Chapter 14

Cancer Gene Therapy with Small Oligonucleotides

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1. Introduction

Although enormous advances have been made in medical research, cancer still remains as one of the leading causes of death. The effects of cancer impacts on many lives and patients' families. Also, this insidious disease represents a huge financial and socioeconomic burden to both the family and health care systems.

Cancer is a multigenetic, multicellular and multisystemic disease. Recently, the International Agency for Research on Cancer (IARC) announced that 7.6 million deaths were due to cancer and that there is on average 12.7 million new cases per year worldwide [1]. Current trends indicate that 63% of cancer deaths are from developing countries [2], [3].

Current conventional treatment options include surgery, chemotherapy and radiotherapy which can be used independently or sometimes, in combination. However, many of these treatment options are restricted to early stage tumours and even after surgery, there is still a high possibility of the tumour recurrence in these patients. In addition to the conventional treatments of cancer, there are also a number of relatively new therapies that include targeted cancer therapy, biological or immunotherapy and gene therapy. In contrast to conventional methods, these newly developed treatments can be more effective and have fewer side effects.

2. Solid tumour and its microenvironment

Cancer has two major forms: haemological cancers which are cancers arising from abnormal blood or bone marrow cells and solid tumours, which are tumours that grow into a solid mass. Traditionally, a solid mass were thought to be all rapidly dividing cells and all therapeutics were designed to stop or reverse cellular proliferation. More recent studies have determined finer details of the nature of solid tumours and their microenvironment in order to

identify more specific targets for therapeutics and potential avenues for new cancer gene therapy. Hanahan and Weinberg [4] identified six hallmarks common to cancer:

Sustaining proliferative signaling: Within normal tissues, there is a carefully controlled production and release of growth-promoting signals. Cancer cells deregulate these signals and use them to their own advantage and may produce growth factor ligands for their own receptors. In addition to this autocrine proliferative stimulation, cancer cells may send signals to stimulate normal cells, within the tumour microenvironment or surrounding tissue, to supply the cancer cells with various growth factors [5], [6].

Evading growth suppressors: Cancer cells must evade the actions of tumour suppressor genes, which limit cell growth and proliferation. The two prototypical tumour suppressors encode the retinoblastoma-associated protein (RB) and tumour protein p53 (TP53) which govern the decision of cells to proliferate or to activate senescence (biological aging).

Resisting cell death: Programmed cell death by apoptosis plays an important role in cells as a natural barrier to cancer development. The structure of the apoptotic machinery and how cancer cells can avoid these mechanisms has been widely studied since beginning of last decade. Tumour cells evolve many strategies to limit or evade apoptosis. The most common one is the loss of TP53 tumour suppressor function [7].

Enabling replicative immortality: Cancer cells must have unlimited replicative potential in order to generate macroscopic tumours. Multiple studies suggest that telomeres, which are repetitive sequences at the ends of chromosomes, are centrally involved in the seemingly unlimited proliferation [8], [9].

Inducing angiogenesis: Like normal cells, tumour cells require the uptake of nutrients and oxygen as well as discharge carbon dioxide and metabolic waste. Since tumour cells grow faster than normal vasculature, tumour cells keep demanding the growth of the surrounding vasculature. The induction of angiogenesis addresses some needs in this vicious cycle, facilitating sustained expansion of neoplastic growth [10].

Activating invasion and metastasis: Metastasis is responsible for as much as 90% of cancer-associated mortality. In order for a primary tumour to metastasize it must achieve the following steps: *1) Intravasation*: At first, a cancer cell locally invades tissues in close proximity and thereby enters the microvasculature of the circulatory and lymphatic systems. *2) Extravasation:* In the microvasculature, a tumour cell maintains itself and begins its movement through the bloodstream to microvessels of distant tissues where it leaves the bloodstream. *3) Colonization:* The migrated tumour cell survives within the microenvironment of its new location and uses the local tissue for cell proliferation and a secondary macroscopic tumour formation [11].

More recent studies showed that, once a solid tumour reaches approximately 2 mm, it contains hypoxic regions as a result of the failure of angiogenesis to keep pace with abnormal tumourous tissue growth. Studies have shown that hypoxia can inhibit tumour cell differentiation and promote maintenance of cancer stem cells. Hypoxia also blocks the differentiation of mesenchymal stem/progenitor cells, which is a potential source of tu-

mour-associated stromal cells [12]. There are also the extracellular matrix in solid tumours which is composed of complex secretions of proteins and proteoglycans produced by both neoplastic and normal stromal cells. This network continuously regulates signaling between tumour and normal stromal cells [13]. Traditionally, this microenvironment limits or prevents the effectiveness of many traditional as well as new therapies. Clearly, researchers need to develop new therapeutic strategies if we wish to successfully cure this disease. One such approach may be cancer gene therapy; however, research needs to also look at effective delivery of these agents in order to overcome the barriers set by these tumours and their microenvironment.

3. Targeted cancer therapy

Targeted cancer therapeutics are chemical agents or monoclonal antibodies that specifically inhibit the growth and spread of cancer by interfering with cell proliferation. These strategies interfere with cancer cell division and spread in different ways. Many of these therapies focus on proteins that are involved in cell signaling pathways, which form a complex communication system that governs basic cellular functions and activities. By blocking signaling pathways that make cancer cells grow and divide uncontrollably, targeted cancer therapies may induce cancer cell death through a process known as apoptosis, thus helping to stop cancer progression [18], [19].

Targeted monoclonal antibodies may also be able to modulate immune responses, which raise the possibility that these treatment strategies can be combined with other therapeutic approaches to improve clinical outcomes [20]. Many targeted therapies against tumours affect pathways that are also crucial for immune development and function. This suggests the possibility that targeted therapies may help to optimize anti-tumour immune responses from immunotherapies. Similarly, immunotherapies may serve to consolidate impressive clinical responses from targeted therapies into long-lasting clinical remissions [21]. Immunotherapy on the other hand, endeavors to stimulate a host immune response that effectuates long-lived tumour destruction.

3.1. Biological therapy

Biological therapy (immunotherapy or biotherapy,) is a method that uses a biological agent or the body's immune system, either directly or indirectly, to fight against cancer whereas traditional therapies target the tumour itself. For this reason, biological therapy can be used to lessen the side effects caused by other cancer treatments. For instance, dendritic cell-activated cytokine-induced killer cells, used after chemotherapy in patients with advanced non-small cell lung cancer, improve immune response associated with up-regulation of cytokines that are involved in the anti-tumour activity [22].

Using sipuleucel T and ipilimumab in phase III clinical trials the principle that immunotherapy can also extend cancer patient survival has been validated [23]. Sipuleucel T, which has recently been approved by the US Food and Drug Administration (FDA) for use in metastat-

ic prostate cancer, aims to stimulate T cells that are specific for prostatic acid phosphatase (PAP), a protein that is overexpressed in prostate carcinoma cells [24]. Although the precise basis of action for sipuleucel T remains under study, treatment with this drug increases survival by an average of 4 months with minimal toxicity.

Ipilimumab, an antibody, bolsters T cell responses and potentiates tumour destruction by blocking an important inhibitory signal for activated T cells. Ipilimumab, which has recently been approved by the FDA for use in patients with advanced melanoma, enhances overall survival compared with standard care and, most notably, achieves durable benefits (more than 2.5 years) for 15–20% of treated patients [26], [27].

Agents that target interleukins have also been used in cancer therapy. Blocking IL-6 signaling is a potential therapeutic strategy for cancer characterized by pathological IL-6 overproduction [27]. Researchers have demonstrated that the recombinant immunotoxin IL6 (T23)-PE38KDEL kills IL6R-overexpressing cancer cells, and causes significant tumour regression [28]. Other studies have shown that using viral and non-viral vectors to overexpress IL-24 in human cancer cells results in inhibition of tumour growth both *in vitro* and *in vivo* [29]. Targeted therapies and cancer immunotherapies have begun to enter clinical practice recently and when they were used together they may become promising treatments; however, these combinations have not been well studied.

4. Gene therapy

Gene therapy is a relatively new method compared to other conventional treatments. It involves a therapeutic gene that is selectively delivered to a specific cell or tissue using a vector or delivery vehicle. The first successful treatment of a human disease using gene therapy techniques (as an *ex vivo* gene replacement therapy) was for the treatment of X-linked severe combined immunodeficiency (X-SCID). The replacement of the wild type gene in the bone marrow stem cells was stably expressed and conferred selective growth advantage over the defective T cells. Following treatment, eight patients were cured of this disease but unfortunately 2 patients developed abnormal white blood cell growth due to the oncogenesis ability of the retroviral vector used for gene delivery [30].

Viral vectors are the most widely used vector system for gene therapy. Within Europe and the United States, gene therapy protocols are mostly used for cancer. Cancer gene therapy research has focused mainly on melanoma, prostate cancer, ovarian cancer and leukemia [31]. Some of these protocols for cancer gene therapy include the thymidine kinase gene and the genes for immunomodulatory cytokines such as IL-2 or granulocyte-macrophage colony-stimulating factor (GM-CSF) and have been met with varying success [32]. Early clinical trials of gene therapy used *ex vivo* delivery of therapeutic genes to patients with monogenetic diseases. Such therapeutic genes, ie.: cytokine genes and viral thymidine kinase genes were transduced into autologous cells, normal cells or/and cancer cells. However, delivery of these therapeutic genes had limited efficacy due to their inability to achieve a pharmacological dose of the gene at the target tissue. *In vivo* gene therapy protocols have used mostly

viral vectors for gene delivery. A large barrier for systemic gene therapy is reduced efficacy of transduction. Some of the other obstacles that affect efficacy of cancer gene therapy include: 1). identification of key target genes responsible for the disease pathology and progression; 2). identification of therapeutic genes that can inhibit disease progression; 3). optimal trans-gene expression for suppressing the target gene; and 4). delivery of therapeutic product to the target tissue at an efficacious dose [33]. Components of gene therapy for cancer can be replacement of tumour suppressor gene (p53), inhibition of oncogenes with antisense oligonucleotides, ribozymes and short inhibitory RNA, and activation of apoptosis genes [33]- [37]. However, sometimes the inhibition of the target gene and its pathway is not sufficient to inhibit the disease process because the cells have built abundant or alternative pathways to compensate [35].

The efficient transgene expression requires appropriate promoters and enhancers in order to extend the duration of transgene expression in the cell or tissue. There are two types of promoters: constitutive or inducible. The constitutive promoters can be either viral or tissue specific promoters, such as melanin for melanoma. Inducible promoters can be induced to express transgenes with hormones or small molecules. Location of enhancers is upstream of the promoters and their function is to increase transgene expression 2-100 fold if the amount of gene product is required in very high amounts in the cell. In cancer cells, the duration of transgene expression can be up to 30 days [33], [38].

5. Gene delivery

Delivery of therapeutic genes is one of the most difficult issues in gene therapy studies. All viral gene therapy strategies have significant delivery limitations and very narrow applications for cells and tissues. The best method for delivering genes will depend on the type of tissue to be targeted [36]. Commonly used vectors are retroviruses, adenoviruses, adeno-associated viruses and herpes simplex viruses. For cancer gene therapy, replication competent viruses such as the Newcastle disease virus offer a promising delivery technology for human tumour therapy [38].

In addition to viral delivery methods there are non-viral technologies that offer several advantages including less toxicity, reduced adverse immune responses and ease of producing larger quantities of vectors [39]. Chemically synthesized nanoparticles like DNA/stearyl polylysine coated lipids or DNA coated with glycine oligomers (Peptoids) offer new advances for systemic gene therapy delivery. These molecules have been shown to be effective in cancer related angiogenesis [40].

In addition, bacterium has been developed for gene delivery purposes in cancer patients. The hypoxia and solid tumour microenvironment provide a living haven for anaerobic bacteria. These so far fall into three classes. 1). Gram-positive lactic acid producing anaerobic bacteria; 2). Gram-negative intracellular, facultative anaerobes; and 3). the Gram-positive strictly anaerobic, saccharolytic/proteolytic Clostridia. Some of these modified bacterial cells, such as *Salmonella* and *Clostridium novyi* are already in phase 1 clinical studies [41].

5.1. Bacterial oncolysis

A surgeon named William B. Coley described for the first time that bacteria could be used as anticancer agents in 1890 [14]. Since then, scientists have been researching, and engineering, microorganisms such as *Clostridium, Bifidobacterium, Salmonella, Mycobacterium*, and *Bacillus* which have the ability to specifically target cancer cells and cause oncolysis. These anaerobic bacteria grow in the hypoxic core of solid tumours, where most traditional and many emerging therapeutics are unsuccessful. Due to their specificity for the tumour microenvironment, these bacteria are also promising vectors for delivering therapeutic genes to the cancer patients [15].

The hypoxic nature of solid tumours is a haven for bacterial colonization and proliferation. It has been suggested that the anaerobic nature of hypoxic-necrotic regions within tumours provide faster growth of anaerobic and facultative anaerobic bacteria. Necrotic areas may also provide purines to the further growth of bacteria [16], [17].

6. The use of small oligonucleotides for gene silencing

In 1998, Fire et al [42] discovered a mechanism, which is called RNA interference (RNAi), that moderates the activity of genes by using small single-stranded ribosomal nucleic acids.. These nucleic acids can bind to other molecules and play important roles in cells. It has been shown that small RNAs have the ability to control gene expression and other activities that were assumed to be carried out only by proteins. As a result RNAi has become a promising tool for researchers in the treatment of genetic diseases and cancer.

RNAi applications have a huge potential for use in inhibiting targets. To compare with molecular drugs, RNAi technology promises more specificity and wide range target capacity. Small RNAs that used in RNAi technology currently have been grouped into four major classes: small interfering RNAs (siRNA), short hairpin RNA (shRNA), microRNAs (miRNAs), and P-element-induced wimpy testis (PIWI) interacting RNAs (piRNA). In addition to these there are also qiRNA and other unknown small RNAs still to be discovered [43], [44].

6.1. siRNA

Synthetic siRNA was used in gene silencing firstly as an RNAi technology [45]. In the process, long dsRNA molecules were cut into 19-23 nucleotide RNAs, called siRNAs, which guide for cleaveage of complementary RNAs [46]. siRNA directly incorporates into RNA-induced silencing complex (RISC), where its guide-strand binds to and cleaves the complementary mRNA. After the cleaved mRNA is released and degraded, the RISC binds to another mRNA and starts a new cycle of cleavage [47]. siRNA can cleave its target RNA in both the cytoplasm and the nucleus [48].

Moreover, siRNAs are able to act as primers for an RNA-dependent RNA polymerase that synthesizes extra dsRNA, that results in additional siRNA, which reinforces the effect of the original siRNA [49], [50].

6.2. shRNA

Short hairpin RNA (shRNA) has been developed for long-term gene silencing [51]- [53]. shRNA is transcribed in the nucleus from short double-stranded DNA sequence with a hairpin loop. After that the shRNA transcript is processed and incorporates with RISC in the cytoplasm in a process that is the same as siRNA. However, there are some differences between siRNA and shRNA. Firstly, less than 1% of duplex siRNA remains in the cells 48 hours after introduction to the cells due to the high rate of degradation and turnover, whereas shRNA is constantly synthesized in host cells, leading to more durable gene silencing. Secondly, vector-based shRNA can only be modified by manipulating the expression strategy because it is firstly synthesized in the nucleus then transported to cytoplasm for further processes. Major component of RISC is the argonaute proteins. Within these protein family only Ago2 shows endonuclease activity to cleave shRNA in order to make it active single stranded [54].

6.3. MicroRNA

MicroRNA (miRNA) is another group of small non-coding RNAs. miRNAs are important for gene regulation and highly conserved in cells. miRNA is firstly transcribed from precursors, that are located within intergenic sequences or introns, as a primary transcript (pri-miRNA) in the nucleus. Secondly, pre-miRNA is processed by an RNase III endonuclease called Drosha and then is exported to the cytoplasm. In the cytoplasm, pre-miRNA is cleaved by Dicer, another RNase III enzyme, to make 20-23 base pair long mature miRNA that consists of both guide and passengers strands with mismatches. Mature miRNA cooperates with RISC to inhibit translation with target mRNA degradation [55].

One major difference between siRNA, shRNA, and miRNA is that both siRNA and shRNA require a complete match with the target mRNA but miRNA does not. Change in expression of a single miRNA may affect more than hundreds different genes [56]. miRNA takes part in gene regulation in different ways. Firstly miRNA binds to the 3′ UTR region of the target mRNA and repress translation [55]. Nevertheless, a number of studies have shown that miRNA can also recognize coding region or the 5′ UTR region to inhibit gene expression, although with less efficiency than at the 3′ UTR [57], [58]. Other studies have also shown that miRNA can bind to the 5′ UTR region of an mRNA and promote protein translation or can bind to DNA and induce gene expression [59], [60]. It has been stated that failure in regulation of miRNA can cause a various human diseases, including cancer [61]. Better understanding of mechanism and regulation of miRNA can contributes to develop effective RNAi therapies of cancer and other diseases.

6.4. piRNA

P-element-induced wimpy testis (PIWI) interacting RNAs (piRNAs) are small non-coding RNAs which interact PIWI proteins. These proteins are clade of argonaute proteins and are expressed predominantly in the germlines of a variety of organisms such as *Drosophila* and mammals. piRNAs help to maintain silence repetitive elements, the integrity of the genome, and the development of gametes. It has been suggested that both PIWI proteins and piRNAs are required for transposon silencing. In addition, a subset of piRNAs in *Drosophila* has been shown to function in silencing protein-coding genes [62].

piRNA–PIWI complexes are assumed to directly control transposon activity. piRNAs bound to PIWI proteins show homology-dependent target cleavage *in vitro*. Therefore, transposons are probably silenced through post-transcriptional transcript destruction [63].

piRNAs are different from siRNAs and miRNAs in several ways: 1) piRNAs consist of mostly 24–31 nucleotides whereas other non-coding small RNAs are approximately 21 nucleotides; 2) opposite to several hundred species of miRNAs, piRNAs have 50,000 cloned species; 3) many piRNAs match to the genome in clusters of 20–90 kilobases in a strand-specific manner. In some clusters, one strand is changed abruptly to another strand which suggest that these bidirectional clusters may be transcribed divergently from a central promoter, however, siRNAs and miRNAs are derived from double-stranded and short hairpin RNA precursors, respectively. 4) Some piRNAs may be involved in epigenetic regulation whereas siRNAs and miRNAs generally target mRNAs [64], [65].

7. RNAi phenomena and its use in cancer therapy

Due to their robustness and specificity, siRNA and shRNA have been extensively used to silence cancer-related gene targets. For instance, metastatic pancreatic cancer is one of the most deadly cancers. The overexpression of pancreatic duodenal homebox-1 (PDX-1) in pancreatic adenocarcinoma has been shown to act as an oncogene. A plasmid vector encoding shRNA was used to target PDX-1 expression in a pancreatic animal model. Further examination showed that the expression of PDX-1 was significantly reduced compared with that of the control group. As a result, silencing of PDX-1 expression inhibited tumour growth in malignant pancreatic cancer [66], [67].

Another example is human enhancer of zeste homolog 2 (EZH2) or p110-alpha silencing by siRNA with A systemic delivery vector in advanced prostate cancer in which tumour cells frequently metastasize to bones and regional lymph nodes. It has been shown that siRNA targeted to these proteins inhibit tumour metastasis in these cells [68]. Ryo *et al* have also shown that retrovirus-encoded shRNA was used to silence Pin1 expression in a prostate cancer model. Pin1 is a peptidyl-prolyl isomerase which catalyzes the cis/trans isomerization of peptidyl-prolyl peptide bonds [69]. It is highly overexpressed in prostate and breast cancers. Pin1 shRNA significantly inhibited tumour growth and tumour metastasis.

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Angiogenesis is a charecteristic for neoplasia and tumour metastasis. The vascular endothelial growth factor (VEGF) pathway is the most important pathway in angiogenesis. siRNA has been used to selectively silence VEGF and VEGF receptors to arrest tumour growth and angiogenesis successfully. Tumour growth was markedly suppressed [70]. Moreover, the siRNA targeting VEGF receptor 2 (VEGFR2) presented a significant inhibition of tumour growth with reduced VEGFR2 expression [71]. miRNAs affect malignant process by either resulting in overexpression or downregulation of a gene product. miRNA has been used as a tumour repressor in tumours with reduced expression of tumour supressor genes or other key genes. For example, miR-26a is highly expressed in normal liver tissues but its expression is downregulated in liver tumours. Patients who have low miR-26a expression have decreased overall survival compared with patients who have high miR-26a expression [72]. Further, miR-34c, miR-145, and miR-142-5p also show tumour suppresion properties in several lung cancers. Replacement of downregulated miRNA causes discontinue the growth of lung cancer cells [73].

Due to miRNAs ability to supress tumours, miRNA gene therapy can be used for retriving miRNA gene expression and prevent tumour development. This approach is principally similar to that used for siRNA/shRNA therapeutics except that miRNAs are used to regain miRNA expression. For instance miR-34a is usually lost in human cancers especially lung cancer and prostate cancer. Using a neutral lipid emulsion (NLE), systemic delivery of synthetic miR-43a causes accumulation of miR-34a in normal lung tissues and lung tumours [74]. Furthermore, miR-34a and miR-16 are tumour suppressors of prostate cancer. miR-34a blocks metastasis of prostate cancer by repressing CD44 while miR-16 uses as target CDK1 and CDK2 genes which involves cell-cycle progression and cell proliferation [75], [76]. Another miRNA subtype miR-22 induces cellular senescence. In a breast cancer xenograft model, synthetic miR-22 induced cellular senescence and inhibited tumour growth by intratumoural delivery [77].

The effective delivery of miRNA for cancer therapy can be achieved with either plasmid or virus. Kota *et al* has shown that miR-26a, of which re-expression in liver cancer cells inhibits cyclin D2 and E2 and induces G1 arrest, was delivered into hepatocellular tumour by using adeno-associated virus where it was sucessful in inhibiting of tumour development [78].

8. Modulating key genes controlling cancer metabolism

In general, normal cells produce most of the ATP from glucose through oxidative phosphorylation [79]. On the contrary, many cancer cells produce ATP by conversion of glucose to lactate and show lower oxidative phosphorylation activity. Tumour cells keep high yields of lactic acid and produce ATP by aerobic glycolysis with or without oxygen. This phenomenon is called "Warburg effect" [80].

Accelerated glycolysis provides ATP levels to the fast proliferating tumour cells in a hypoxic environment. Along with increased glutaminolysis, it also supplies metabolic intermediates that are essential for macromolecule biosynthesis and necessary for cell growth and division

[81]. Although the conversion of pyruvate into lactate occurs in normal cells in hypoxic conditions, tumour cells produce excessive amounts of lactate even when oxygen is not a limiting factor. It has been stated that this glycolytic phenotype results from the adaptation of premalignant lesions to spasmodic hypoxia [82].

Down-regulation or completely silencing genes that are related with cancer metabolism may be the key of future methods of cancer treatment. Hexokinase II and pyruvate kinase M_2 are some of metabolic genes that have been focused on in siRNA studies. It has been shown that down-regulated hexokinase II by RNA interference resulted in increased apoptosis rate in colon cancer cells [83]. Inhibition of Pyruvate Kinase M_2, a metabolic enzyme whose expression in cancer cells results in aerobic glycolysis causes substantial tumor regression [84]. Another study have indicated that combined therapy with siRNA and cisplatin drug resulted in enhanced antitumor activity [85].

9. Silencing telomerase activity by RNAi

There are specialized, repeated structures called telomere which protect the ends of all chromosomes in eukaryotic organisms [86]. Telomeres are essential for chromosome stability. Also, it is suggested that telomeres are responsible for cellular aging since it acts as a mitotic clock [87], [88]. Telomere shortening triggers the senescence check point so-called Hayflick limit in human somatic cells [89]. Escape from this check point is the first step in cellular immortalization [90].

In most organisms the main mechanism of telomere length maintenance is carried out by telomerase, a ribonucleoprotein complex [91]. This enzyme elongates the telomeres at the 3′ end of the DNA [92]. Although the telomerase complex contains a number of components that provide telomerase activity *in vivo*, the basic components of telomerase enzyme are telomerase reverse transcriptase (TERT) and telomerase RNA [93]. Increase expression of these Proteins results in high telomerase activity and has been demonstrated in 85-90% of all human tumours [94].

Currently, attempts are underway for reducing telomerase activity which may provide a potential avenue for cancer gene therapy. Kosciolek *et al* has shown that telomerase activity in human cancer cells can be inhibited by siRNAs targeting telomerase components [95]. Human cancer cell lines were transfected with 21 nucleotide double-stranded RNA homologous to either the catalytic subunit of telomerase (hTERT) or to its template RNA (hTR). Both agents reduced telomerase activity in a variety of human cancer cell lines which included both carcinomas and sarcomas.

10. Other gene silencing approaches in cancer therapy

B cell lymphoma 2 (BCL2) is an important gene in eukaryotic cells as its expression causes uncontrolled growth by inhibiting cell death [96]. Overexpression of BCL2 protein has been

reported in many types of human cancers, including leukemias, lymphomas, and carcinomas [97]. Cimmino *et al* demonstrate that miR-15a and miR-16-1 expression is inversely correlated to BCL2 expression in chronic lymphocytic leukemia (CLL) [98]. Both these miRNAs negatively regulate BCL2 at a posttranscriptional level. BCL2 repression by miR-15a and miR-16-1 induces apoptosis in a leukemic cell line model. As a result, miR-15 and miR-16 are natural BCL2 inhibitors that could be used for therapy of tumours in which BCL-2 overexpresses.

Another protein which is cyclooxygenase-2 (COX-2) enzyme has been involved in the tumourgenesis and in the progression of colorectal cancer (CRC) [99]. The use of developing RNAi-based techniques allowed researchers to better study the molecular and phenotypical loss of function of COX-2 gene by doing experiments based on a strong COX-2 silencing effects. Denkert and colleagues [100] tested the effect of an anti-COX-2 siRNA (siCOX-2) on OVCAR-3 cells derived from human ovarian carcinoma. A comparison with the COX-2 inhibitory drug NS-398 has shown that a different effect of siCOX-2 occured due to its highly specific mechanism of action. Even though COX-2 protein levels significantly reduced in both cases, NS-398 treatment induced a G0/G1 cell cycle arrest in OVCAR-3 cells but only after another factor stimulation. This effect was probably due to the action of NS-398 on other cellular targets involved in cell proliferation.

Research performed by Charames *et al* and Kobayashi *et al* demonstrated that an siRNAs can efficiently knockdown COX-2 in HT-29 human colon cancer cells and bovine Cumulus-Granulosa (CG) cells [101], [102]. Based on their results, it is clear that RNAi, compared with non-steroidal anti-inflammatory drug (NSAIDs), are more powerful and selective tools for studying *in vitro* COX-2 functioning [103]- [106].

RNAi-mediated COX-2 silencing proved to be highly effective using anti-COX-2 shRNAs (shCOX-2). In 2006, Strillaci *et al* have illustrated that an *in vitro* strategy in which COX-2 is stably knockdowned in colon cancer cells (HT- 29) [107]. There are several studies that have implicated failure of miR expression in carcinogenic mechanisms [108], [109]. miR concentrations may be increased or repressed in hepatocellular carcinoma, which suggests that these sequences may act as oncogenes or suppressors of hepatocyte transformation. Recent studies using miRNA microarrays showed that high expression of miR-21 can contribute to growth and spread of human hepatocellular cancer (HCC) by inhibiting phosphatase and tensin homolog (PTEN) tumour suppressor, whereas low levels of miR-122a which target to cyclin G1 mRNA result in increased HCC [110], [111].

One of the cancer-related genes is the multiple drug resistance (MDR1) gene which provides resistance to vinca alkaloids (vinblastine, vincristine), anthracyclins (adriamycin, daunorubicin), etoposide and paclitaxel. In order to reverse the MDR1 gene-dependent multidrug resistance (MDR), two siRNA constructs were designed to inhibit MDR1 expression by RNA interference. Some data indicate that this approach may be applicable to cancer patients to change from tumouric P-glycoprotein-dependent MDR phenotype back to a drug-sensitive one [112]. An Eppstein Barr Virus (EBV)-encoded product, latent membrane protein (LMP-1), is considered to be an oncogene playing an essential role in cell transformation and metastasis. EBV-encoded LMP-1 was inhibited by RNAi and selective inhibition of LMP-1

had anti-proliferation effect on Nasopharyngeal carcinoma (NPC) cell. RNAi could be a powerful method in further investigations of LMP-1 [113]. A recombinant adeno-associated virus type 2 vector was used to deliver shRNA targeting EBV-LMP-1 into the EBV-positive human NPC C666-1 cells. Results showed that long-term suppression of EBV-encoded LMP-1 *in vivo* is an effective way for preventing NPC metastasis [114].

One of the most important signaling pathways to control growth and proliferation of our cells is the mitogen-activated protein kinase (MAPK) pathway. Ras, which is an enzyme in this pathway, is turned to an oncogenic form in about 15% of human cancer. Suppression of tumourgenicity was done by virus-mediated RNAi to inhibit specifically the oncogenic allele of K-ras (K-rasV12) in human tumour cells [115]. Other studies have reported that the use of siRNA can further block the Ras to Map kinase cascade, at either the Raf level or through NADPH oxidase1 (Nox1) [116]- [118].

11. Conclusion

Cancer is widely recognised as one of the largest burdens to health world wide. Some main features of cancers are its ability to sustain proliferative signaling, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, and activate invasion and metastasis. Until now, many methods have been developed for the treatment of cancer. Conventional treatment methods, ie.: surgery, chemotherapy and radiotherapy, are still widely used in the treatment of most cancers. However, these methods result in a high recurrence of cancer in patients. Clearly, there is an urgent need for the development of new therapies. In contrast to conventional methods, targeted gene therapy, immunotherapy, and gene therapy offer promising alternatives that are more effective and produce less side effects. Both targeted therapies and cancer immunotherapies have recently been used in clinic and these therapies can be succesful when used together, nevertheless, there are still limitations with these therapies.

Gene therapy has already begun to show great promise and is expected to be more effective in curing cancer. Targets for cancer gene therapy may include tumour suppressor genes (e.g. p53), oncogenes,and apoptosis genes. The most problematic issue for cancer gene therapy studies is the delivery of the therapeutic gene to the tumour cells. Although viral delivery methods are widely in use, there are non-viral technologies that offer several advantages that include less toxicity, reduced adverse immune responses and easier to producing large amounts of gene products. More recently, bacteria have also been used in cancer treatment. The hypoxic nature of solid tumours provides considerable conditions for growth of bacteria and bacterial colonisation. Necrotic areas can also supply purines to further facilitate growth of bacteria.

Bacterial delivery of RNA silencing tools combined with benefit of bacterial oncolysis can contribute to the treatment of cancer. Exploiting of small oligonucleic acids which are carried by spesific bacteria to cancer cells can be an effective way to cut energy supply and lysis of tumor cells.

Small oligonucleic acids can form complex secondary and tertiary structures. These nucleic acids can bind to other molecules and play an important role in cells. It has been shown that small RNAs have the ability to control gene expression and other activities that previously were assumed to be carried out only by proteins. As a result, small fragments of RNA may be tools for researchers to cure cancer. Small RNAs that are used in RNAi technology currently have been grouped into three major classes: small interfering RNAs (siRNA), microRNAs (miRNAs), and PIWI interacting RNAs (piRNA).

siRNA and shRNA have been extensively used to silence cancer-related targets. miRNA, as a tumour supressor, can be used in gene therapy for retrieving miRNA gene expression and preventing tumour development. Tumour cells keep high yields of lactic acid and produce ATP by aerobic glycolysis with or without oxygen. Accelerated glycolysis provides ATP levels to the fast proliferating tumour cells in a hypoxic environment. It is proposed that with RNA interference technologies metabolic genes in cancer cells can be silenced. Therefore tumour proliferation can be inhibited. Beside that, studies have shown that genes which are related to cancer such as Telomerase, BCL-2, COX-2 can be silenced for preventing cancer.

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