the development and progression of diseases like cancer. RNAi technology is used to increase the precision, effectiveness and stability of the treatments especially gene therapies. A fast and easy research technique for inhibiting the expression of a gene of interest is RNAi technology. In the mechanism, RNAi involves some siRNA, shRNA, miRNA. The aim is to degrade the mRNA and hence, inhibit diseased protein production. When dsRNA is introduced in the cells, tan endonuclease enzyme Dicer gets activated. Dicer will break the dsRNA into short fragments. These short fragments will bind to Slicer or Argonaut II and will form a complex called RISC. These RISC will guide those short fragments of RNA toward mRNA that needs to inhibit translation. The strand which has complementary bases to target mRNA will bind to target mRNA. Then, an enzyme called RNaseH will degrade mRNA involved in cancer causing protein production. The mechanism by which RNAi technology works has been depicted in Figure 1.

Furthermore, by specifically inhibiting a cancer-associated target, RNAi provides a new opportunity for gene therapy to work against certain neoplasms (Lage, 2005).

4.1 siRNA Induced Gene Silencing

The two stages of siRNA production are the initiating stage and the effecting stage. In the initial stage, Dicer cleaves a long double stranded RNA (500–200 bp) into small fragments and siRNA is formed. In the affecting stage, helicase separates double-stranded siRNA before endogenous demarcation of the sense strand. The RNA-induced silencing complex (RISC) binds to endonucleases and an antisense strand. This complex then binds with the target mRNA. Argonate, a RISC element, breaks down the target mRNA through its ribonuclease activity. As long as RISC breaks the target mRNA, gene expression stops (Mansoori et al., 2014).

The main benefit of siRNA therapy in the treatment of cancer is its capacity to precisely inhibit a broad range of cancer-associated genes without considering the draggability of protein. siRNA therapy may be used to cure cancers that are currently incurable. siRNA and microRNA are two different categories of small RNA molecules. siRNA needs mRNA complementarity to be

perfect, whereas miRNA can target mRNA with less perfection. The main comparison is that miRNAs primarily mute their target genes by translational repression, whereas siRNAs often shut down gene expression at a post-transcriptional level through mRNA degradation. Post-transcriptional gene silencing and transcriptional gene silencing are the two basic phases of the siRNA-mediated gene silencing mechanism. Both have a particular repressive impact. The direct sequence-specific cleavage translation inhibition and subsequent degradation is one of two additional post-transcriptional gene silencing methods (Subhan & Torchilin, 2019).

siRNA medicines provide advantages over chemotherapy anti-cancer drugs, especially for resistant cancer treatment. First, it is incredibly safe. Second, siRNA does not interact with DNA, avoiding the mutation and teratogenic concerns associated with gene therapy. Instead, it acts on the post-translational stage of gene expression. Third, siRNA is incredibly effective. Fourth, even in a single cell with a small number of copies, siRNA can significantly decrease gene expression. Fifth, the greatest benefit of siRNA over other small molecule medications or antibody-based medications is the freedom to target any gene and the specificity that results from complementary base pairing (Subhan & Torchilin, 2019).

4.2 shRNA Induced Gene Silencing

In contrast to siRNA, shRNA is formed in the nucleus and then transferred to the cytoplasm for processing before binding to RISC and carrying out its functions. RNA polymerase II or III promoters on the expression cassette work together to transcribe shRNA. The stem-loop-like structure that initiates the transcription of the RNA polymerase II promoter is processed by a complex that includes the RNaseIII family, Drosha and double-stranded RNA binding protein domain (DGCR8). The enzyme breaks down the shRNA hairpin complex into individual shRNAs by adding two overhang nucleotides to the 3' end. Pre-shRNA, which is carried into the cytoplasm by exportin5/RanGTPase, is processed during this stage. Pre-shRNA is transported onto RNaseIII complexes in the cytoplasm that also contain Dicer, TRBP and PACT. The loop is then cleaved, resulting in the production of double-stranded siRNA with a two-nucleotide overhang at the 3'. siRNA can be loaded onto the RISC's Ago2 protein with the support of the complex comprising Dicer (Mansoori et al., 2014).

4.3 miRNA Induced Gene Silencing

In the nucleus, miRNAs are transcribed by RNA polymerase II which are several hundred nucleotides long pri-miRNAs. Pri-miRNAs have bulges and mismatches and one or more hairpin structures with a length of roughly 70 nucleotides. The bulges might help avoid the long dsRNA-induced interferon response. Additionally, this structure shields miRNAs from RNase degradation. Pri-miRNA is then cut by the intra-nuclear RNaseIII type endonuclease Drosha to a stem-loop hairpin structure known as precursor (pre)-miRNA. Pre-miRNAs are specifically exported to the cytoplasm by binding to the nuclear export receptor Exportin-5. The pre-miRNA is then converted by Dicer into a mature miRNA, which has roughly 21 double stranded nucleotides. Similar to RISC in the siRNA pathway, one strand of miRNA is incorporated into the micro-ribonucleoprotein complex (miRNP) and unwound by the helicase activity of miRNP. RISC and miRNP share several basic components. The molecular structure and size of RISC and miRNP forms are different. The miRNA-induced miRNP mostly suppresses mRNA translation by binding to the 3' UTR of the relevant mRNAs through improper base pairing. A few target mRNAs can also be cleaved by mammalian miRNAs. Only a 7 base complementary seed region (positions 2–8 of the miRNA) is usually needed for miRNA binding to the 3' UTR of mRNAs, but this is enough for translation. A significant number of mRNAs that share the same short motifs in their 3'UTR regions can be impacted by a single miRNA since miRNAs require less complementarity than generic siRNAs. It is predicted that miRNAs control at least 30% of all proteins. As a result, dysregulation of miRNAs has an impact on a variety of cellular processes, including organogenesis, cell differentiation, proliferation, and death. miRNA expression profiles in cancers have been examined, and several of these miRNAs are thought to promote or inhibit tumor growth. In cancer cells, miRNA expression levels are usually downregulated altogether. The majority of miRNAs thus appear to have a tumor-suppressive impact. In fact, non-specific miRNA degradation is caused by dysfunction of the miRNA processing system, including that of Dicer (Okamoto & Murawaki, 2012). Table 1 shows the comparison of the general properties among siRNA, miRNA and shRNA.

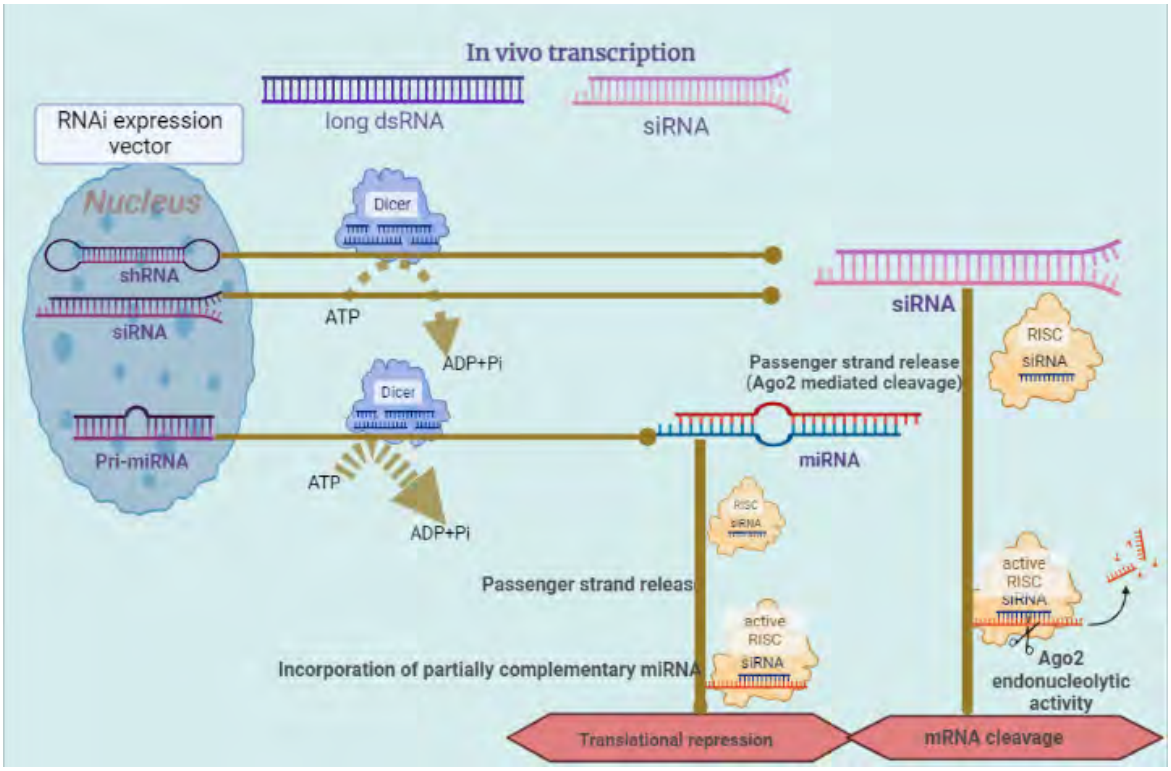

Figure 1: Mechanism of action of RNAi technology. Long double-stranded RNA (dsRNA), small interfering RNA (siRNA), short hairpin RNA (shRNA), microRNA (miRNA) all affect RNAi. Here, Dicer breaks down long dsRNA, shRNA, and pre-miRNA. An RNA-induced silencing complex is produced when processed siRNAs come along with cellular proteins (RISC). One strand passenger is removed during RISC assembly, whereas the other strand forms an active RISC that eventually causes translational repression or the breakdown of sequence-specific mRNA (Adapted from de Paula et al., 2007).

Table 1: Comparison among siRNA, miRNA and shRNA (Adapted from Li et al., 2021; Lam et al., 2015)

Features	siRNA	miRNA	shRNA
Source	Chemically synthesized; processed from long dsRNA dsRNA that contains 30 to over 100 nucleotides or pre-shRNA	Endogenic; chemically synthesized; pre-miRNA contains 70-100 nucleotides with interspaced mismatches and hairpin structure	Expressed from shRNA vector
Structure	21-24 nucleotide RNA duplex with 2 nucleotides 3ˈ overhang	18-25 nucleotide RNA duplex with 2 nucleotides 3ˈ overhang	A stem of 19–29 nucleotides with a loop of at least 4 nucleotides and a dinucleotide overhang at the 3′ end.
mRNA target	Inhibit the expression of one specific target mRNA	Regulate the expression of multiple mRNAs.	Inhibit the expression of one specific target mRNA
Complementary	Fully complementary to mRNA	Partially complementary to mRNA, typically targeting the 3ˈuntranslated region of mRNA	Fully complementary to mRNA

4.4 RNAi Therapy Delivery Methods

i. siRNA-Ligand Conjugates

The direct attachment of ligands to siRNA is a potential delivery method. To enhance cellular absorption and target particular cell types, siRNA has been covalently attached to a variety of

ligands, such as small molecules, carbohydrates, aptamers, peptides, and antibodies. As siRNA conjugates there is the benefit of requiring additional delivery components that also enhances the delivery formulation's tolerance and safety profile. Conjugation is normally carried out on the sense strand or 3' end of the antisense strand since the 5' end of the antisense strand is necessary for silencing activity. Parallel synthesis and linear synthesis are the two synthetic methods that have been used. A variety of chemical conjugations to siRNA are frequently made using linear synthesis in which functional groups are added consecutively (Dong et al., 2019).

ii. Nucleotide Derived Nanoparticles

The use of nanoparticles for disease detection, monitoring, prevention and treatment is known as nanomedicine or nanotechnology. Three areas of nanomedicine where nanomaterials can be used extensively include diagnosis, drug delivery and regenerative medicine. The use of nanocarriers as drug delivery methods is growing rapidly, especially for the treatment of cancer. Drugs systemically delivered have enhanced bioavailability, safety and pharmacokinetic characteristics due to the use of nanoparticle-drug compositions. A wide range of nanoparticles can be produced using nucleotides as building blocks. A siRNA delivery system must fulfill a number of requirements that take into account the difficulties siRNA faces in cancer cells. Effective and safe distribution of siRNA is a major difficulty since the big (13 kDa) and negatively charged molecules cannot diffuse across cancer cell membranes on their own. Nanoparticles based siRNA delivery is beneficial as they

- have less immunogenicity
- minimize interactions with serum proteins and non-cancer cells
- resist renal clearance
- increase vascular permeability
- permit cell entry and endosome escape to enter the RNAi machinery
- have low toxicity
- are biocompatible and biodegradable (Subhan & Torchilin, 2019).

On the surface of the nanoparticle, folate-a cancer targeting ligand was placed to identify tumor cells from healthy cells. At a dose of 2.5 mg/kg (anti-luciferase siRNA), the tailored siRNA-DNA structure revealed significant suppression in tumor cells in a mouse xenograft model. Moreover, compared to free siRNA (t1/2 ≈ 6 min), it showed a longer blood circulation time (t1/2 ≈24.2 min) (Dong et al., 2019).

iii. siRNA-Polymer Bioconjugates

Solid, biodegradable, colloidal systems that have been thoroughly explored as drug vesicles are polymer-mediated delivery systems, also known as polymeric nanoparticles. Polymer-mediated delivery methods can be categorized into two groups, water soluble cationic polymers and polymer nanoparticles, depending on the substance utilized. Polymer nanoparticles are often based on polycaprolactone (PCL), poly(D, L-lactide) (PLA) and poly(D,L-lactide-co-glycolide) (PLGA), while water-soluble cationic polymers for anticancer siRNA delivery include cyclodextrin or polyethyleneimine (PEI) .

Polyethylenimine (PEI) is one of the most studied cationic polymers for siRNA and miRNA delivery, with straight and branched forms. PEI has a high cation charge density, which is considered as one of its advantages. Capability to complex siRNA is one of the initial needs for polymeric carriers. Electrostatic interactions between positively charged groups on polymer chains and the negatively charged phosphates in siRNA molecules have been used most commonly to effect this. siRNA directly conjugates to polymers to enhance stability, pharmacokinetics, cellular uptake and transport. *In vitro* gene silencing in hepatoma cells and in a tumor model was found to be facilitated by PEG conjugated to siRNA via an acid-labile linker (Dong et al., 2019).

iv. Lipid Based Delivery System

Small spherical vesicles with an aqueous core inside and a bilayer lipid membrane that protects pharmaceuticals from oxidation, have the benefits of low toxicity and non-immunogenicity are known as liposomes (Chen et al., 2010). For RNAi delivery, cationic liposomes are attractive due to their easy RNAi complexation and vascular targeting. Using a rotary evaporator, cationic lipids that have been dissolved in a solution of organic solvents are evaporated to a thin film

layer to produce cationic liposomes. The lipid film is rehydrated in a buffer to allow for liposome formation after any remaining solvent has been removed. RNAi complexation can arise after this liposome suspension has gone through an extruder. RNAi delivery methods consider cationic liposomes to be particularly intriguing due to how easily RNAi molecules can mix with them. Many different anionic lipid-based formulations have been developed as alternatives to positively charged lipid nanoparticles due to their systemic toxicity. When compared to their cationic liposomes, anionic liposomal delivery systems exhibit superior biodistribution, effectiveness, and less toxicity (Ward et al., 2021).

Chapter 5

Therapeutic Potential of RNA Interference Technology in Cancer

RNAi's effectiveness in a cancer treatment is highly efficient that induces gene silencing during advanced phases of growth because it transmits silenced genes to the next generation, affordable than other forms of gene therapy and more specific than other forms of cancer treatment. Cancer is one of the main targets for RNAi-based therapy (Mansoori et al., 2014). RNAi is a post-transcriptional technique that inhibits gene expression by stimulating cleavage on a particular location of a target messenger RNA (mRNA). This technique has demonstrated improved treatment effects for a variety of diseases, particularly cancer. Small regulatory RNAs, microRNAs or siRNAs, can silence target messenger RNAs (mRNAs) by a sequence-specific process known as RNA interference (RNAi), an endogenous post-transcriptional control process. Recently, RNAi has been used in therapeutic applications and has developed into a potent method for evaluating gene function. The down-regulation of particular proteins is the primary function of RNAi in cells. When the reference strand sequence is paired with an mRNA complementary sequence, Argonaute 2 (Ago2) cleaves the target mRNAs, resulting in the siRNA passenger strand being unwound and the siRNA guide strand being loaded into the RNA-induced silencing (RISC) complex. Through utilizing the mRNA transcript sequences identified in the adequate human genomic data to produce oligonucleotide compounds, this essential mechanism has made it possible to develop novel therapeutic approaches (Mahmoodi Chalbatani et al., 2019).

5.1 RNAi Therapeutics for Pancreatic Cancer

Pancreatic cancer is expected to be the second leading cause of death from malignancy by 2025. One of the most aggressive cancers is marked as pancreatic cancer that has a five-year survival rate of 8%. Chemotherapy, radiotherapy and molecularly targeted therapies are preferable to surgical treatment because of the aggressive perineural and vascular local growth. There is an urgent need for novel therapeutic alternatives for patients with pancreatic cancer because chemotherapy typically only increases life expectancy from 8 to 16 weeks. Over the past ten years, there has been extensive research in new anticancer medications and evaluated successful

treatment methods, but the overall patient survival rate has remained poor. Because of genetic abnormalities and dysregulated signaling it is the main cause of tumor formation to conventional therapy in pancreatic cancer. Interference of RNAi with expression of specific genes has gained attention as a promising treatment option. Despite their therapeutic potential, RNA-based therapeutics still face challenges like tumor vulnerability to serum breakdown. These limitations could've been removed by using nanocarriers in clinical applications. (Kim et al., 2021)

The initial stages of pancreatic cancer are frequently symptomless which delays identification. Pancreatic ductal adenocarcinoma (PDAC), representing 90% of cases of pancreatic cancer, is an exocrine cell carcinoma that develops in the pancreatic duct. For PDAC patients, there are numerous experimental therapy options available, however the 5-year survival rate is very low (only 3-6%). It also has a low incidence of excision, a poor prognosis and chemotherapy and radiotherapy medication resistance. Target gene knockdown therapy, a novel treatment approach has demonstrated significant therapeutic potential. Further, when radiotherapy and chemotherapy are combined with RNAi, pancreatic cancer cells become less resistant to these treatments (Chang, 2007). The studies researchers have been carrying out are: frequent uncontrolled microRNA in PDAC, potential molecular targets in signaling pathway, use of nano-gene-silencing drugs to target patients who are resistant to treatment and duodenal homeobox 1 as a specific and potential RNAi target and use of siRNA to silence or inhibit the kirsten rat sarcoma viral oncogene (KRAS). Pancreatic, colon and lung cancer often have mutant variants of the oncogene KRAS. Despite the potential of siRNA therapy, there are challenges that limit clinical applications. These challenges are brought on by the high genetic heterogeneity in pancreatic tumors. However, nanotechnology offers a wider platform for upcoming research to address these issues (Tian et al., 2021).

In combination therapy, use RNAi drugs with acquired genetic or epigenetic mechanisms rather than additional pharmacologic inhibitors which increase toxicity. As Pancreatic cancer is one of the most prevalent cancer types with a very low survival rate, it can be distinguished from other cancer types by the thick tumor microenvironment that surrounds it and restricts the therapeutic impact of available medications. Dysregulated expression of genes associated with cancer causes both the thick stroma and acquired resistance to standard therapy. RNAi-based techniques have gained popularity for targeting these important genes because they can both directly attack

tumors and change the tumor microenvironment to break down obstacles to chemotherapeutic action. According to Zeng et al., the synergistic effects of siRNA-induced cell cycle block and arsenic-induced apoptosis significantly reduced the growth of pancreatic tumors when used together (Kim et al., 2021).

PDAC (pancreatic ductal adenocarcinoma) is caused by the cumulative accumulation of critical driver mutations, which typically result in the activation of the KRAS oncogene and the deletion of the tumor suppressor gene TP53. Invasive adenocarcinoma is brought on by these genetic anomalies, which also alter the stromal structure in the area to make it more conducive to tumor growth. Because they are challenging to suppress using tiny pharmacological molecules, the genes identified as novel cancer-related oncogenes that promote tumor development are frequently found to be "undruggable." Many oncogenes are difficult to suppress with small-molecule drugs because they lack appropriate ligand binding sites or because they have extensive protein-protein interaction surfaces (Kim et al., 2021).

The originating role of the KRAS mutation in PDAC and its involvement in the development of the malignancy are supported by genetically modified mice models. The emergence of metastatic PDAC occurs frequently when pancreatic intraepithelial neoplasia is followed by mutational inactivation of TP53 (TP53R175H), CDKN2A, or SMAD4. KRAS has been a difficult problem in recent decades because there are no specific inhibitors for it as a therapeutic target. KRASG12C inhibitors were recently developed, and Phase I clinical studies on non-small cell lung cancer patients showed they were beneficial. However, only 2% of PDAC contain KRASG12C-specific mutations, as opposed to the more frequent KRASG12D mutation. As a result, even if KRASG12C inhibitors are a good proof of concept, the need to find alternative KRAS-targeting molecules like RNAi only grows. Other abnormalities in gene expression in PDAC, in addition to the conspicuous undruggable KRAS mutation, highlight the necessity of an RNAi-mediated therapeutic strategy. siRNA therapies are used to treat pancreatic cancer by focusing on a variety of "undruggable" genes. Most crucially, the inclusion of delivery mechanisms such extracellular vesicles and nanoparticles has made it easier to deliver siRNA to cancer cells. There are multiple convincing examples of dysregulated miRNA levels in pancreatic cancer from both in vitro models and PDAC patient samples. Eight miRNAs—miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b, and miR-95—were

significantly up-regulated in the majority of pancreatic cancer samples after 95 miRNAs were analyzed for expression levels in pancreatic cancer tissues and cell lines. In contrast hand, PDAC commonly exhibits down-regulation of the tumor suppressor miRNAs miR-148a, miR-217, miR-34a, and miR-375 (Kim et al., 2021).

5.2 RNAi Therapeutics for Breast cancer

The most common cancer in women is breast cancer. The emergence and evolution of various breast cancer subtypes by the response to treatment are all influenced by various genetic modifications and gene expression profiles. In this regard, gene therapy is emerging as a viable treatment option for breast cancer due to its potential to fix damaged genes and control gene expression (Zuo et al., 2017). Transferring genes, gene fragments or oligonucleotides including siRNAs and miRNAs, *in vivo* or *in vitro* allows for the genetic manipulation of target cells (Bottai et al., 2017). RNAi-based treatment options for HER2+ (human epidermal growth factor receptor 2) breast cancer have been investigated in a few preclinical studies. Two of these have shown successful targeted injection, combining siPLK1 with a peptide fusion protein carrying HER2 scFv and employing PEG-PLA-based nanoparticles that bind to HER2 scFv for targeted siRNA delivery (Ngamcherdtrakul et al., 2016). Additionally, many researchers are working on the concept of targeted siRNA delivery to treat and eradicate cancer's chemoresistance, employing miRNAs as a functional marker to identify cell properties and control the biological activity of breast cancer cells (Tian et al., 2021).

5.2.1 Dual Hypoxia-Targeting RNAi Nanomedicine for Breast Cancer Therapy

Hypoxia is a tumor-specific stimulus that has been used to generate bio reductive prodrugs and sophisticated drug delivery devices. As hypoxia gives an indication of solid tumors, it promotes tumor growth and controls expression of genes related to hypoxia due to its therapeutic resistance. In one of the most tumor-specific aspects, hypoxic microenvironment is prevalent in the majority of solid tumors for its imbalance between the fast-growing tumor cells' high oxygen consumption and insufficient oxygen supply provided by aberrant vasculatures. Cell division cycle 20 (CDC20) is an oncogene that plays a role in tumorigenesis. CDC20 mRNA is significantly upregulated in breast cancer patient tumor tissues as opposed to para-tumor tissues

and that this overexpression is positively correlated with tumor hypoxia. Hypoxia-responsive nanoparticle (HRNP) was developed by the self-assembly of a polypeptide modified with 2-nitroimidazole and a cationic lipid-like molecule for the delivery of siRNA to target CDC20, a hypoxia-related tumor-promoting gene, in the treatment of breast cancer. The targeted delivery of siCDC20 by HRNP exhibited strong antitumor activity and effectively repressed the expression of CDC20. Therefore, a novel and precise approach of treating breast cancer includes employing hypoxia-responsive siRNA nanoparticles to silence pro-tumorigenic genes associated with hypoxia (Y. Li et al., 2020).

In one of the latest studies, the anticancer effectiveness of HRNP/siCDC20 *in vivo* was examined. First, BALB/c nude mice models containing MCF-7 tumors were developed. After that, PBS, siCDC20, HRNP/siCtrl, or HRNP/siCDC20 were given, with the dose of siRNA fixed at 1.0 nmol/mouse. When compared to PBS, siCDC20, and HRNP/siCtrl, HRNP/siCDC20 effectively suppressed tumor growth in the xenograft breast tumor model with an initial tumor volume of 55 mm^3, and the antitumor efficiency was up to 85.6%. There were no appreciable changes in body weight throughout therapy. Then, using western blot and immunofluorescence labeling, CDC20 and its downstream gene expression levels were examined in the tumor tissues. In the HRNP/siCDC20 group, more than 90% of CDC20 knockdown at the protein level was seen. The upregulation of cyclin B expression and the downregulation of Mcl-1 expression upon silencing of CDC20 expression suggested that cell cycle arrest and apoptosis may be responsible for the considerable suppression of tumor growth. HRNP/siCDC20 may function as an effective nanoplatform for breast cancer therapy at various stages, as evidenced by the tumor inhibition curves and tumor inhibition rate that followed a similar pattern to those from animals with smaller tumor volumes (Y. Li et al., 2020).

5.3 RNAi Therapeutics for Colorectal Cancer

One of the most frequent cancers in the western world is colorectal cancer (CRC). Colorectal cancer (CRC) is a complex and heterogeneous cancer because it is characterized by a broad range of genetic and epigenetic alterations together with environmental variables. CRC slowly grows and develops in the colon and rectum from adenomatous polyps. And these polyps are a result of the intestinal mucosa's excessive development. The serrated pathway and the conventional adenoma-carcinoma sequence are particularly noticeable among the several

pathways for CRC development that are taken into consideration. RNAi technology treatment can be combined with immune modulators to enhance the efficacy of cancer treatment. The sensitivity of CRC cells to conventional treatments is also enhanced due to the synergistic effects of RNAi-mediated gene knockdown and inhibitors/chemotherapy drugs (Jebelli et al., 2021).

The effect of siRNA is tested according to the size and growth of tumors is on xenografts mice. Tumor volume was calculated after the inoculated mice were split into two groups: control and siRNA-20 mol/L treatment. Comparing the tumor volume to the controlled group, a significant reduction has occurred. Then 3 groups were made into: (i) Control group (ii) group treated with 20 mol/L of siRNA (iii) firstly inoculated 4 groups of mices which is one controlled and three treated and then these are treated with siRNA doses of 10, 20, and 50 mol/L weekly for 4 weeks. From two weeks after transfection, the tumor volumes are significantly reduced in more than 20 mol/L siRNA compared to the control group ($p = .03$). RNA interference (RNAi) has shown significant improvement in the inhibition of tumor progression for both in vitro and in vivo models of colorectal cancer (CRC), but its use in clinical therapy is still in the early stages, mostly due to the lack of efficient delivery mechanisms. The best carriers for delivering RNAi drugs to mCRC cells are cationic polymers and liposomes. Both vehicles can effectively silence genes but they can also be harmful and do not perform the continuous release of siRNA. The final point is essential for maintaining the therapeutic effects. The long-term release of the cargo is made possible by encapsulating siRNA in less harmful and biodegradable matrices, such as poly (lactic-co-glycolic acid) (PLGA) polymers. Only a small number of clinical trials in CRC are being conducted at the moment using the RNAi approach (ClinicalTrials.gov). In one clinical experiment, patients with stage IV CRC, mCRC and recurring colorectal cancer who cannot undergo resectable surgery were given siRNA. Patients received peripheral blood mononuclear cells ex vivo treated with siRNA targeting the E3 ubiquitin ligase casitas B-lineage lymphomab gene (Cblb) in this phase I trial with 11 participants. A significant intracellular checkpoint called Cblb limits lymphocyte activation. Natural killer cells and T cells that stop the expansion of tumor cells are strengthened when Cblb is inhibited (Jebelli et al., 2021).

5.4 RNAi Therapeutics for Lung Cancer

One of the most common cancer-related deaths in both men and women worldwide is lung cancer. Treatment success is very low in lung cancer because cancer is detected at an advanced stage. The three main therapeutic approaches currently used to treat lung cancer are surgery, chemotherapy, and radiotherapy. Non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC) and lung carcinoid tumor are the three subtypes of lung cancer recognized by the American Cancer Society. Squamous cell carcinoma, adenocarcinoma and large cell carcinoma are the three types of NSCLC but squamous cell carcinoma and adenocarcinoma receive the most diagnoses. A cancerous tumor in epithelial cells gave rise to SCLC, which comprises tiny cells with very little cytoplasm, imprecise cell membrane borders and undetectable or absent nucleoli. Reduced survival rates are caused by the aggressiveness and delayed diagnosis of this kind of lung cancer (Magalhães et al., 2018). Through the use of nanocarriers, lung cancer biomarkers and critical regulatory molecules involved in cellular pathways includes cell proliferation, migration and death in which RNAi therapies successfully target and deliver therapeutic genes to lung cancer cells. (Tian et al., 2021).

The let-7, miR-34, miR-126, miR-195 and miR-200 acting as tumor suppressor genes, are downregulated and the miR-21, miR-17-92 cluster, miR-221/222, miR-155 and miR-31 acting as oncogenes are upregulated in lung cancer. miRNAs get involved in the epithelial-mesenchymal transition (EMT) and the inflammatory process in lung cancer. EMT is essential for the metastasis and invasiveness of epithelial cancer which is a complicated process that involves polarizing epithelial cells into a mesenchymal state by a variety of biochemical pathways that promote cell motility, invasion and resistance to apoptosis. (Magalhães et al., 2018).

siRNAs and miRNAs are used in RNAi-based therapies for cancer. Multiple genes can be targeted by miRNAs, which allows for the synchronized regulation of many genes implicated in tumor systems. Due to its ability to overcome the multidrug resistance that frequently develops after chemotherapy, this method is now thought to be a realistic and effective cancer treatment alternative. The distribution of RNAi molecules to the target site must overcome a number of pertinent difficulties, including avoiding serum nuclease destruction, evading renal clearance, crossing the plasma membrane, avoiding endosomal entrapment and competing with the natural RNAi system. Applying the recognized lung cancer biomarkers, RNAi-based therapy delivers

therapeutic genes into lung cancer cells in a targeted and effective manner using nanocarriers. These therapeutic genes are important regulators of vital biological processes that control cell division, migration and apoptosis. Thus, the biology of lung cancer and its features and functionalization of the nanocarrier have a direct impact on increasing the therapeutic effect of this therapy (Magalhães et al., 2018).

5.4.1 RNAi-Mediated Targeted Immune Checkpoint Nanoblocker in Lung Cancer

One of the most potent tools against advanced tumors has been the use of immune checkpoint blockers to maintain the damaged host immunological defense against tumor cells. Currently, checkpoint blockade therapy has produced clinical advancements with long-lasting responses and increased survival in a variety of cancer types especially when targeting the PD-1/PD-L1 axis. To restore the tumor-killing capacity of T cells, it typically relies on antibodies to inhibit checkpoints on T cells. The landscape of cancer treatment has undergone a revolution in the past ten years with the development of immune checkpoint blocking, which activates host T cells to assault tumor cells. However, only a small percentage of individuals have also shown prolonged response. Uncommon but effective tumor-targeted checkpoint blocking method using RNAi nanoengineering for T-cell-independent cancer therapy is using non-small cell lung cancer (NSCLC) as the cancer model. Such nano blockers, unlike antibodies, silences cancer cells' membrane and cytoplasmic PDL1, obviating the need for binding. Furthermore, it is shown that the nanoblocker's silencing of PD-L1 can directly trigger programmed cell death in NSCLC H460 cells without the aid of T cells. The tumor homing peptide alteration allows the nanoblocker to aggregate in the tumor tissue, downregulate PD-L1 expression and inhibit the tumor growth. It is observed and tested which says that the tumor growth is more effective than before according to in vivo studies from xenograft tumor models. Innovative immune checkpoint blocking method is using RNAi nanotechnology for tumor-targeted and improved cancer therapy. The RNAi-driven checkpoint nanoblocker is fundamentally distinct from traditional cancer immunotherapeutic approaches that heavily rely on activating T cells to combat tumor cells. It could effectively induce cell apoptosis and long-term proliferation suppression in NSCLC H460 cells upon delivery of PD-L1 siRNA. Findings from xenograft tumor models demonstrated that the nanoblocker could successfully deliver PD-L1 siRNA to tumor, effectively

silencing PD-L1, and reducing tumor development without unfavorable systemic immune activation (Magalhães et al., 2018).

A therapeutic siRNA that targets VEGF receptor 1 was utilized in the first RNAi-based therapy study on humans, which was carried out in 2004. Following the success of that study, some research has been conducted to create more effective RNAi-based treatments for a range of pathologies, including lung illnesses. Number of siRNA and miRNA-based therapies have been designed to treat lung cancer. These treatments have shown encouraging results in in-vivo lung cancer models and are qualified to participate in clinical trials for the treatment of lung cancer. Some of the most important RNAi-based treatments have already been created and are being tested in lung cancer patients' medical tests. Lung cancer was one of the solid tumors that CALAA-01 was tested to treat in a Phase I clinical trial that has been terminated. A siRNA against ribonucleotide reductase can be delivered to a specific location using the nanocomplex CALAA-01 which is made of cyclodextrin containing polymer (CAL101) coupled with the stabilizing agent PEG and functionalized with protein transferrin (R2). The major objective of CALAA 01 are to safeguard anti-R2 siRNA and deliver it in a targeted and effective manner. In a Phase I clinical trial (NCT00004604), a CEA RNA-pulsed DC cancer vaccine (carcinoembryonic antigen (CEA) RNA-pulsed autologous dendritic cells (DC)) was investigated to activate the immune system and reduce tumor development and proliferation in patients with metastatic tumors expressing CEA. The biological therapy AGS-003-LNG has been studied in an open Phase II clinical trial (NCT02662634) to see how well it works in treating stage 3 NSCLC when combined with traditional anticancer drugs. RNA from the tumor was isolated and electroporated into fully developed autologous dendritic cells to create AGS-003-LNG. The primary objective of this trial's first stage is to assess the efficacy and viability of this therapy for treating patients with resectable NSCLC. The treatment of lung cancer using gene therapy and RNAi-based therapies is emphasized as a potential and innovative new strategy. Pharmaceutical companies are now interested in using these therapies to treat a variety of cancers as a result of the advancements made in RNAi-based formulation in terms of transport and delivery efficacy and several clinical trials to treat lung cancer are already ongoing (Magalhães et al., 2018).

5.5 RNAi Therapeutics for Brain Cancer

Neoplasms that primarily develop in the brain and spread there are known as brain tumors. Invasive cancerous cells that penetrate the CNS's surrounding tissues are the cause of malignant primary brain tumors. Because drugs cannot cross the blood-brain barrier, there are currently few therapy options for malignant brain tumors. The development of current research has allowed for the identification and characterization of specific molecular markers that are important for the survival, development, metastasis, and angiogenesis of tumors. For RNAi-based therapeutics, which allow for site-specific silencing of the gene responsible for tumor proliferation, these molecular markers have acted as therapeutic targets. However, an effective delivery vehicle that can pass the blood-brain barrier and reach the desired location is required for therapeutic success. Blood-brain barrier structural anomalies at the tumor site: The BBB plays an important role in ensuring the brain microenvironment's homeostasis. The CNS and the circulatory system are separated by it. Development of disease-modifying therapies in various therapeutic areas is the potential for artificially interfering with endogenous gene expression through the RNAi pathway (Malhotra et al., 2015).

RNAi therapies in brain glioma tumors have gene targets. Constant advancements made it possible to identify specific dysregulated molecular targets that may make good candidates for RNAi treatments. Oncogenesis-related gene products, cell cycle regulators, the apoptotic pathway, cell senescence and protein stability and degradation are some of the therapeutic targets in the carcinogenic pathway. RNAi therapies provide advantages over traditional treatments. RNAi therapies have a significant advantage over traditional cancer therapies since they can almost certainly target any gene with a known sequence that has been associated with brain cancer. To increase the effectiveness of current conventional therapies based on chemotherapy and/or radiotherapy, RNAi therapeutics have been used in conjunction with such approaches (Malhotra et al., 2015).

Their studies showed improved siRNA delivery in an in vitro 3D brain cancer spheroid model. Endothelium, basement membrane, pericytes, astrocytes, and tight junctions were present in a transverse segment of the blood-brain barrier (BBB). The aquaporin localization around the blood vessels on the endfoot of the astrocytes is seen in the healthy BBB. Due to the migration of glioma cells along blood arteries, which displaces the astrocytic endfeet and ultimately causes

leaking of serum components (edema) into brain parenchyma, the leaky BBB indicates the breakdown of the basement membrane and loss of tight junctions. Other forms of cancer have also shown the efficacy of nanodelivery systems for RNAi therapeutic delivery, and in certain cases, these systems have reached the stage of clinical trials. The treatment of brain cancer is not now in a condition where the scientific gains produced have not yet been used in clinical settings. The lack of convincing levels of BBB penetration at the adequately high doses required for the desired duration of activity is one of the factors that has hampered clinical advancement. The long-term toxicity of multi-functional bioactive delivery systems is also unknown, despite the fact that numerous chemical modification procedures have been employed to reduce the negative immune activation effects of siRNAs and enhance systemic stability (Malhotra et al., 2015).

Chapter 6

Limitations and Future Prospects

6.1 Limitations of RNAi Technology

i. Modes of administration

siRNA has been administered locally to tissues that are accessible or external i.e., through eye drops and nasal sprays. One of the first clinical trials of siRNA local delivery was the intravitreal injection of naked siRNA with VEGF mRNA as the target. Many significant target sites are not immediately accessible and can only be accessed through systemic injection of RNAi therapies rather than topical administration. Effective transport of siRNA to the target sites is a challenge due to inherent characteristics of siRNA for being negatively charged and easily broken down by nucleases (D. Li et al., 2021).

ii. Renal clearance and size dependency

When siRNA is administered systemically, renal clearance leads to short half-life and poor effectiveness. While the hydrodynamic diameter of a siRNA drug delivered intravenously into the circulatory system is greater than 6 nm, kidney filtration impact can be effectively avoided. To increase the particle size of siRNA delivery nanoparticles, an appropriate modification which will lengthen the residence time of siRNA in the circulatory system is required (D. Li et al., 2021).

Because a significant number of alterations can inhibit RISC from binding to siRNA and therefore prevent the RNA interference mechanism. siRNA molecules applied for therapeutic purposes are only partially altered. These alterations are frequently seen close to the 3' and 5' ends of the passenger and guide strands where it has been shown to have a significant effect on the following properties:

· Exonuclease degradation activity should be avoided

· RISC affinity should be increased

- Passenger strand loading should be prevented
- Innate immune response should be reduced
- miRNA-like effects should be avoided. (Mahmoodi Chalbatani et al., 2019).

iii. Nuclease degradation and immune system recognition

An intravenous siRNA therapeutics' potential for degradation by endogenous nucleases has always been a concern. The short half-life of intravascular naked siRNA arises from its instability. Due to the high activity of ribonucleases and the features of siRNAs, it is vital to protect them from degradation. The siRNA therapy is further limited by the fact that toll-like receptors can mediate an innate immune response through certain siRNA oligo patterns. A modification for siRNA delivery nanoparticles has been utilized to enhance siRNA nanoparticles escape in order to decrease the potential toxicity of RNAi therapy and limit the uptake by immune cells (D. Li et al., 2021).

iv. Heterogeneity of tumor vasculature

If the concentration of nanoparticles in tumors can be enhanced by the effects of EPR (enhanced permeability and retention), total tumor eradication is a problem since not every region of the tumor shares the same concentration of nanoparticles due to the inherent properties of tumor vasculature. The permeability of the arteries in solid tumors may differ and the EPR effect may not be present, which could lead to a variety of limitations on the passive targeting method. (D. Li et al., 2021).

v. Endosomal Escape

The most important cellular uptake mechanism for all biological agents, including siRNA is endocytosis, which takes place at the cell surface. The lack of an endosomal escape mechanism is one of the major challenges to creating an effective siRNA therapy. Nanocarriers that can enhance the endosomal escape have been developed in order to limit lysosome destruction. (D. Li et al., 2021).

6.2 Future Perspective

Clinical trials must always be expanded in the future with better methods for encapsulating RNAi therapeutics. These problems may indeed be rectified through developments in nanotechnology and pharmaceutical chemistry and RNAi-based therapies which will gain wider acceptance. Essentially all genes connected to disease can be targeted using the siRNA molecule. Major improvements in siRNA delivery for cancer therapy have been documented in recent years. The extensive use of RNAi in the clinic is nevertheless limited by a number of fundamental problems. Due to their poor uptake effectiveness and high cytotoxicity, current approaches for RNA therapeutic treatments have difficulty being used in a health context. The most extensively used methods for overcoming the difficulties and prohibitions that prevent siRNA from reaching the target cells are chemically altered siRNA backbone and using viral and non-viral delivery vehicles. Currently, a promising strategy for overcoming the extracellular limitations that prevent siRNA from being delivered precisely to the target site is the invention of nano-based carriers. siRNA delivery systems must successfully cross extracellular and intracellular barriers with minimal systemic impact (Subhan et al., 2021). This is necessary to molecularly develop new cationic carriers to enable the distribution and transport of RNAi and siRNA with specificity. It is important to offer improved biodegradable carriers that are chronologically regulated since the carriers will later need to be removed from the cells and perfused from the body. This means that in order to remove the carrier in a programmable manner, the degradation process must be highly carefully regulated and likely divided into numerous stages. Furthermore, it is necessary to molecularly build multifunctional cationic carriers so that they can likely transport and direct two separate siRNA kinds that can be administered at two noticeably different speeds. That will make it possible for specific diseases to be treated gradually and chronologically. Scientists are now researching a number of novel ways to accomplish being able to report on them soon. Finally, improved nanosized carriers are required to ensure appropriate stability during the systems' therapeutic lifetimes (Ward et al., 2021).

Chapter 7

Conclusion

RNAi technology has an extensive therapeutic potential against each type of cancer. This review is predominantly based on RNAi technology involving siRNA, shRNA and miRNA induced gene silencing method by degradation of mRNA. Furthermore, this RNAi technology is used in cancer when there is a diseased protein produced and found in the body then we target and use siRNA, shRNA and miRNA according to complementary bases. The extent of RNAi-mediated gene therapy will translate into clinical practice that will be determined by the evaluation of RNAi-based gene therapeutic techniques. The advancement of delivery methods for gene therapy will define how well the RNAi technology will be used in the clinical practice. It is still challenging to identify the targets of RNAi molecules and the signaling mechanisms that control the ways they are expressed in tumor development. There have been overwhelming evidences over the past few years that RNAi-based technology has potential for treating cancer. In this regard, a better understanding of the characteristics of cancer as well as the unique biomarkers of each form of cancer is essential for the accurate diagnosis as well as the development of more targeted therapies.

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