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Review Article

ONCMIRS: FROM BENCH TO BED SIDE

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ABSTRACT

The biomedical research community now focuses its attention to miRNA due to its significant advantages when compared to siRNA and they are believed to be emergent class of therapeutant. Innumerable evidences support the remarkable link between miRNA and cancer. Hence introducing oncomiRs as therapeutant is certainly an important milestone in cancer therapy. MiRNA replacement therapy and miRNA antagonists have been successful in basic research. But the main drawback to actualize these Superdrug's is the delivery systems. For miRNA based therapeutics several pre- clinical and clinical based trials were initiated and in this review the status of miRNAs in pre- clinical and clinical trials are described. This review focusses on the journey of miRNA through various phases. In its initial phase it describes the link between miRNA and cancer as well as highlighting its role in cancer stem cells. The journey continues by narrating its introduction to therapeutic field especially the two main strategies miRNA antagonists and mimics with special emphasis on the pharmacological property of miRNA. The journey reaches its destination by introducing the various delivery systems for effective transport of miRNA to target cells and moreover discussing the miRNAs under clinical trials and the various pharmaceutical companies involved in it and furthermore opening new dimensions to small RNA biology.

Keywords: antagonists, replacement therapy, cancer stem cells, Delivery Systems, therapeutic miRNA.

INTRODUCTION

Small non coding RNAs of about ~22 nucleotides long are called microRNA (miRNA), which are cleaved from its hairpin precursors of about 70 to 100 nucleotides length by a complex protein machinery including drosha, pasha, exportin-5, dicer and RNA-induced silencing complex [1]. Since its discovery in *Caenorhabditis elegans* [2], it was found in a variety of organisms including humans. The *let-7* miRNA was the first to be identified in humans and they are found to be highly conserved [3]. The endogenous miRNA could cause gene silencing [2,3,4,5,6] in all the organisms. It has been estimated that the total number of miRNA identified in human exceeds 1500 [7]. It plays a significant role in gene regulation in health and diseases by targeting the specific mRNA [8]. The *Lin-4* miRNA was the first to validate its regulatory role by repressing the Lin-14 translation [2,4]. In human genome, it was found that about 1- 4% of genes are miRNA and the more surprising fact is a single miRNA could regulate nearly 200 genes. Moreover it could regulate nearly 30% of the human protein coding genes [9]. It has a remarkable role in processes such as cell growth, cell proliferation, cell differentiation, embryonic development, apoptosis, cellular signaling network, cross-species gene expression variation and co- regulation with transcription factors [10, 11, 12, 13]. With a vast array of regulatory functions it contributes critically to ~70 diseases including different forms of cancer, asthma, cholestetoma, spinal cord injuries, heart failure, periodontitis, inflammation, lens opacity, muscular disorders, endometriosis, lupus vulgaris, Crohn disease, cardiomegaly [14]. Moreover, its significance in various other diseases is yet to be identified. Probing the function and therapeutic significance of miRNA in all these diseases is beyond the scope of this review. As a consequence, its potential as a therapeutant in cancer was detailed. Those miRNA which contributes its regulatory role in cancer was termed as OncmiRs and it shows differential expression levels in tumor cells [10]. Our analysis on PubMed data, shown in Figure: 1 indicates an alarming increase in miRNA related research in cancer. It was found that not only in solid tumors it shows apparent expression in blood- borne malignancies also [15]. Distinctive pattern of miRNA expression were found in samples from different cancer types. Intriguingly such miRNA expression could serve as prognostic markers [10, 15]. The introduction of a single miRNA against its target oncogene can effectively contribute to tumor regression and it has been proved both *in vitro* and *in vivo* [16]. This undoubtedly reveals the therapeutic value of oncmiRs in cancer.

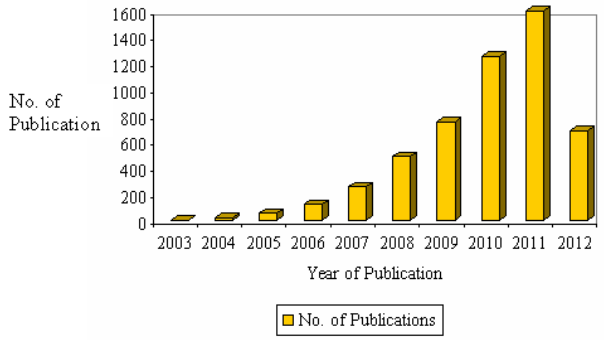

Figure 1: Publication since June 2012 relating miRNA and Cancer*

***** Key word used for search is microRNA+ miRNA+ tumor+ cancer

JOURNEY BEGINS: ONCMIRS IN CANCER

Evidence of oncmiR in cancer

The remarkable discovery happened in the year 2002 [17] which reported for the first time the link between miRNAs in human cancer. The chromosome 13q14 was the most frequently deleted chromosoml region in most of the B cell chronic lymphocytic leukemias (B-CLL). This region was suspected to have a connection with the progression of leukemiasis. While studying this region, surprisingly Calin and colleagues found two miRNAs: miR- 15 and miR-16 which are located exactly in this deleted chromosomal portion of about 30 kb. Moreover they concluded that in about ~68% of CLL, miR-15 and miR-16 genes are either deleted or down-regulated clearly showing its function in leukemiasis. Apparently its contribution in several cancer such as breast cancer, basal cell carcinoma, Burkitt lymphoma, renal cell carcinoma, squamous cell carcinoma, lung cancer, liver cancer and so on [14] was revealed. However till 2005 the mechanisms behind its regulatory function are not known and later several independent studies [18,19,20] provides attention to it. One of the studies, in lung tumor cells regarding Let-7 family revealed it negatively regulates let-60/RAS and it in turn suppresses Let-7. It was found that 3' UTR of let-60/RAS contains multiple Let-7 complementary sites which restrict reporter gene expression in a let-7-dependent manner [18]. c- MYC, a proto-oncogene encodes a transcription factor which regulates various cellular processes and found to be associated with abnormalities in

human malignancy. c- MYC also targets E2F1, a transcription factor associated with cell proliferation. It was found that the two miRNA clusters: miR-17-5p and miR-20a negatively regulates the expression of E2F1. This shows that regulated miRNAs in c- MYC targeted gene network could transfer proliferative signals [19]. In another study, mir-17-92 polycistron located region of chromosome is frequently amplified in human B- cell lymphomas. Moreover in a mouse B-cell lymphoma model, higher expression of mir-17-92 along with c- MYC expression to speed up tumor development was found. Together these indicate specific miRNAs could modulate tumor progression [20]. As in Figure: 2 miRNAs could involve in various processes of cancer development such as cell proliferation, cancer metastasis, tumor vascularization, apoptosis as well as in modulating the tumor environment [21]. Such global regulation of miRNA in cancer prone the scientific community to probe its importance in the clinical level.

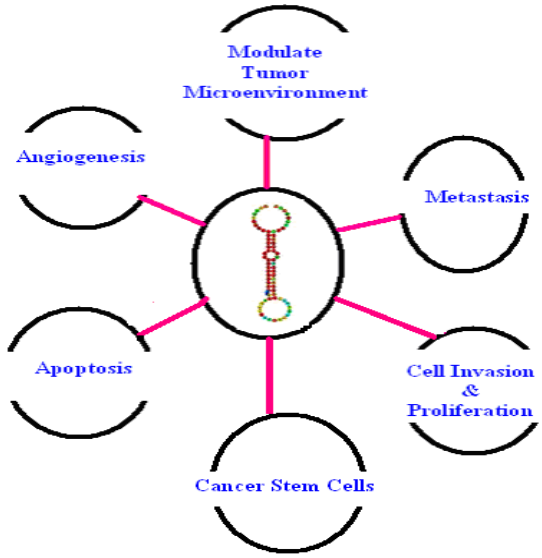

Figure 2: miRNAs and its role in cancer

An interesting feature of miRNA was disclosed when its critical role in Cancer Stem Cells (CSC) was found. A group of scientists considers CSC as the root of cancer cause and re-occurrence, and the hypothesis were further proved through *in vivo* and *in vitro* experiments [22]. In an immunodeficient mouse the self-renewal of such cells could promote tumor was proved [23]. The CSCs present in solid tumors including head and neck, small intestine, stomach, colon, brain, pancreas, breast, liver, prostate, ovary and bladder have been identified using either CD44, an adhesion molecule, or in combination with other markers [24]. The self- renewal and differentiation are the key characteristics of CSC in spite the mechanisms behind it are complicated. Protein coding genes are regularly haunted previously to prove this mechanism but once the role of miRNA in cancer becomes clear, scientists turned their attention towards it. Evidences indicate that huge numbers of miRNAs are associated with CSC [25]. A study showed that nearly 37 miRNAs were found to be differentially expressed in human breast cancer stem cell (BCSC) [26] and moreover, miR-200c-141, miR-200b-200a-429, and miR-183-96-182 clusters were found down regulated in BCSC. A usual regulator of stem cell self- renewal was BMI1and its expression was modulated by miR- 200c. It was also found that down- regulation of this miRNA could link BCSC with normal stem cells. It was already found that the prostate cancer stem cells with high tumor inducing and metastasis capabilities are with increased content of CD44. But the miRNAs role in CD44 prostate cancer stem cell metastasis was unclear. A recent report [24] state that miR-34a, a p53 target was under expressed in CD44 prostate stem cells and the enhancer expression of this miRNA could inhibit cell proliferation, metastasis and tumor regeneration. In vivo studies in mice model which suffers from prostate tumors, after systemic delivery of miR-34a, shows surprising result of huge reduction in metastasis and increased survival. Hence the study concluded that miR-34a as the key negative regulator of CD44 prostate cells and confirms it as a perfect therapeutic candidate for prostate cancer stem cells.

Side Populations (SP) are considered as a sub- population of cells that distinguishes it from the main population of cells by markers. Usually these cells are identified using flow cytometry technique. Interesting these cells is considered to possess stem- cell like biological property including self- renewal, tumor forming capacity and chemoresistance [27]. A comparative analysis on SP and non- SP cells by microarray shows that miR-21 and AP1 are frequently up-regulated in SP cells. Inhibition of miR- 21 by anti-miR-21 locked nucleic acid increased the drug sensitivity and decreased colony forming ability. This shows the remarkable role of it in chemoresistant phenotype of SP cells and provides new vision for therapy [28]. There are insufficient studies relating colorectal (CRC) cancer and SPs. In a study on colorectal cancer, the SPs were identified from CRC cell lines and primary cell cultures and microarray analysis was performed. A significant reduction of miR-328 expressions was noticed in SP cells when compared with non-SP cells. Intriguingly, functional studies shows there are significant changes in number of SP cells in correlation with expression of miR-328. Moreover over expression of miR- 328 inhibited the cell invasion as well as drug resistance property of SPS. Even the two direct targets of miR- 328: ABCG2 and MMP16 were also found in CRC, indicating it as a potential target for therapy [29]. All these instances certainly prove the link between cancer and miRNA and more over it could be a better candidate as therapeutant. Targeting such malignant cancer stem cells makes a critical challenge in cancer research due to its property of drug resistance.

JOURNEY CONTINUED: ONCMIRS ENTRY IN TO THERAPY

Strategy for using OncmiRs in therapy

Intriguingly, the role of miRNA as an oncogene as well as tumor suppressor are utilized in cancer therapy. Few of the tumor suppressor miRNA include Let-7 [30], miR-26a [31], miR-16-1[32], miR-34 [33]. Analysis of available cancer associated miRNA by Lu and team found that miR- 195 is down regulated in all the reported cancers [14]. Even though it is practically pending, gene therapy approaches had been implied for tumor suppressor genes which are protein coding. Eventually this creates an idea of using tumor suppressor miRNA as a candidate for cancer therapy [34]. It was widely accepted that the probability of miRNAs as oncogene is very less when compared to the probability of it as tumor suppressors. Because in comparison with normal cells, the most differentially expressed miRNAs are suppressed in majority of tumor cells [35]. More over another study proved that the global repression of miRNAs could enhance tumorigenesis [36]. One of the strategy of using miRNA in therapy aims to inhibit the one which are up- regulated in tumor cells. This is possible through the introduction of anti-miRs, locked-nucleic acids (LNA), or antagomiRs. The second is through reintroduction of tumor suppressor miRNA mimic which renew a loss of function, miRNA replacement [37,38,39,40](Table: 1). Among the two the inhibitory method is widely accepted since similar to siRNA based approach. But the other paves a challenging opportunity to explore the tumor suppressive property in therapy. Either method can be chosen depending on the miRNA function and the type of cancer.

Antagonists and Mimics

Antagonists are small single stranded miRNAs which inhibits the miRNAs with gain of function in cancer. Its concept is similar to the inhibitory approach of siRNA and other small molecules. They are foreign molecules of ~7 kDa and could be delivered either locally or systematically [41]. The oligonucleotide antagonists are chemically modified, cholesterol conjugated moreover complementary to miRNAs. A study [42] published in Nature journal showed that they are specific as well as efficient in silencing endogenous miRNA in mice. Two types of antagonists are available: synthetic and expressed inhibitors. Synthetic inhibitors are generally a non- hydrolysable, single stranded reverse complement to the mature miRNA. The mechanism of inhibition is likely mediated by irreversible binding of the inhibitor to mature microRNA- loaded RISC, thus preventing interaction of the mature microRNA to its endogenous targets. Expressed inhibitors are sometimes referred to as miRNA sponges

are generally artificial miRNA constructs with multiple miRNA sites that compete with natural miRNA target for binding of miRNAs. A combination of these approaches and tools in loss- of- function assays increase the likelihood of observing the otherwise subtle phenotypes that often associated with miRNA inhibition. Different antagomirs including antagomirs- 16, antagomirs- 122, antagomirs- 192, antagomirs 194 were shown to decrease the level of corresponding miRNAs in various organs including liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries and adrenals when injected intravenously. Detailed analysis of liver specific miR- 122 through gene expression and bioinformatics studies reveals more about its functional annotation. It was clear that cholesterol biosynthesis gene is affected by the differential expression of miR- 122. Hence showing its therapeutic strategy to silence specific miRNA in diseases [42]. In general antagomirs target single miRNA product similar to the small molecular ligands by introducing a passenger strand named anti-miR which specifically binds to active miRNA forming a duplex. The RISC compound now unable to process this double stranded new miRNA duplex leading to duplex degradation.

Similar to antagonists, mimics are short single stranded miRNAs which restores the miRNAs showing loss of function in human cancer. They are ~15 kDa and could be delivered either locally or systematically to the target cell. MiRNA replacement therapy is the other term for mimics, which is based on a concept that the re-introduction of miRNA which is eliminated in cancer can be restored thus activating cellular pathway leading to suppress tumor [41]. Two types of miRNA mimics are used to conduct gain- of- function experiments: synthetic mimics and overexpression constructs. Synthetic miRNA mimics are chemically synthesized double stranded RNA molecules that are intended to mimic the transient duplexed product of dicer complex processing. Like naturally occurring miRNA, the two strands of synthetic mimics separate, the single stranded mature miRNA is incorporated in to RISC and thereby regulates down regulation of transcript levels. Synthetic miRNA mimics require delivery in to cells via transfection. Over-expression constructs are plasmids that encode native microRNA sequences to achieve exogenously introduced miRNA expression. Similar to shRNA constructs, plasmid DNA can be directly transfected in to the cells , or plasmid vectors can be engineered to encode miRNA sequences in the in the context of a viral backbone. Thus, by introducing these molecular mimics in to a cell type of interest one can enhance or supplement endogenous microRNA activity. In contrast to siRNA, miRNA mimics could target multiple miRNA and moreover the sequence of mimics is similar to that of endogenous miRNA. Most of the available miRNA therapy concentrates on gain- of- function whereas nowadays companies including MiRNA therapeutics are concentrating on replacement therapy. Despite the presence of miRNA in normal cells the introduction of mimics may not cause any adverse effects in the pathways as it was already controlled by the endogenous miRNA with the similar sequence.

Table 1: Antagonists versus Mimics

	Antagonists	**Mimics**
Molecular Weight	~7 kDa	~15 kDa
Configuration	Short single stranded miRNAs	Short single stranded miRNAs
Status of drug target	Gain of function	Loss of function
Body Recognizes	Foreign	Native
Therapeutic Application	Inhibition	Replacement
Mode of Delivery	Locally/Systemically	Locally/Systemically
Endogenous processing in to final therapeutic	None	RISC
Structure of active pharmaceutical ingredient	short ss RNA	short ss RNA

Pharmacological Property of OncmiRs

Pharmacological property of miRNA includes stability, size, cellular delivery and tissue availability. As per World Health Organization (WHO) the definition for Active Pharmaceutical Ingredient (API) is "A substance used in a finished pharmaceutical product (FPP), intended to furnish pharmacological activity or to otherwise have direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease, or to have direct effect in restoring, correcting or modifying physiological functions in human beings". In accordance with miRNA development of API includes developing oligonucleotides with favourable pharmacological and pharmacokinetic properties. Antisense oligonucleotides that are partially or completely complementary to specific miRNA sequences were considered for formation of miRNA antagonists. 2'-O-methyl, 2'-O-methoxy ethyl and locked nucleic acids were the nucleotide analogs which are incorporated in to the antisense molecules to ensure prevention of miRNAs from interacting with mRNAs subsequently selective hybridization with endogenous miRNA [43]. These chemical modifications in which the 2'-*O*-oxygen is bridged to the 4' position via a methylene linker to form a rigid bicycle, locked into a C3'-endo (RNA) sugar conformation. Another chemical modification applied to enhance oligonucleotide stability is the balance between phosphodiester and phosphorothioate linkages between the nucleotides, with phosphorothioate providing more stability to the oligonucleotide and making it more resistant to nucleases. The 2'-*O*-methyl group modification is used most often to improve nuclease resistance and improve binding affinity to RNA compared with unmodified sequences. Optimised length and chemical composition of antisense oligonucleotides ensures improved circulation time, cellular internalization and activity.

In case of miRNA mimics, its activity can be mimicked using a synthetic, single-stranded RNA molecule that contains the same sequence and chemistry as the mature endogenous miRNA. The potency of single stranded miRNA mimics is comparatively far less and hence double stranded miRNA mimics are preferred. Here, the passenger strand either makes perfect complementary to the mature miRNA or it can include mismatches similar to the precursor's form that is produced naturally. In order to improve the half- life and activities of miRNA mimics was possible through a variety of sugar and phosphate modifications can be incorporated in both the active and passenger strand, including 2'-O-methyl, 2'F, 2'NH2, 2'H, phosphorothioates and locked nucleic acids. Terminal modifications such as inverted bases, biotin, alkyl groups and others can be added in the passenger strand without negatively affecting the activity of the miRNA mimic. Often the passenger strand is typically linked to a molecule such as cholesterol for enhanced cellular uptake [44]. However, it should be noted that although this method would replace the miRNA levels lost during disease progression, it will also result in the uptake by tissues that do not normally express the miRNA of interest, resulting in potential off target effects. Regarding delivery of miRNA, the size of miRNA is less when compared to the size of plasmid vectors hence comparatively they are easy to deliver and manipulate. In case of cellular delivery the following characteristics should be taken to be account. The prime characteristics of delivery of small RNA include biocompatible, biodegradable and nonimmunogenic. Secondly, the delivery system should protect the active small RNA product from attack by serum nucleases. As the next step, its target tissue specific distribution after systemic delivery should avoid rapid hepatic or renal clearance. Finally, after successful delivery in to target cells through endocytosis, the system should promote the endosomal release of its miRNA content in to the cytoplasm allowing the interaction of the small RNA with the endogenous RNA Inducing Silencing Complex. In addition, aberrant miRNA expression was showed in almost all the tumors investigated either it is solid or the fluid filled. Such abnormal expression could help to classify tumor prognosis as well as its response to therapy. Furthermore it can contribute significantly to the signalling pathway of oncogenes and tumor suppressor genes controlled by p53 and c-Myc [19,45]. Specific miRNAs

are shown to be contributing significantly in terms of functional for cellular transformation and tumorigenesis.

JOURNEY TO THE DESTINATION: ONCMIRS AS THERAPEUTANT

Delivery of OncmiRs to target region

There are two strategies of in vivo delivery named localized delivery and systemic delivery. As the name suggests, localized means directly injecting in to the target areas including: intratumoral, intraretinal, intramuscular and it produce a lesser biodistribution. As a contrary systemic means injection in to systemic circulatory system and provides a widespread biodistribution [46]. Systemic delivery of miRNAs in to target tissues are really challenging because of the small size of the therapeutic RNA nearly 7- 20 kDa. Molecules less than 50 kDa if found in the blood stream are usually filtered of by the kidney. Hence while transferring a therapeutic miRNA through blood due to its small size, it cannot pass through capillary endothelium and retain in circulatory blood, which is being filtered off by kidney and subsequently excreted [47]. Moreover the macrophages, monocytes, phagocytic immune cells and all could invade them once in the blood. If luckily it escapes and cross the cell membrane, the endosomal vesicles may destroy them. Through localized delivery its bioavailability can be increased to target tissues including tumor, skin and eyes. Such delivery minimizes its uptake to a non- target normal tissues. Liver cells can accept molecules up to 200nm and hence remains as a perfect target site for therapeutic miRNA delivery. In case, local delivery is not possible many alternative delivery systems are available.

Physical Delivery

Like siRNA, one among the strategy of introducing miRNA in to cells and tissues are through physical methods. This method could avoid the possible non- specific immune stimulations, which may occur in some chemical delivery methods. Among the physical methods electroporation is the most widely implied one. It increases the electrical conductivity and permeability of the cellular plasma membrane by an externally applied electric field. They are relatively cytotoxic; the electrical impulse that induces pore formation in cellular membranes could kill a higher percentage of cells. Nowadays companies introduced specialized consumable pipette tip instead of the standard electroporation cuvette which results in higher cellular viability. For *in vivo* delivery, the optimization of pulse conditions and the reduction of cell damages in pulse- applied sites are required.

PEGylated liposome

They are 50- 500 nm molecules with an aqueous core which contains miRNA [47]. They are composite structures made of phospholipids and it can encapsulate various kinds of nucleic acids. The cationic lipids in them mimic physical characteristics of natural phospholipid bilayer of the cellular membrane hence forming a synthetic analogue. The success of liposome depends on the lipid formulation, charge ratio, particle size and method of its preparation. It provides protection due to efficient miRNA- liposome encapsulation. It produces less innate immunity response and low cytotoxicity due to biodegradable PEG (Polyethylene Glycol) modification. PEG allows longer circulatory life.

Lipoplexes

It was introduced to improve the delivery of nucleic acids to target cells. Usually there are three types of lipids: anionic, neutral and cationic. The cationic properties of lipids are utilized for the formation of lipoplexes. The positively charged cationic lipids can easily bind with negatively charged nucleic acids. Moreover it can bind with the negatively charged cell membranes. The liposomes composition facilitates it to bind with nuclear membrane, endosomal and cytoplasmic membrane and moreover it can be released in to cells through endosomes [47]. In a recent report [48] in lung cancer cells cationic lipoplexes were used. miR-133b was one among the tumor suppressor miRNA and it was selected as a therapeutic target because it target MCL- 1 gene. It was found that lipoplexes delivered pre-miR-133b in an efficient manner compared to other transfection agent used for the study. Moreover the production of mature miR-133b was ~52 fold higher in mice treated with pre-miR-133b containing lipoplexes when compared to untreated mice showing lipoplexes as best carrier system for miRNA based therapeutics in lung cancer.

Neutral Lipid Emulsion

Neutral Lipid Emulsion (NLE) has a unique composition based on neutral lipids and hence it overcomes some of the problem related to charged lipids. It enables efficient delivery systemically and less harm in composition to cationic lipid delivery systems. It was found that *in vitro* siRNA delivery using liposome with higher proportion of neutral lipids leads to less cellular toxicity without compromising much of its ability to down regulate gene expression. Still a thorough understanding of the best delivery systems for small RNA is under research, DOPC (1, 2-dioleoyl-sn-glycero-3-phosphatidylcholine) evolves as one of the best delivery systems. It allows an efficient uptake of small RNA in to it, further uptake of liposome in to cell and subsequent breakdown of intracellular liposomes with the release of its contents in to cellular cytoplasm. Since it is neutral it can overcome the challenges due to charge such as forming aggregates in biofluids, be filtered by the liver, adhere to the endothelium, or to be taken up by scavenging macrophages. In addition it proves to be less toxic in mice models by showing no elevated liver and kidney enzymes in serum and also did not induce non- specific immune response [49]. A study [50]using NLE as delivery system for miRNA mimics was performed in mouse model for lung cancer. miR- 34a and *let-7* mimics were used for the study and the neutral lipids shows efficient delivery.

Polyethylenimine (PEI)

The method once extensively used for plasmid vector delivery has now been used for small RNA delivery which usually involves a cationic moiety. Cationic polymers usually fall in to either synthetic or natural polymers. Polyethylenimine (PEI) is synthetic polymers which is used extensively and have a wide range of molecular weights and many protonable amino acids leading to high cationic charge density at physiological pH.

Viral vectors

The viral life cycle could be divided in to two phases: lytic and lysogenic. The lytic phase is the mode of replication of virus where the destruction of host cell occurs. The latter is utilized in gene therapy methods and this non- invasive infection that introduces functional genetic information in to the target cell is termed as transduction. Viral genes include coding genes (trans) and the regulatory cis- acting regions. Although some overlap exists, most cis acting sequences map outside of the viral coding sequences. This spatial segregation is exploited in designing the recombinant viral vectors. The therapeutic nucleic acid is always linked to the cis region. Maintaining separation of viral trans genes and cis regulatory elements will determine the efficiency and safety of a viral vector system. Retroviruses, lentiviruses, adenoviruses, adeno- associated viruses are generally considered as viral vector systems for enabling high levels of trans gene expression [51]. Adeno- associated virus (AAV) derived gene delivery systems could package single-stranded DNA genome. It has transduction properties including increased efficiency and independence from targeted cell DNA synthesis [52]. In a report on miRNA replacement therapy, adeno-associated viruses were used as delivery agent [40].

Aptamers

They are short stranded DNA or RNA molecules that have the ability to fold into complex three dimensional structures which bind to target molecules with high affinity and specificity [53]. SELEX (Systemic Evolution of Ligands by Exponential Enrichment) is the method by which aptamers are selected to bind to a particular target from a combinatorial library of synthetic oligonucleotides consisting of a pool of single stranded DNA fragments with enormous repertoire and functionality. RNA aptamers provide much higher level of structural diversity when compared to DNA aptamers. In a therapeutic point of view aptamers possess both advantages and disadvantages. In *in vitro,* as small molecules with non-modified nucleic acids, they have a half-life of minutes to hours due to

nuclease degradation and can be cleared rapidly by the kidneys. However, these benefits become disadvantages when considering their use as in vivo therapeutic agents, due to their inherent instability. In a recent report RNA aptamer A10 – 3.2, a second generation RNA aptamer was found to be more effective to deliver miRNAs such as miR-15a and miR-16-1 to prostate cancer cells [48]. As a vehicle for miRNA target delivery, this aptamer uses polyethyleneglycol (PEG) as a spacer and PAMAM (polyamidoamine) was conjugated to aptamer (PAMAM-PEG-APT).

Nanoparticles

Nano sized carrier molecules possess important technological advancements in the scenario of delivery vehicles. They show high stability, high carrier capacity, possible to incorporate both hydrophilic and hydrobhobic substances and the likelihood of various administration routes [54]. Since the gold nanoparticle means of delivery was successful for siRNA delivery it can be used for miRNA also. It can protect the nucleic acid from RNases. Crew *et al*., [55] demonstrated that in multiple myeloma cells, thiol-functionalized miRNAs on gold nanoparticles shows efficient knockdown in the functional luciferase assay. Even though further studies in animal models need to validated, previous studies with siRNA and gold nanoparticles proves they are effective delivery systems.

Dendrimers

Dendrimers have some unique properties because of their globular shape and the presence of internal cavities. They consist of three major architectural complexes: core, branches and end groups. The most important one is the possibility to encapsulate guest molecules in the macromolecule interior. The dendrimers are usually prepared by using divergent or convergent method. Dendrimers have some unique properties because of their globular shape and the presence of internal cavities. The most important one is the possibility to encapsulate guest molecules in the macromolecule interior. PMAM dendrimers are of considerable importance because it is a reliable and efficient carrier system. The terminal amine groups of PAMAM dendrimers can be modified with different functionalities and can be linked with various biomolecules. In order to co- deliver antisense-miR- 21 (as-miR-21) oligonucleotides and 5- fluorouracil (5-FU) to achieve delivery of as-miR-21 to human glioblastoma cells and enhance the cytotoxicity of 5-FU antisense therapy PAMAM dendrimers were used [56].

Localized delivery system such as intratumoral is not successful for a metastatic tumor. Hence choosing the appropriate delivery system is necessary. Most of the delivery systems support basic research but on the other hand for commercialization and for treatment purpose in depth research should be performed.

OncmiRs under therapeutic Development

Pre-clinical development is a stage under drug development that begins before clinical trials and one of its main goals being determining the product's ultimate safety profile before conducting human studies by characterizing the pharmacokinetic profile, to identify potential adverse drug effects and to define a safety margin for human clinical studies. Both *in vivo* and *in vitro* test were conducted. Even though nowadays animal testing has been reduced due to ethical and cost reasons, researchers still need them for safety testing. Choice of the animals for various experiments depend on which will best correlate to human trials. Since miRNAs are chemically synthesized, they are reviewed under the guidelines of small molecules. One of the advantages of using miRNA in clinical diagnosis is its high stability. They are highly stable *in vitro* and long lived *in vivo* [57,58].

Table3: Few Pharmaceutical Companies involved in small non coding RNA drug development

Cancer type	Company	web address
Breast cancer	City of Hope Medical Center	http://www.cityofhope.org/Pages/default.aspx
Skin Cancer	Ruhr-University Bochum, Germany	http://www.ruhr-uni-bochum.de/index_en.htm
Renal cell carcinoma	The 1st Affiliated Hospital, Sun Yet-sen University, China	http://eng.sysu.edu.cn/medical/affilatedhospitals/thefirstaffiliatedhospital/index.htm
Prostate cancer	Mirna Therapeutics	http://www.mirnarx.com/
Naevi malignant melanoma	Ruhr-University Bochum, Germany	http://www.ruhr-uni- bochum.de/index_en.htm
Melanoma	Rigshospitalet, Denmark	http://www.rigshospitalet.dk/menu/
Lungs and non-small cell cancer	Mirna Therapeutic	http://www.mirnarx.com/
Cancer and liver infection	Rosetta Genomics	http://www.rosettagenomics.com/
Cancer, acute leukemia	Mirna Therapeutic	http://www.mirnarx.com/myelogenous
Leukemia	National Cancer Institute	http://www.cancer.gov/

Table: 4 Institutes focusing on pre- clinical and clinical trial of OncmiRs

Company	Delivery Systems	Clinical Trial
Alnylam Pharmaceuticals	SNALP	Phase I Clinical trial
Alnylam Pharmaceuticals	ALN-RSVO1	Phase II Clinical trial
Transderm	TD101	Phase I Clinical trial
Opko Health's	Bevasiranib(naked siRNA)	Phase III Clinical trial
Santaris Pharma	SPC3649 LNA based antisense molecule targetting miR-122	Phase I Clinical trial
Silence Therapeutics	siRNA-lipoplex technology	Phase I Clinical trial
miRNA Therapeutics	SMARTICLES oligonucleotide delivery technology	Phase I Clinical trial

There are various companies globally undergoing research in miRNA drug development most of them utilizes the antagomir technology. While the miRNA Therapeutic took a daring step ahead, they utilize the miRNA mimics by replacing the tumor suppressor miRNA. Most of the companies are using the delivery systems that are either approved for clinical trials or those that are already in the clinic. Apart from cancer, few miRNAs are under clinical trial for other diseases including miR- 122 (Hepatitis C Virus), miR- 208/499 (Chronic heart failure) and miR- 195 (Post-myocardial infarction remodelling). The miRNA mimics which were under pre- clinical development against cancer are miR- 34 and let- 7. The tumor suppressor miRNAs were shown to block tumor growth in a number of different pre- clinical animal studies [59, 60]. Proprietary miRNA, miR- 34, formulated with SMARTICLES oligonuleotide delivery system is called MRX01. The efficacy of MRX01 is proved from preclinical studies in mouse model of hepatocellular carcinoma. The replacement therapy works by replenishing the miRNA once it is lost or reduced. Because of their inefficient delivery or degradation, single dose of miRNA may not allow sustainable target regulation hence multiple doses are mandatory. Experiences with multiple doses of siRNA shows three to five doses of replacement miRNA could provide sufficient miRNA for about twenty to thirty days.

CONCLUSION

The discovery of miRNA has changed the fundamental concept of gene expression but *in vivo* function of miRNA is still hidden. The

impact of miRNA in therapy is readily visible as its acceptance as therapeutant under clinical trial since its discovery a decade ago. In addition the delivery methods of siRNA were already in hand, this strategy never proves to be a hindrance for miRNA therapy. Studies revealed that miRNA therapy is already a step ahead of siRNA therapy. Utilization of naturally occurring cellular nanoparticles which are involved in *in vivo* communications may overcome some of the challenges of delivery systems. Combination of miRNA replacement therapy with gene therapy may further enhance the chance to strengthen nucleic acid based therapeutants. Moreover the main challenges for commercializing miRNA antagonistics are due to highly expensive nature. There is an increased proportionality of growth and development in pharmaceutical related miRNA companies. But there are certain drawbacks faced by them including the lack of proper delivery systems, the level of miRNA in cell cultures and in tissues differs results in adverse effects *in vivo* treatment, multiple targets for a single miRNA and choosing the appropriate technology to modulate miRNA expression. Overcoming all these hurdles which were once faced by its therapeutic counterparts could result oncmiRs as a promising therapeutant.

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REFERENCES

1. Bartel, DP. MicroRNAs: Genomics, biogenesis, mechanism and function. Cell. 2004; 116: 281-97.
2. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* Heterochronic Gene lin-4 Encodes Small RNAs with Anitsense Complementary to lin-14. Cell.1993; 75: 843-854.
3. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Müller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E, Ruvkun G. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature. 2000; 408: 86-89.
4. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. Cell. 1993; 75: 855-862.
5. Olsen PH, Ambros V. The lin-4 regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. Dev. Biol. 1999; 216: 671-680.
6. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. Nature. 2000; 403: 901-906.
7. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res. 2006;34(Database issue):D140-4.
8. Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, Barzilai A, Einat P, Einav U, Meiri E, Sharon E, Spector Y, Bentwich Z. Identification of hundreds of conserved and nonconserved human microRNAs. Nat Genet. 2005;37(7):766-70.
9. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005;120(1):15–20
10. Esquela-Kerscher A, Slack FJ. Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer. 2006; 6: 259–269.
11. Cui Q, Yu Z, Purisima EO, Wang E. Principles of microRNA regulation of a human cellular signaling network. Mol Syst Biol. 2006; 2: 46.
12. Cui Q, Yu Z, Purisima EO, Wang E. MicroRNA regulation and interspecific variation of gene expression. Trends Genet. 2007a; 23: 372–375.
13. Cui Q, Yu Z, Pan Y, Purisima EO, Wang E. MicroRNAs preferentially target the genes with high transcriptional regulation complexity. Biochem Biophys Res Commun. 2007b; 352: 733–738.
14. Lu M, Zhang Q, Deng M, Miao J, Guo Y, Gao W, Cui Q. An analysis of human microRNA and disease associations. PLoS One. 2008;3(10):e3420.
15. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer. 2006;6(11):857-866.
16. Medina PP, Nolde M, Slack FJ. OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. Nature. 2010;467(7311):86-90.
17. Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM. Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci U S A. 2002;99(24):15524-9
18. Johnson SM, Grosshans H, Shingara J, Byrom M, Jarvis R, Cheng A, Labourier E, Reinert KL, Brown D, Slack FJ.RAS is regulated by the let-7 microRNA family. Cell. 2005;120(5):635-47.
19. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature. 2005;435(7043):839-43.
20. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ, Hammond SM. A microRNA polycistron as a potential human oncogene. Nature. 2005;435(7043):828-33.
21. Joseph Baby and Nair Vrundha M. OncmiRs: Small Noncoding RNA with Multifaceted Role in Cancer. Research Journal of Recent Sciences. 2012; 1(11), 70-76.
22. Wicha MS, Liu S, Dontu G. Cancer stem cells: an old idea – a paradigm shift. Cancer Res. 2006;6: 1883-1890.
23. Webb S. MicroRNA reins in tumor-initiating cells. Nature Reports Stem Cells. 2008. doi:10.1038/stemcells.2007.137.
24. Liu C, Kelnar K, Liu B, Chen X, Calhoun-Davis T, Li H, Patrawala L, Yan H, Jeter C, Honorio S, Wiggins JF, Bader AG, Fagin R, Brown D, Tang DG. The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44. Nat Med. 2011;17(2):211-5.
25. Xia M.H. P. Great Potential of MicroRNA in Cancer Stem Cell. Journal of Cancer Molecules. 2008; 4(3): 79-89.
26. Shimono Y, Zabala M, Cho RW, Lobo N, Dalerba P, Qian D, Diehn M, Liu H, Panula SP, Chiao E, Dirbas FM, Somlo G, Pera RA, Lao K, Clarke MF. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell. 2009;138(3):592-603.
27. Takubo K, Ohmura M, Azuma M, Nagamatsu G, Yamada W, Arai F, Hirao A, Suda T. Stem cell defects in ATM-deficient undifferentiated spermatogonia through DNA damage-induced cell-cycle arrest. Cell Stem Cell. 2008;2(2):170-82.
28. Misawa A, Katayama R, Koike S, Tomida A, Watanabe T, Fujita N. AP-1- Dependent miR-21 expression contributes to chemoresistance in cancer stem cell-like SP cells. Oncol Res. 2010;19(1):23-33.
29. Xu XT, Xu Q, Tong JL, Zhu MM, Nie F, Chen X, Xiao SD, Ran ZH. MicroRNA expression profiling identifies miR-328 regulates cancer stem cell-like SP cells in colorectal cancer. Br J Cancer. 2012;106(7):1320-30.
30. Trang P, Medina PP, Wiggins JF, Ruffino L, Kelnar K, Omotola M, Homer R, Brown D, Bader AG, Weidhaas JB, Slack FJ. Regression of murine lung tumors by the let-7 microRNA. Oncogene. 2010;29(11):1580–7.
31. Kota J, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, Chang TC, Vivekanandan P, Torbenson M, Clark KR, Mendell JR, Mendell JT. Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. Cell. 2009;137:1005–17.
32. Takeshita F, Patrawala L, Osaki M, Takahashi RU, Yamamoto Y, Kosaka N, Kawamata M, Kelnar K, Bader AG, Brown D, Ochiya T. Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors *via* downregulation of multiple cell-cycle genes Mol Ther. 2010; 18181–187
33. Wiggins JF, Ruffino L, Kelnar K, Omotola M, Patrawala L, Brown, Bader AG. Development of a lung cancer therapeutic based on the tumor suppressor microRNA-34 Cancer Res. 2010; 705923–5930.
34. Bader AG, Brown D, Winkler M. The Promise of MicroRNA Replacement Therapy. Cancer Res. 2010; 70:7027-7030

35. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. Nature. 2005;435:834–8.
36. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet. 2007;39(5):673-7.
37. Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, Wilson M, Wang X, Shelton J, Shingara J, Chin L, Brown D, Slack FJ. The let-7 micro- RNA represses cell proliferation pathways in human cells. Cancer Res. 2007;67:7713–22.
38. Esquela-Kerscher A, Trang P, Wiggins JF, Patrawala L, Cheng A, Ford L, Weidhaas JB, Brown D, Bader AG, Slack FJ. The let-7 microRNA reduces tumor growth in mouse models of lung cancer. Cell Cycle. 2008;7:759–64.
39. Youngster ST, Corey DR. Transcriptional gene silencing in mammalian cells by miRNA mimics that target gene promoters. Nucl. Acids. Res. 2011; 39(13):5682-91.
40. Chorn G, Klein-McDowell M, Zhao L, Saunders MA, Flanagan WM, Willingham AT, Lim LP. Single-stranded microRNA mimics. RNA. 2012; 18(10):1796- 804.
41. Bader AG, Brown, D Stoudemire J, Lammers P. Developing therapeutic microRNAs for cancer. Gene Therapy. 2011; 18, 1121–1126
42. Krutzfeldt J, Rajewsky N, Braich R, Rajeev K. G, Tuschl T, Manoharan M, Stoffel M. Silencing of microRNAs in vivo with 'antagomirs'. Nature. 2005; 438, 685–689.
43. Elmén J, Lindow M, Schütz S, Lawrence M, Petri A, Obad S, Lindholm M, Hedtjärn M, Hansen HF, Berger U, Gullans S, Kearney P, Sarnow P, Straarup EM, Kauppinen S. LNA-mediated microRNA silencing in non-human primates. Nature. 2008; 452: 896–899.
44. Petri A, Lindow M, Kauppinen S. MicroRNA silencing in primates: towards development of novel therapeutics. Cancer Res. 2009; 69: 393–395.
45. Chang TC, Wentzel EA, Kent OA, Ramachandran K, Mullendore M, Lee KH, Feldmann G, Yamakuchi M, Ferlito M, Lowenstein CJ, Arking DE, Beer MA, Maitra A, Mendell JT. Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. Mol. Cell. 2007; 26(5): 745–752.
46. Fletcher S, Honeyman K, Fall AM, Harding PL, Johnsen RD, Wilton SD. Dystrophin expression in the mdx mouse after localised and systemic administration of a morpholino antisense oligonucleotide. J Gene Med. 2006;8(2):207-16.
47. Broderick JA , Zamore PD. MicroRNA therapeutics. Gene Therapy. 2011; 18, 1104-1110.
48. Wu Y, Crawford M, Yu B, Mao Y, Nana-Sinkam SP, Lee LJ. MicroRNA delivery by cationic lipoplexes for lung cancer therapy. Mol Pharm. 2011;8(4):1381-9.
49. Landen CN Jr, Chavez-Reyes A, Bucana C, Schmandt R, Deavers MT, Lopez-Berestein G, Sood AK. Therapeutic EphA2 gene targeting *in vivo* using neutral liposomal small interfering RNA delivery. Cancer Res. 2005; 65: 6910–6918.
50. Trang P, Wiggins JF, Daige CL, Cho C, Omotola M, Brown D, Weidhas JB, Bader AG, Slack FJ. Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. Mol Ther. 2011;19(6):1116-22..
51. Mark AK, Joseph CG, Luigi N . Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics . Nature Medicine. 2001;7(1): 33-40.
52. McCarty DM, Fu H, Monahan PE, Toulson CE, Naik P, Samulski RJ. Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo. Gene Therapy. 2003; 10: 2112–2118.
53. Nimjee, SM, Rusconi CP, Sullenger BA. Aptamers: an emerging class of therapeutics. Ann. Rev. Med. 2005; 56: 555-83.
54. Gelperina S, Kisich K, Iseman MD, Heifets L. The potential advantages of nanoparticle drug delivery systems in chemotherapy of tuberculosis. Am J Respir Crit Care Med. 2005;172(12):1487-90.
55. Crew E, Rahman S, Razzak-Jaffar A, Mott D, Kamundi M, Yu G, Tchah N, Lee J, Bellavia M, Zhong CJ. MicroRNA conjugated gold nanoparticles and cell transfection. Anal Chem. 2012;84(1):26-9.
56. Zhou X, Yu Ren, Xubo Yuan, Peiyu Pu, Chunsheng Kang. PAMAM Dendrimer as Potential delivery System for Combined Chemotherapeutic and MicroRNA-21 Gene Therapy, Non-Viral Gene Therapy, Prof. Xubo Yuan (Ed.), 2011, InTech 499-514.
57. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature. 2005; 433:769–773.
58. Tang F, Hajkova P, Barton SC, Lao K, Surani MA. MicroRNA expression profiling of single whole embryonic stem cells. Nucleic Acids Res. 2006; 34:e9.
59. Craig VJ, Tzankov A, Flori M, Schmid CA, Bader AG, Müller A. Systemic microRNA 34a delivery induces apoptosis and abrogates growth of diffuse large B-cell lymphoma in vivo. Leukemia. 2012. doi: 10.1038/leu.2012.110.
60. Wu X, Ding B, Gao J, Wang H, Fan W, Wang X, Zhang W, Wang X, Ye L, Zhang M, Ding X, Liu J, Zhu Q, Gao S. Second-generation aptamer-conjugated PSMA-targeted delivery system for prostate cancer therapy. Int J Nanomedicine. 2011;6:1747-56.