MINI-REVIEW

RNA Interference as a Plausible Anticancer Therapeutic Tool

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Abstract

RNA interference has created a breakthrough in gene silencing technology and there is now much debate on the successful usage of RNAi based methods in treating a number of debilitating diseases. Cancer is often regarded as a result of mutations in genomic DNA resulting in faulty gene expression. The occurrence of cancer can also be influenced by epigenetic irregularities in the chromatin structure which leads to alterations and mutations in DNA resulting in cancer cell formation. A number of therapeutic approaches have been put forth to treat cancer. Anti cancer therapy often involves chemotherapy targeting all the cells in common, whereby both cancer cells as well as normal cells get affected. Hence RNAi technology has potential to be a better therapeutic agent as it is possible to deactivate molecular targets like specific mutant genes. This review highlights the successful use of RNAi inducers against different types of cancer, thereby paving the way for specific therapeutic medicines.

Keywords: siRNA treatment - miRNA - lung cancer - liver cancer - gynecologic cancers - urologic cancers

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Introduction

RNA interference has emerged as an important tool in molecular medicine. This innovative technique which has been in existence in the biological machinery of organisms can be honed effectively as therapeutic modules in treating many debilitating diseases. Cancer is mostly considered as a genetic disease as it caused due to alterations in the genetic program involving a number of oncogenic pathways resulting in cancer cell transformation. Investigations on gene expression profiles have shown a number of alterations confirming the fact that many genes and pathways play a prominent role in carcinogenesis. RNAi can be used as a therapeutic agent as it can be used to target disease causing mutant genes as well as oncogenes. A number of *in vitro* as well as *in vivo* experiments have been successfully carried out using RNAi inducers which opens an insight on to develop RNAi based techniques in anti-cancer therapy.

Mechanisms of RNAi Action

RNA interference involves the silencing of a gene by the use of dsRNA with homologous sequences to that of the target mRNA. Small interfering RNAs (siRNAs) are generated by the cleavage of endogenous as well as exogenous dsRNA molecules with the help of an RNAse III enzyme called Dicer (Hamilton and Baulcombe, 1999; Zamore et al., 2000; Bernstein et al., 2001). Human dicer are proteins about ~200kDa in size with about 1,922 amino acids consisting of two RNase III domains (RIIIDa and RIIIDb) and a double stranded RNA binding domain (dsRBD) along with an N terminal segment that consists of a PAZ domain, RNA HELICASE DOMAIN and DUF283 domain. The 3' ends of small RNAs bind to the PAZ domain and the RNA helicase domain hydrolyzes ATP resulting in unwinding of RNA duplex. The RISC complex consists of Argonaute family of proteins about ~100kDa in size. It contains two domains namely the PAZ domain as well as the PIWI domain. PAZ domain is ~130 amino acids placed at the centre of the RISC to which the 3' end of the anti-sense RNA gets attached. The PIWI domain consists of ~300 amino acids and helps in the cleavage of the target mRNA (Hutvagner, 2005).

This dicer processes the dsRNA into short duplexes of about 19-21 nucleotides with symmetric 2 nucleotide overhangs along with hydroxyl group at the 3' end and a phosphate group at the 5' end. These siRNAs contain a sense strand as well as an anti-sense strand. The anti-sense strand alone gets incorporated into the RISC assembly and cleaves the complementary target mRNA (Alquist, 2002).

siRNAs

siRNAs are 19-21 nucleotides long duplexes which are produced from ds RNAs (double stranded RNAs) by the action of an enzyme RNase III Dicer as shown in Figure1. These siRNA duplexes consist of a sense strand and a complementary anti-sense strand with a phosphate group at both the 5'ends along with hydroxyl groups as well as two nucleotide overhangs at the 3'ends. The siRNAs unwind due to helicase activity of the RISC assembly after which the anti-sense strand attaches to the RISC and targets the specific mRNA (Zhang et al., 2004).

shRNAs

Short hairpin RNAs (shRNAs) are a group of siRNAs which can be expressed with the use of U6 or H1 promoters (Brummelkamp et al., 2002a; Paul et al., 2002). Sh RNAs have a plethora of advantages when compared to siRNAs like long time silencing effects as well as efficient and easy

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delivery methods. These shRNAs contain a single stranded RNA molecule of about 50-100 bases. The complementary regions are spaced with the help of a hairpin loop which allows the transcript to fold back on itself thereby getting the name "short hairpin" RNA. This shRNA is processed by Dicer and gets converted to siRNAs which unwind due to helicase activity of the RISC assembly after which the anti-sense strand gets incorporated into the RISC which cleaves the target mRNA due to its endonuclease activity (Bernstein et al., 2001).

miRNAs

The microRNAs (miRNAs) are another group of RNAi inducers that play an important role in development of an organism. They form an integral part as an endogenous gene silencing machinery as seen in Figure 1. The primary precursors of miRNAs which are referred to as primary miRNAs (pri-miRNAs) are present in the genome and get transcribed by RNA pol II (Kim, 2005). The pri-miRNAs consists of 33 base stem-loop structures in which the miRNA is present in the 5' or 3'end of the stem. These pri-miRNAs get processed by Drosha an RNAse III enzyme , which cleaves the pri-miRNAs to form 70 nucleotide long precursor miRNAs (pre-miRNAs), which get transported from the nucleus into the cytoplasm with the help of a protein called Exportin-5. These pre-miRNAs get cleaved in the cytoplasm by Dicer to form functional miRNAs which are about 21-25 nucleotides in length. Further processing allows the antisense strand of the miRNA to get incorporated to the RISC assembly and leads to translational repression of the target mRNA (Zeng et al., 2005). MiRNAs play a major role in proliferation as well as apoptosis (Cheng et al., 2005). MiRNAs are also found to regulate a number of oncogenes and tumour suppressor genes.

Studies by Calin et al. (2004) have indicated that about 50% of miRNA genes are localized in genomic regions associated with cancer.

Another study by Calin et al. (2002) showed that miR15 as well as miR16 genes were found to be deleted in chronic lymphocytic leukemias (CLL). Another member of the miRNA family, miR-155 was overexpressed in Hodgkin's lymphomas, B-cell lymphomas, Burkitt's lymphomas as well as in human breast cancers confirming the fact that this miRNA acts as an oncogene (Metzler et al., 2004; Eis et al., 2005; Iorio et al., 2005; Kluiver et al., 2005). Another class of miRNAs namely the miR-21 miRNA was found to be overexpressed in highly malignant human brain tumor as well as in glioblastoma (Chan et al., 2005).

Based on this information we can precisely come to the conclusion that miRNA profiling can be very useful in Cancer diagnosis (Li et al., 2005b).

Delivery Methods for RNAi Inducers

The most important criteria for the success of RNAi based strategy depends on the efficient delivery of these biomolecules into the specific cell, tissue or organ. *In vitro* delivery of siRNAs is brought about using cationic liposome based methods whereas the same strategy cannot be successfully incorporated for *in vivo* delivery due to rapid clearance of these lipid molecules in the Liver. Studies by Sorenson et al. (2003) as well as those by Sioud and Sorenson, (2003) have reported that the successful systemic delivery of lipid based siRNA complexes and siRNAs using cationic polymer.

A number of successful *in vivo* delivery methods have been devised like combining siRNAs to antibody protamine fusion (Song et al., 2005),using the sense strand

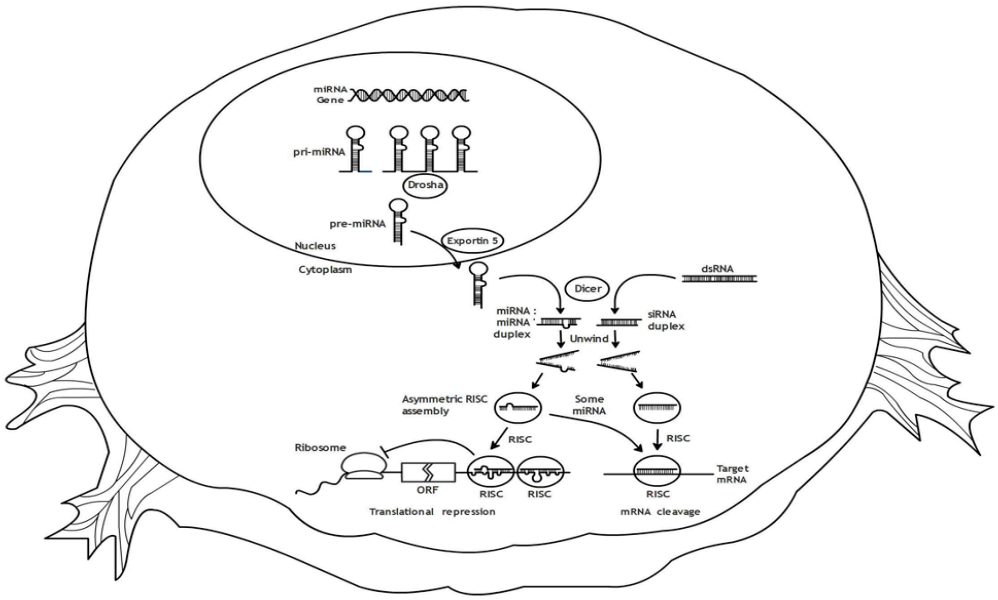

Figure 1. Mechanisms of siRNA and miRNA. The dsRNA molecules are processed by Dicer into short interfering RNA (siRNA) duplexes. The Anti-sense strand of the siRNA duplex is assembled into the RNA-induced silencing complex (RISC) and results in the cleavage of the target mRNA. The primary microRNA (pri-miRNA) transcripts get processed by drosha into 70-nucleotide precursor microRNAs (pre-miRNAs). These pre-miRNAs get transported from the nucleus to the cytoplasm with the help of Exportin 5 and get processed by Dicer to form dsRNAs. The anti-sense strand of this duplex gets incorporated to the RISC complex and results in translational repression or cleavage of the target mRNA.

of the siRNA in combination with Cholesterol conjugate (Soutschek et al., 2004), incorporating siRNAs into cyclodextrin nanoparticles (Hu-Lieskovan et al., 2005) as well as using aptamer siRNA conjugates (McNamara et al., 2006). Each of these combinations can be used to bring about successful *in vivo* delivery of siRNA biomolecules targeting specific genes.

Short hairpin RNAs (shRNAs) containing DNA-based expressed expression cassettes containing a sense and anti-sense strand along with Pol III promoters can also be successfully used as RNAi inducers (Brummelkamp et al., 2002a; Lee et al., 2002; Paddison et al., 2002; Sui et al., 2002).

Vector-based shRNA systems can be successfully delivered using viral vectors. Adenovirus and Adeno associated viral vectors (AAV) provide an excellent platform for delivery of shRNAs (Li et al., 2005a; Osada et al., 2005; Ragozin et al., 2005). Retroviral vectors like MLV(Murine leukemia Virus) (Hemann et al., 2003) and lentiviral vectors like HIV (Human Immunodeficiency Virus), FIV (Feline Immunodeficiency Virus), EIAV (Equine infectious anemia virus) have also been successfully used to deliver shRNAs to induce RNA interference (Dittgen et al., 2004; Bahi et al., 2005).

RNAi in Cancer Therapy

Cancer is a complex genetic disease which involves a multitude of oncogenic pathways. Many oncogenes and tumour suppressor genes play an important role in carcinogenesis. In order for the development of cancer, the cells proliferate uncontrollably, they resist apoptosis, tend to draw nutrients via angiogenesis and also invade the other parts of the biological entity by metastasis. Since cancer involves many pathways it becomes quite essential to target multiple genes. RNAi has emerged as a potent method to bring about gene silencing and can be used as a therapeutic tool to treat many diseases (Hosono et al., 2005). In majority of cancers RNAi can be used to specifically target mutant genes, cancer associated genes, receptors involved in oncogenic pathways as well as signaling molecules thereby opening new avenues in anti-cancer therapy.

A study by Brummelkamp et al. (2002b) showed that sequence specific siRNAs can inhibit mutant K-Ras gene excluding the wild type gene in human pancreatic carcinoma. Similarly specific siRNAs have been successfully used to target AKt isoforms 2 and 3 in uterine cancers thereby sensitizing the cells to cisplatin (Gagnon et al., 2004).

Cancer associated genes which are not mutated but overexpressed in a majority of cancers can also be potential target in RNAi based techniques. Clusterin is an antiapoptotic gene which is expressed in most cancers was successfully targeted by specific siRNAs which resulted in increased chemosensitivity of lung cancer cell lines to chemotherapeutic agents (July et al., 2004).

Oncogenic pathways include a large network of receptors which play an important role in malignancy. These receptors can be used as potential targets for gene silencing. The use of siRNAs to target chemokine receptor chemokine (C–X–C motif), receptor 4 (CXCR4) on breast cancer cell line MDA-MB-231 resulted in marked reduction in cell proliferation (Lapteva et al., 2004).

Signalling molecules can also be potential targets for RNAi based therapy. B-RAF is a serine/threonine-specific protein kinase which is found to be mutated in most human melanomas when targeted by siRNAs inhibited cell growth and enhanced apoptosis in melanoma cell lines (Karasarides et al., 2004).

RNAi in Tumorigenesis

Many genes have been targeted using RNAi based technology in different tumour cell models and the knock down of these genes have made new inroads in therapy. Some of the genes that have been studied include oncogenes, telomerase, growth factor receptor genes, signalling molecules and other genes.

An important oncogene Bcl-2 is over expressed in many human tumours. A study by Fu et al. (2005) demonstrated that siRNA targeting Bcl-2 induced apoptosis in 50% of the cells *in vitro* and shRNAs against Bcl-2 suppressed tumour growth by 60% in mice with xenograft tumour.

In vitro studies using synthetic siRNA specific for Bcl-2 when introduced in combination with cationic liposomes inhibited the expression of Bcl-2 protein and inhibited growth of human tumour cell lines. This combination of liposome complexed bcl-2 siRNA also exhibited strong anti-tumour activity in mouse models having liver metastasis as well as in xenograft models of human prostate cancer (Yano et al., 2004). A study by Lima et al. (2004) demonstrated that down regulation of bcl-2 or another antiapoptotic gene, X-IAP (X-linked inhibitor of apoptosis) by using specific siRNAs sensitized breast cancer MCF-7 cells to anticancer drugs like etoposide and doxorubicin.

In vitro studies by Ling and Li, (2004) showed that shRNAs specific against survivin, a gene which is up-regulated in many cancers (Kim et al., 2003), silenced the expression of survivin and resulted in apoptosis of the transfected cells. RNAi induced down regulation of survivin in esophageal squamous cell carcinoma and caused significant inhibition of cancer cells both *in vitro* and *in vivo* (Wang et al., 2005). Human rhabdomyosarcoma xenografts when treated with a cocktail of survivin-shRNA encoding plasmids over two weeks resulted in 70% reduction in tumour growth (Caldas et al., 2006). *In vivo* studies by Takei et al. (2004) showed that siRNAs have been successfully used to reduce angiogenesis by targeting vascular endothelial growth factor (VEGF).

A member of the signal transduction and activation of transcription (STAT), the STAT3 gene is frequently activated in different types of cancer. RNAi specific to STAT3 resulted in inhibition of DU-145 prostate cancer cell line by inducing apoptotic cell death (Lee et al., 2004). STAT3 siRNA inhibited the growth of Hep2 human laryngeal cancer cell line, resulting in apoptosis and down regulation of Bcl-2 expression (Gao et al., 2005).

The multiple drug resistance (MDR1) gene product P-glycoprotein is over expressed in cancer and poses a

major problem in chemotherapeutic treatment of cancer. Retroviral mediated shRNA specific to MDR1 sensitized cancer cells to cytotoxic drugs (Pichler et al., 2005). Similarly a study by Hua et al. (2005) reported that the suppression of MDR1 gene using siRNA expression vector reversed drug resistance to doxorubicin in human uterine sarcoma cell line.

Polo-like kinase 1(PLK1) is a serine/threonine kinase which plays an important role in mitosis as well as in malignant transformation.It has been found that siRNAs specific against PLK1 on Non-small cell lung cancer(NSCLC)cell lines resulted in reduced cell proliferation as well as increased cellular apoptosis and sensitized the cells to chemotherapy.

An important genetic alteration in the protein tyrosine kinase pathway leads to a disease called chronic myeloid leukemia (CML) which occurs as a result of recurrent chromosomal translocation between chromosome 9 and 22 leading to the formation of a hybrid BCR-ABL gene. This gene encodes for a deregulated protein tyrosine kinase which plays an important role in the pathogenesis of chronic myeloid leukemia. Sequence specific siRNAs were used to target BCR-ABL activity (Wilda et al., 2002; Wohlbold et al., 2003; Scherr et al., 2005; Withey et al., 2005). RNAi inhibited BCR-ABL dependent cell growth and induced apoptosis in CML cells. Further studies revealed that siRNAs against BCR-ABL increased the sensitivity of leukemia cells to a drug called imatinib which targets the deregulated protein tyrosine kinase (Wohlbold et al., 2003).

Another important gene cyclophilin A (CypA) is over expressed in most non-small lung carcinomas. Down regulation of CypA using specific siRNAs in human lung tumour cells resulted in reduced growth of Xenograft tumours along with decreased cancer cell proliferation and increased apoptosis both *in vitro* and *in vivo* (Howard et al., 2005).

The potential anti-tumour applications of RNAi based techniques offer great hope in designing effective anti-cancer therapy.

Lung Cancer

Lung cancer is the most common of all cancers. There are two types of lung cancer namely small cell lung carcinoma and non-small cell lung carcinoma. The use of siRNA technology has increased the chances of combating lung cancer.

The use of specific siRNAs targeting survivin encapsulated in PEGylated LPD nanoparticles resulted in down regulation of survivin gene and showed pronounced anti-tumour effect as well as enhanced apoptosis along with inhibition of tumour cell growth in lung cancer cells (Li et al., 2006). Similarly the use of epidermal growth factor receptor specific siRNAs in combination with LPD nanoparticles in Lung cancer xenograft mice model resulted in tumour growth inhibition (Li et al., 2008).

In another study, the use of Akt1specific siRNAs into urethane induced lung cancer mice model showed downregulation of Akt1 gene thereby resulting in inhibition of tumour growth (Xu et al., 2008).

Liver Cancer

A known risk factor for liver cancer is infection with HBV or hepatitis C virus. Chronic infection with HBV could result in Hepatocellular carcinoma. RNA interference based techniques provide an effective strategy to target HBV infection (Arbuthnot et al., 2007). Specific siRNAs that target the HBV RNA were intravenously injected into mice infected with HBV which resulted in drastic reduction in serum HPV DNA levels (Morrissey et al., 2005). *In vivo* studies were carried out in liver metastasis nude mice by incorporating Bcl-2 specific siRNAs along with a cationic liposome LIC-101 which resulted in reduction of tumour size (Yano et al., 2004).

Gynecologic Cancers

Breast Cancer

Breast Cancer is the second most common of all Cancers and results in high mortality among women in western countries (Jemal et al., 2010). This disease condition is characterized by uncontrolled cell growth in the tissues of the breast. A plausible treatment for breast cancer is surgery in the case of localized tumour along with additional therapies such as chemotherapy as well as radiotherapy.

Sequence specific siRNA targeting cyclin D1 were seen to increase cellular apoptosis in MCF-7 breast cancer cell lines. siRNAs specific for plasminogen activator inhibitor type I also resulted in increased level of apoptosis in MDA MB 231 cells (Meryet-Figuières et al., 2007).

Successful use of siRNAs that target signaling peptide of secretory clusterin in combination with Copolymers of PEI and PEG (PEI-g-PEG) have resulted in reduced expression of clusterin thereby sensitizing human MCF-7 breast cancer cells to ionizing radiation (Sutton et al., 2006).

In another study the use of HER2/neu specific siRNA when complexed with chitosan nanoparticles encapsulating quantum dots resulted in the silencing of the HER2/neu gene and increased apoptotic levels in SKBR3 breast cancer cells (Tan et al., 2007).Bcl-2 targeting siRNAs when introduced into MDA-MB-231 human breast cancer cell line sensitized these cells to paclitaxel and increased cellular apoptosis (Wang et al., 2006).

An Important study was carried out by Valdehita et al. (2012) in which siRNAs were used to target VPAC1 in T47D as well as MDA-MB-468 breast cancer cell lines thereby inhibiting vasoactive intestinal peptide (VIP) which is responsible for stimulation of VEGF an important factor in angiogenesis.

In vivo delivery of c-raf specific siRNAs packed in synthetic cationic cardiolipin analogue (CCLA) liposomes into SCID mice with human breast xenograft tumours resulted in successful suppression of tumour (Chien et al., 2005).

Ovarian Cancer

RNA interference has been proposed as a therapeutic tool to fight different cancers. Many specific genes have been targeted using siRNA technology as possible

anticancer therapy. Ovarian cancer is considered to be the most deadly among other gynecologic cancers. A potential target has been the Her-2/neu gene. Invitro studies using sequence specific siRNAs to target Her-2/neu gene in ovarian cancer resulted in decreased cell proliferation, apoptosis and reduced tumour growth (Yang et al., 2004). siRNAs targeting the H-ras gene decreased the tumour volume in ovarian cancer models as well as increased apoptosis in ovarian cancer cell lines (Miyamoto et al., 2004).

Glutathione-S-transferase (GST) and p-glycoprotein (p-gp) are over expressed in ovarian cancer and are responsible for multiple drug resistance. Invitro studies in ovarian cancer cell lines revealed that sequence specific siRNAs targeting GST and p-gp resulted in sensitivity to Cisplatin (Zhang et al., 2005).

Cervical Cancer

Human papillomavirus (HPV) is a virus that belongs to the papillomavirus family and HPV infection is a cause of nearly all cases of cervical cancer. RNAi technology has been used to target HPV associated genes as potential targets against cervical cancer. Specific siRNAs that targeted HPV E6 gene expression resulted in the accumulation of p53 protein resulting in reduced cell growth whereas silencing of the HPV E7 gene by siRNAs resulted in apoptosis (Jiang et al., 2005; Yoshinouchi et al., 2003). *In vivo* studies using siRNAs targeting HPV18 E6 and E7 resulted in tumour suppression in cervical cancer xenograft mice model (Fujii et al., 2006).

Urologic Cancers

Prostate Cancer

Prostate Cancer is one of the most lethal cancers owing to male related cancer death. New modes of therapy become the need of the hour to treat this deadly form of cancer.

Prostate cancer cells show increased expression of pro-oncotic genes like polo-like kinase 1 (Eckerdt et al., 2005) and Bcl-2 (Cory and Adams, 2005). *In vitro* studies using siRNAs that specifically target polo-like kinase 1 and Bcl-2 have shown successful silencing of the targeted genes along with decrease in cell proliferation. *In vitro* studies using polo-like kinase-1 and Bcl-2 specific siRNAs in human prostate cancer cell induced athymic mice lead to decrease in tumour volume (McNamara et al., 2006).

An important receptor which is over expressed in prostate cancer is the insulin-like growth factor receptor which can be potential target for gene therapy studies. *In vitro* studies using sequence specific siRNAs targeting the type 1 insulin-like growth factor receptor in DU145, LNCaP and PC3 prostate cancer cell lines resulted in reduced cell proliferation and increased rate of apoptosis (Rochester et al., 2005).

In vivo studies using Raf-1 specific siRNAs in Prostate cancer xenograft mice model resulted in successful inhibition of tumour growth (Pal et al., 2005).

Another *in vivo* study using anti-integrin alpha V siRNA along with liposomes in human PC3 Prostate cancer cell line induced Xenograft mice models resulted in inhibition of tumour growth (Bisanz et al., 2005). Similarly siRNAs targeting Bcl-2 when introduced into PC3 cell line induced Xenograft mice models resulted in decrease in tumour size (Yano et al., 2004).

siRNAs were successfully used to target the c-myc gene, a key regulator of cell

proliferation and death, which resulted in decreased growth of prostate cancer cells (Green et al., 2011).

Bladder Cancer

Bladder Cancer can be treated by transurethral removal of the tumour along with chemotherapy. The use of RNAi based technology has been successful in controlling the cell proliferation in a number of bladder cancer cell lines.

siRNAs that specifically target survivin showed a marked decrease in cell proliferation and increased apoptosis in bladder cancer cell lines (Ku et al., 2010).

In vitro studies in which many antiapoptotic proteins have been used as potential targets in bladder cancer cells to bring about some clinical significance which would pave the way for a successful therapeutic approach (Kunze et al., 2008).

Renal Cancer

In Renal cell carcinoma (RCC) the foremost therapeutic strategy involves surgery. Gene therapy especially those involving the use of RNAi technology have been the forerunners in the treatment of RCC. A plausible target is HuR gene which is an mRNA stabilization protein and is over expressed in RCC (Ronkainen et al., 2010). *In vitro* studies using siRNAs targeting HuR gene in RCC cell lines resulted in marked growth inhibition of RCC cells. Similarly *in vivo* studies with the same siRNAs in xenograft mice models resulted in reduced tumour growth (Danilin et al., 2010).

Another important gene namely osteopontin plays a significant role in metastasis and tumorigenesis of RCC. *In vitro* studies using siRNAs specific for osteopontin in Caki-1 human renal carcinoma cell line resulted in reduced cell proliferation and increased cellular apoptosis (Zhang et al., 2010).

Conclusion

RNAi technology has been an important tool in the analysis of gene function and in reverse genetics. The past era has witnessed a number of clinical trials involving RNAi as a potential therapeutic agent against a number of debilitating diseases. Cancer is a complex disease involving a network of oncogenic pathways. These pathways are regulated by a number of genes which can be used as potential targets using RNAi based techniques. There have been a number of *in vitro* studies that indicate that RNAi technology can bring about cell death in cancer cell lines but few to elaborate the success of this technique to destroy tumours *in vivo*. The effectiveness of RNAi in Cancer therapy is bound to increase as novel efficient methods of delivery have been devised which offer accurate delivery of the RNAi inducer to the target system. With the advent of RNAi based gene therapy, it is possible to combat cancer at the molecular level thereby

opening new avenues for an effective anticancer therapy.

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