RNA Interference-weapon against cancer

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ABSTRACT

Cancer is the second principal death cause, in the recent scenario 1 out of every 4 people die out of cancer. Not more than 50% advanced cancer subjects live a year ahead. RNA interference (RNAi) a phenomenon which serves for shutting off the expression of a specific sequence has proved itself a finest weapon against cancer. This mechanism involves silencing of the expression of desired mRNA sequence by degrading it using a dsRNA (double stranded RNA) molecule having complementarity to the desired mRNA. However a major challenge in proficient implementation of this therapy against cancer is lack of efficient delivery methods for the dsRNA to the target tissue. Various delivery methods had already been developed for efficient and effective delivery of the dsRNA and many are under trial. This review is going to present the great efficacy of RNAi against cancer and the various delivery methods performing capable work in accurate application of this therapy.

Key words: Cancer, RNA interference (RNAi), double stranded RNA (dsRNA), small interfering RNA (siRNA), microRNA (miRNA), mRNA, RNA induced silencing complex (RISC).

INTRODUCTION

RNAi phenomenon was first observed in 1998 to be occurring naturally in Caenorhabditis elegans (a nematode) by Criag C. Mello and Andrew Fire [1]. In this process a specific mRNA sequence is silenced by using a fragment of double stranded RNA (dsRNA) which has complementarity to the mRNA sequence under concern and leads to cleavage of mRNA by nuclease enzyme which targets where dsRNA is attached. It's a post transcriptional process which results in blocking the translation of mRNA [2].The dsRNA used for this purpose may be endogenously expressed (miRNA or micro RNA) or be transferred exogenously (siRNA or small interfering RNA).

The exogenous long dsRNA molecules are broken down into small fragments of 21-23 nucleotides and possess 2 overhanging neucleotides on 3' unphosphorylated ends of each strand. This small fragments formation incorporates the use of Dicer enzyme (RNAase III family) which cleaves long ds RNA into small fragments and these small fragments so formed are known as siRNA (small interfering RNA) [3].

The endogenous ds RNAs are expressed as pre miRNAs and then processed through various enzymes Drosha (RNAase III family), Pasha, Exportin and Dicer to form mature miRNAs, which are equivalent in action to siRNAs mentioned above. The pre miRNA is a short hairpin RNA (shRNA) which is acted upon by the enzymes Drosha and Pasha which cleave it to remove undesired sequence, then this cleaved pre miRNA is transported to cytoplasm with the aid of enzyme Exportin. There it encounters another enzyme, the Dicer, which cleaves it into small fragments to form mature miRNA or potential siRNA. These endogenous RNAs may be transcribed naturally under the stress conditions or by the effect of several biological processes [3, 5]. But they can also be expressed by inserting a 22-bp single or double stranded RNA sequence which after reverse transcription gets incorporated in the host DNA and then after transcription produce pre miRNA which undergoes same enzymatic process as the miRNA which is naturally formed endogenously, that is by the above mentioned enzymes and thus forms the potential siRNA fragment [5, 6].

The siRNAs or mature miRNAs are then subjected to endonuclease Argonaute-2 which unwinds these double stranded RNAs. Then and there the guide strand or the antisense strand of siRNAs gets integrated with RISC complex (RNA induced silencing complex), while the passenger or the sense strand is released. This guide or antisense strand directs the complex to the sequence on mRNA which has complementarity to guide strand and there nuclease enzyme act upon the mRNA to cleave it in case of

siRNA, while miRNAs contain incomplete sequence homology and recognise sequence in the untranslated 3' end only thus avoids the degradation or cleavage of the mRNA, instead they obstruct the translation or leads to bypass some genes [5].

Cancer is uncontrolled growth of cells, which is the outcome of several mutations in genes responsible for expression or regulation of cell apoptosis, proliferation and differentiation [2]. These mutations often activate the potential oncogenes which are present in our genome in inactive form. These mutations may be genetic mutations, epigenetic mutations (due to suppression of DNA repair system) or viral transformation [4, 5]. Just a single mutation is not capable to cause cancer instead several combinations of mutations together form favourable conditions for cancer. The use of chemotherapeutic agents like doxorobucin, taxenes, camptothecin, platinating agents have proved to be efficient but they have some drawbacks like they effect both cancerous and normal cells and also have toxic side effect for vital parts of the body like heart, nephron, nervous system and other cells of the body [12].

It is evident that RNAi phenomenon has great potentiality to be efficiently used against cancer. It has achieved high success rate in regulating the expression of genes that are responsible for, cell proliferation, metastasis, invasion, differentiation and sensitivity to chemotherapy and radiotherapy [12]. RNAi in cooperation with RNA polymerase II promoter can knockdown gene expression up to 95 % as is permitted in a drug inducible system [8]. According to recent studies human genome is capable for encoding some 1500 miRNAs which can alter the expression of about 60% of the human genes [5]. A particular miRNA have efficiency to join several mRNAs, some mi RNAs are also effective as tumor suppressor genes [12]. Among the advantages of using miRNA instead of siRNA, one is that siRNA target single gene while miRNA can focus multiple genes at one go [6]. But siRNA promise effective target mRNA cleavage as is suggested by application of next generation sequencing scrutiny on 5' RNA ligase-mediated rapid amplification of cDNA end products (5'RACE by sanger) that siRNA is capable to direct cleavage at 5 distinct position in target mRNA in a manner which is sequence dependent[20]. RNAi technique has been implemented for refusing the expression of B-cell CLLlymphoma-2 gene, insulin like growth factor 1 receptor, epidermal growth factor 1 and 2, epsilon 4 (p12 subunit) etc. [8]. With the application of genetics and bioinformatics approaches it is possible to go through the gene expression and signalling pathway and also the effect of RNAi technique. Even expression of many genes can be determined by using DNA microarray and it can give conformations about genes which are differentially expressed in two cancer patients so help in comparative study too [8]. But some limitations in systematic administration of siRNA like short half-life, lack of ability to penetrate plasma membrane, activation of immune response, rapid renal clearance and potential toxicity makes its delivery to the target tissue a major challenge [10, 11]. Exact and specific delivery techniques are required for delivery of siRNA to specific cell. Many new techniques have been developed for the purpose and they are use of recombinant adenoviruses, atelocollagen, cancer cell specific antibodies, nanoliposomes etc. We will be discussing about the following later in the text.

DISCOVERY OF RNA INTERFERENCE

RNAi first became eminent an era ago when Richard Jorgensen and colleagues were experimenting for extra intense purple petunias. They integrated additional pigment gene to petunias but this surprisingly resulted in irregularly coloured petunia or white petunia [9]. This process then termed as co-suppression was similar to RNAi which turned off the expression of already existing genes by the addition of foreign RNA, as this foreign RNA was complementary to that existing gene it get attached to it leading to the existing gene degradation. Same results were found in fungi Neurospora crassa [9].

During the same period Guo and Kemphues were working on finding the par -1 gene function in Caenorhabditis elegans ,they first employed antisense strand (complementary to the par -1 gene) for the purpose and then the sense strand (same sequence as that of par-1 gene), surprisingly both resulted in the degradation of the desired mRNA[9].

Then Andrew Fire and Craig Mello got to see the similar observations in Caenorhabditis elegans. They then tried for ds RNA which gave more potent result, resulting in ten times greater result then either of the strands alone. This kept the foundation of the

discovery of RNA interference [1]. This was later also observed in fruit flies and mammals too.

MECHANISM OF RNA INTERFERENCE

RNA interference is a post transcriptional process that regulates the expression of gene with the help of RNA molecules[1]. It is a process that involves use of small complementary RNA sequences (20-22 bp long), which blocks the translation of specific genes on mRNA by degradation of the mRNA or by bypassing the translation of that gene, after attaching to that specific gene sequence with the help of RNA induced silencing complex (RISC) [8].

These small sequences can be obtained either exogenously or endogenously as siRNA or miRNA respectively. Both are used for the same purpose that is, silencing the mRNA, but there source of derivation are different.

The source of siRNA may be endogenous or exogenous dsRNA. The dsRNA can be created endogenously by transcription of transferred gene by RNA directed RNA polymerase, these genes are transferred via viral replication intermediate and by transcription of both strands is done simultaneously from specific loci on genome or exogenously via introducing genes, which encodes inverted repeats, through genetic engineering using vectors. These dsRNA are then treated with RNase III family enzyme Dicer which breaks these dsRNA into small interfering RNAs which are 20-22 base pair long and have 5'phosphorylated ends and 3' non phosphorylated ends with two nucleotides unpaired on both strands [2, 3].

MicroRNA (miRNA) is specifically endogenous in generation. Their gene are present in inter genic regions but can be derived from introns also. These miRNAs results from single stranded RNAs formed after transcription of these source genes by RNA polymerase II and some by III [5]. The single stranded RNA forms hair pin structure which makes it double stranded and then processed through various enzymes to form mature miRNA [7]. The hairpin or pre miRNA is processed by RNase III family enzyme Drosha and Pasha and matured mi RNA thus formed contain 2 nucleotide unpaired at 3' ends and 5' phosphorylated end. This mature mi RNA is then transported to cytoplasm by Exportin 5 protein. Mature mi RNA in cytoplasm is acted upon by Dicer protein to convert it into an efficiently small mature miRNA [6, 7].

The siRNA or miRNA in the cytoplasm remains associated to Dicer through interaction with some other partner proteins of Dicer, these partner proteins differ with species and circumstances. These partner proteins involve TRBP in humans [8]. Dicer interact with these small RNA binding proteins to form RNA loading complex (RLC) which help in making the small interfering RNA single stranded by separating it into sense and antisense strand, hence making it capable for loading and then antisense or guide strand is taken up by RISC[7, 9]. RISC contains member of Argonaute family where the antisense strand binds in RISC, as in case of synthetic siRNAs they are so designed that they are thermodynamically stable and can direct loading through their stable duplex ends [20]. This guide strand guides the complex to the desired site on the mRNA as it is complementary to that site and leads to the degradation of mRNA at that position as shown in figure 1. If the participating member of the argonaute family is argonaute-2 then this will direct endonucleolytic phosphodiester bond cleavage of the mRNA (target) at 10-11 nucleotides from 5'end [7,20].

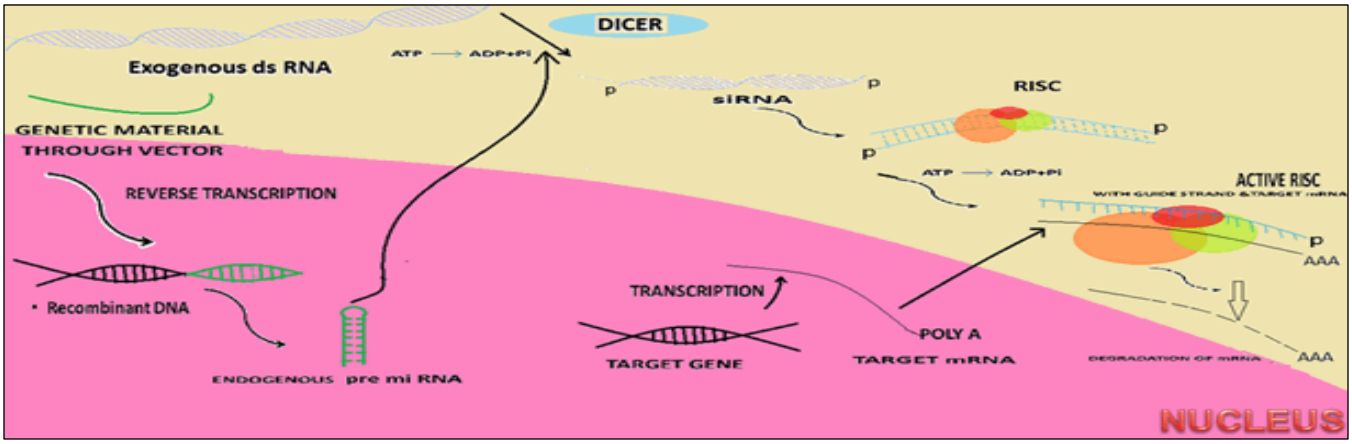

Figure 1: Mechanism of RNA interference through exogenous and endogenous ds RNA.

CANCER and RNAi

RNAi as an efficient weapon against cancer focuses on the specific mutated genes which cause cancer and the complementary small sequences which can be used for silencing these genes [7, 8]. These genes vary according to the mutagen thus there is great variability in the genes responsible for cancer in each case. Some genes which can be potentially involved to cause this deadly disease if mutated or over expressed are Epidermal Growth Factor receptor 1 and 2(EGRF 1 & EGRF 2), heat shock 27kDa protein 1 (HSP27), ferritin (FTL),insulin like growth factor 1 receptors (IGFIR), polymerase (DNA directed), epsilon 4 (P12), breast cancer 1, Mdm2 p53 binding protein homolog (MDM2) (mouse), early onset (BRCA1), Human papillomavirus proteins (HPV E6 & E7),vascular endothelial growth factor VEGF, transforming growth factor α and β, Estrogen receptors, Multiple-drug resistant, protein kinase A, iron-responsive element (IRE), c-fos FBJ murine osteosarcoma viral oncogene homolog (C-fos), B-cell CLL/lymphoma 2 (Bcl-2, RAF proto-oncogene serine/threonine-protein E6 and E7), v-MYC myelocytomatosis viral oncogene homolog (avian) (C-myc) and metallothionein (MT) genes[8]. Various cell cycle associated genes of kinesin spindle protein (KSP) and polo like kinase 1(PLK1) as targets of RNAi have proved efficient curing power of RNAi [17]. Recently RNAi therapy is under phase 1 trial for degradation of MYC gene which is the major oncogene known. Mutant gene of k-RAS is now being targeted by siRNA loaded with millimeter sized LODER (local drug elute R) which release siRNA over time and has passed phase 1 trial for pancreatic cancers [16, 19]. It is quite difficult to obtain these sequences endogenously, so genetic engineering techniques are used to introduce these sequences inside the cells. Exogenous dsRNA are effective to be used via RNAi to control metastasis, cellular proliferation, invasion, differentiation and sensitivity to therapies (radiation & chemotherapy therapy) in various types of cancer cells [4]. As the delivery of these siRNA for RNAi to be implemented is facing great challenge so evokes the need to develop efficient delivery techniques for the purpose [7]. These upcoming sections are all about the possible ways of introducing these complementary sequences (dsRNA) inside the cell efficiently [12].

ADENOVIRUS

Adenovirus infects the adenoid lymphatic tissue in nasopharyngeal cavity [8]. It is icosahedral particle having no envelope that encapsulate upto36 kilo base double stranded DNA. It has clinical importance as it can be transformed to replication deficient adenovirus vector (deficient of E1 and E3genes) and by utilising RNA polymerase III H-1 RNA promoter of mammalian host cell (other promoters can be used for the purpose) can be used to derive efficient expression of siRNA [14]. It has ability to infect a broad cell type spectrum and is independent to cell division. Initial high affinity of the virion for the cell occurs via direct binding of the fibre knob domain to its cognate receptor on cell, which is the 46 kilo Dalton coxsackie and adenovirus receptor (CAR) [14].Adenovirus intra tumoral injection coding the hypoxia inducible factor -1(HIF-1)-targeted siRNA had a noteworthy influence on tumor growth when integrated with ionizing radiations [8]. Adenovirus vector TT-034 or PF-05095808 have hepatic tropism due to its serotype 8 capsid, is an example in recent research [19, 20].

ATELOCOLLAGEN

Atelocollagen has great access to work for the purpose. It is highly purified type I collagen (fibrous protein in the connective tissue) from calf dermis which is pepsin treated [11, 15]. It has sequence of amino acids known as telopeptide at N and C terminals which causes antigenicity. Pepsin treated atelocollagen are less immunogenic as they lack telopeptide [13]. It allows increase in cellular uptake, prolonged release of oligonucleotide and genes and nuclease resistance. It has the capability of being in liquid state at room temperature [15]. Atelocollagen and siRNA are mixed in equal amount or volume for 20 min at 4degree Celsius and then kept at same temperature for 16 hr before use [15]. As they have positive charge on the surface they can easily form electrostatic bonding with nucleic acid which are negatively charged [13]. The size of atelocollagen and siRNA can be altered by altering the ratio of both in complex [13]. Thus it can work for efficient delivery of siRNA *in vivo*.

NANOLIPOSOME

Nanomedicine is playing a great role in overcoming the existing challenges various nanoparticles and

nanotechnology related techniques are proving themselves efficient in delivering siRNA to the target. Recently multi drug delivery associated with nanoparticles has given great outcomes in treating breast cancer [4]. Layer by layer doxorubicin and siRNA or only siRNA delivery linked with nanoparticles is shaking hands with researchers [18]. Use of "nanoliposomes" as "nanomedicine" is a major topic of concern for siRNA delivery due to its high bioavailability and safety associated after administration inside the body (less toxic). They are artificially prepared vesicles of lipid bilayer and were first discovered by Alec D Bangham a British haematologist in 1961 [10]. Nanoliposomes are of size 30–100 nm and are formed by phospholipids, such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol and others as cholesterol self-organization [12]. They are formed of aqueous solution, and capable to carry hydrophobic and hydrophilic molecules [11]. Hydrophobic can be carried by embedding into the bilayer that is between the two layers of bilayer and hydrophilic by encapsulating in liposome core which is aqueous cavity. These liposomes are mixed in solution with the nucleic acid in the required ratio as they are cationic so can easily encapsulate negatively charged nucleic acid [4]. They are often layered with diffusible PEG-lipid conjugate which shields the cationic liposome making it stable or transforming into neutral liposome against systematic clearance *in vivo.* When liposomes are used in transformation and transfection of DNA it is known as lipofection [10]. These nanoliposomes can be engineered with N-acetylgalactosamine whose receptors are present in liver and make it easily accessible to liver cells in case of liver cancer even the pore size of liver endothelium is 100nm so it is easy for 70-80nm liposomes to gain entry inside it [16]. They can be directed through targeting ligands and then they can diffuse with cells lipid bilayer or endocytosis to deliver the required nucleic acid [16]. Some use antibodies or their fragments for targeting them to the specific cell type [18, 19]. The siRNA can also perform supportive work for the treatment of cancer, as through these liposomes many drugs can be delivered and the role of siRNA can be in increasing the efficacy of these drugs, by silencing the translation of such proteins which can interrupt in smooth attacking by the drug for example, doxorubicin delivered with MDR-1 siRNA, this siRNA suppress the P-glycoprotein expression thus enhance the ability of doxorubicin in the cancer cells which are multi drug resistance [18, 19]. In this way RNAi can also support other treatments of cancer like chemotherapy.

CONCLUSION

Though various developments in therapeutics for cancer are in battle field, RNAi has proved itself a powerful weapon against cancer by its wide scope to cure all types of cancer and researcher are expecting great improvement in potentiality of this therapy in coming future. Already the researches are assuring great potential for clinical application of RNAi in silencing cancer concerned genes and its ability to promote sensitivity of cancer cells to chemotherapy. Most of the delivery methods described above as adenovirus, atelocollagen and nanoliposomes have proved themselves quite efficient, safe and specific *in vitro*. Various other delivery methods for siRNA are being discovered and many are under examination to be used in vivo. Thus RNAi is having great promising future as a therapeutic for cancer and will soon land up in clinics for improving life of cancer patients.

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