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Exploring miRNA based approaches in cancer diagnostics and therapeutics

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ABSTRACT

MicroRNAs (miRNAs), a highly conserved class of tissue specific, small non-protein coding RNAs maintain cell homeostasis by negative gene regulation. Proper controlling of miRNA expression is required for a balanced physiological environment, as these small molecules influence almost every genetic pathway from cell cycle checkpoint, cell proliferation to apoptosis, with a wide range of target genes. Deregulation

Abbreviation: miRNA, microRNA; TRBP, Transactivating Response RNA-Binding Protein; RISC, RNA induced silencing complex; UTR, untranslated region; DDX, DEAD box protein; TS, tumor suppressor; OG, oncogene; TGF β, Transforming Growth Factor β; RT-PCR, Real Time-polymerase chain reaction; ER, Estrogen receptor; C13orf25, Chromosome 12 open reading frame 25; HSC, hematopoietic stem cell; VHL, Von-Hippel Lindau; HIF, Hypoxia Inducing Factors; CEA, carcinoembryonic antigen; AFP, alpha-fetoprotein; PSA, prostate specific antigen; USPTO, United States Patent and Trademark Office; EMT, Epithelial-to-Mesenchymal Transition; HPV, Human Papilloma Virus; THBS1, thrombospondin-1; HER-2, Human Epidermal Growth Factor-2; TGFβR2, Transforming Growth Factor B Receptor 2; DAPK1, Death-Associated Protein Kinase 1; APC, Adenomatous Polyposis Coli; NR5A2, Nuclear Receptor Subfamily 5, group A, member 2.

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in miRNAs expression correlates with various cancers by acting as tumor suppressors and oncogenes. Although promising therapies exist to control tumor development and progression, there is a lack of efficient diagnostic and therapeutic approaches for delineating various types of cancer. The molecularly different tumors can be differentiated by specific miRNA profiling as their phenotypic signatures, which can hence be exploited to surmount the diagnostic and therapeutic challenges. Present review discusses the involvement of miRNAs in oncogenesis with the analysis of patented research available on miRNAs.

1. Introduction

Uncontrolled proliferation of damaged cells, as a result of deregulation of genes involved in the cell cycle machinery and apoptosis, leads to tumor formation (Vecchione and Croce, 2010). Accounting for approximately 3% of the human genome, miRNAs are 22 nucleotides long, single stranded RNAs found in both plants and animals (Setoyama et al., 2011). The biogenesis of miRNAs is well defined in literature. Briefly, a stem-loop structure of pri-miRNA (primary miRNA) of about 1 Kb in size, transcribed by RNA polymerase II undergoes a two step maturation phase. Initially, the RNase III enzyme Drosha and ds-RNA binding endonuclease Pasha (DGCR8) process it into a 70 nucleotides pre-miRNA by cleaving both the strands and generating a stem loop structure (Krishnan et al., 2011; Zhiguo et al., 2008). The pre-miRNA is then transported to the cytoplasm with the help of a RAN GTP-dependent transporter exportin 5 for further processing by RNA III enzyme Dicer and ds-RNA binding protein TRBP (Transactivating Response RNA-Binding Protein), hence generating a 22 nucleotides miRNA: miRNA* duplex. The duplex is incorporated to the RISC (RNA induced silencing complex) complex wherein the mature miRNA strand is retained and the miRNA* fragment is degraded, guiding the RISC to the target mRNA molecule for gene regulation, which is a sequence complementarity dependent process. Perfect complementarity of the "seed region" with target mRNA results into its RISC associated degradation while imperfect matching leads to translation repression as 5′ end of miRNA binds to the 3′ UTR (untranslated region) of the target gene (Zhang et al., 2007; Xiangyang et al., 2008; Stefanie et al., 2008) [Fig. 1].

Miss-expression or mutation of factors involved in miRNA biogenesis could result into alterations in miRNA processing, stability, and targeting, hence causing serious ailments including cancers. The potential role of miRNAs in cancer is suggested owing to their involvement in the regulation of cell proliferation and apoptosis by controlling the expression of tumor suppressor genes and oncogenes. Additionally supporting this, about 50% of miRNAs are found to be located at "fragile sites" in the genome, which are the sites mostly amplified or deleted in cancer. Microarray, bead-based flow cytometry, sequencing and RT-PCR (Real Time-polymerase chain reaction) are some of the techniques used for analyzing the differential expression of genes in normal and cancerous cells, to elucidate the exact role of miRNAs in carcinogenesis (Monya, 2010). It has been reported that miR-10b, miR-125b and miR-145 are down-regulated while miR-21 and miR-155 are up-regulated in cancer development, hence playing roles as tumor suppressors and oncogenes, respectively (Thalia et al., 2011). Moreover, rearrangements in the gene regions containing miRNAs have been identified indicating their altered expression levels in the neoplastic cells. Their differential expression profiling thus gives information both on the differentiation state and developmental lineage of tumor, thereby paving way for cancer diagnosis and therapy (Niamh et al., 2009).

2. Role of miRNAs in cancer development

Studies suggest that miRNAs are majorly involved in the onset and progression of cancer. In lung cancer, the expression of dicer is found to be down-regulated, further decreasing the post-operative survival rate as a result of truncated miRNA maturation process (Ruan et al., 2009). Knock down studies with DICER, DGCR8 and TRBP2 genes shows enhanced tumor formation while re-introduction of these genes cause reduction in tumor growth. Genes from other pathways such as LIN28A block processing of let-7 resulting into cancer, are also involved in disrupting miRNA processing. Moreover, helicases DDX5 (DEAD-box protein 5) and DDX17 (DEAD-box protein 17) induce the processing of several tumor suppressor miRNAs by interacting with TP53 gene, wherein a mutation drives the miRNAs to remain in their immature state, blocking their tumor suppressor function (Suzuki et al., 2009). Defects in miR-21 biogenesis resulting in its over-expression, due to mutation in SMADs (an effector of TGFβ superfamily which carries out RNASEN-mediated miRNA maturation through interaction with DDX5 helicase), cause brain and pancreatic cancers (Nikitina et al., 2012).

miRNAs have differential expression in cancers related to different tissues, as a result of hundreds of targets affecting multitude of transcripts in cancer-related signaling pathways. Down-regulated miRNAs lead to an increased expression of oncomers, while those up-regulated cause suppression of tumor suppressor genes, indicating that miRNAs may act as both tumor suppressors and oncogenes. Some of the majorly studied miRNAs regulating cancer associated genes are discussed further [Fig. 2].

2.1. Oncomirs as tumor suppressors

2.1.1. miR-15 and miR-16

Homozygous loss in 13q14 region involving LEU1 and LEU2 genes is associated with the deletion of miR-15 and miR-16, which map within the introns of non-protein coding gene LEU2, is reported in more than half i.e. in about 68% of B-CLL (B-cell chronic lymphocytic leukemia) indicating their role as tumor suppressors associated with over 50% of mantle cell lymphoma, 16–40% of multiple myeloma and 60% of prostate cancers (Aqeilan et al., 2010). These miRNAs negatively regulate the anti-apoptotic gene BCL2, associated with various cancers. Therefore a loss in the activity of BCL2 regulatory miRNAs results into uninhibited proliferation of cells leading to cancer progression.

2.1.2. miR-142

miR-142 gene, located at chromosome 17, is also responsible for aggressive B-cell leukemia due to the *t*(8;17) translocation of MYC gene to its upstream strong promoter resulting into the loss of a conserved region of 20 nucleotides present downstream of miR-142 precursor, which hence disrupts miRNA processing leading to the up-regulation of MYC gene (Kwanhian et al., 2012).

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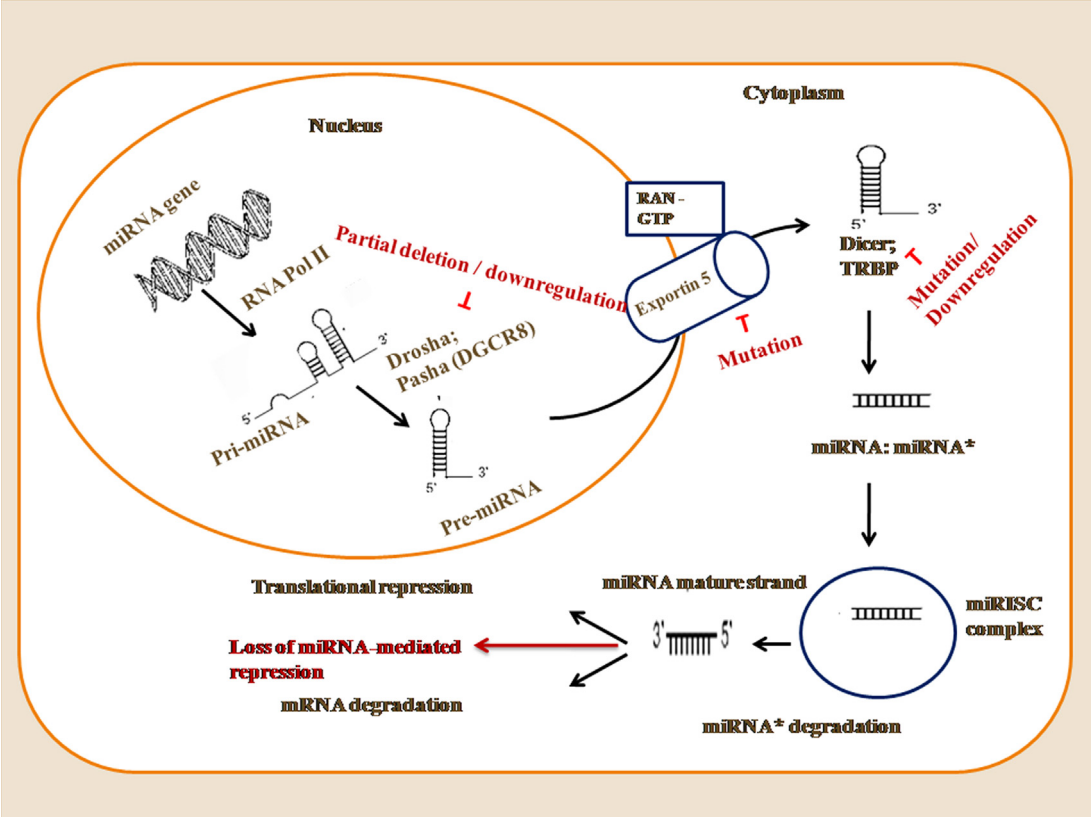

Fig. 1. Biogenesis of miRNA: depicting the events occurring in the nucleus and cytoplasm during the synthesis of an miRNA. In the nucleus, pre-miRNA is formed after the activity of Drosha and Pasha enzymes on pri-miRNA. Subsequently, pre-miRNA is exported to the cytoplasm by RNA-GTP associated exportin 5, where it is acted upon by Dicer and TRBP to generate a miRNA: miRNA* duplex structure. The role of RISC complex in miRNA biogenesis then comes into picture, when it degrades the unstable miRNA* strand and the mature strand is utilized further in gene regulation.

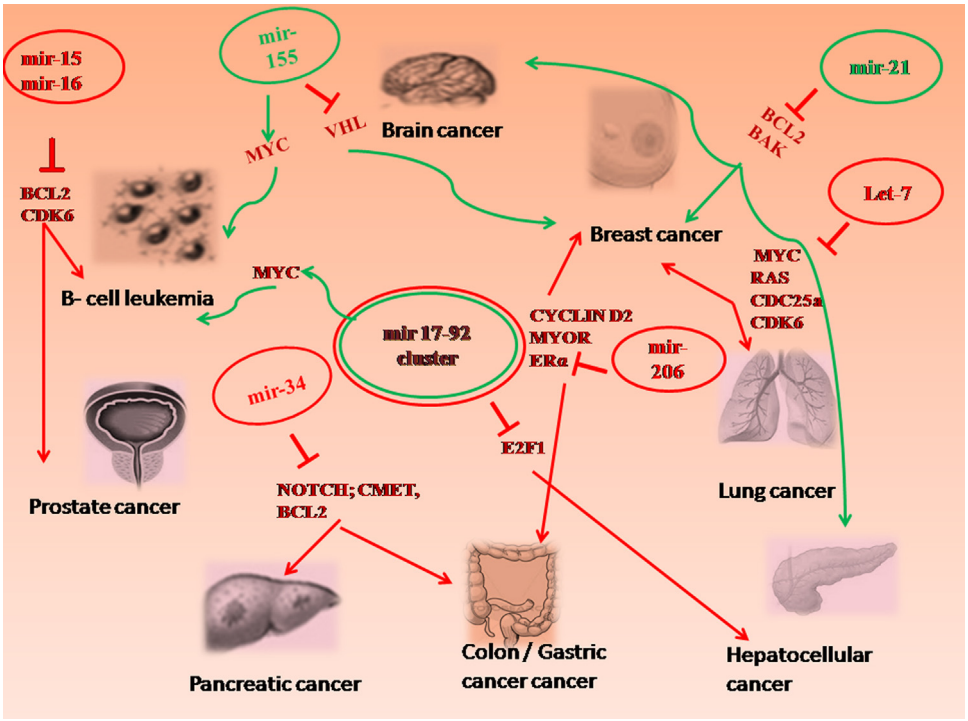

Fig. 2. Cancer association of miRNAs: the involvement of various miRNA is shown in cancers associated with different organs. Red colored miRNAs depict the tumor suppressor activity and green color shows their oncogenic role in cancer. miR-17-92 cluster acting both as tumor suppressor and oncomer in cancer associated with different organs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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MYC, normally responsible for cell cycle transition, when gets over-expressed results into sustained angiogenesis which is one of the hallmarks of cancer. During tumorigenesis it involves bypassing of apoptotic pathways which are crucial for a cell to avoid cancer progression (Hoffman and Liebermann, 2008).

2.1.3. Let-7

Let-7 is the best studied candidate tumor suppressor controlling lymph node metastasis and proliferation capacity. Cells with mutant Let-7 fail to exit cell cycle and remain undifferentiated. Studies show that the over-expression of Let-7 is correlated with inhibition of cell growth and reduction in cell cycle progression (Boyerinas et al., 2010). The family having 12 human homologues is mapped to the fragile sites linked to lung, breast, urothelial and cervical cancers, wherein it negatively regulates the oncomer Ras (membrane-associated GTPase signaling proteins for cell growth and differentiation). The 3′-UTR of Ras contains multiple complementary sites for interaction with Let-7 family and a repression of Ras by this family of miRNA is found to be reversed by the Let-7 inhibitors. The Let-7 and Ras correlation is further elucidated in lung cancer showing that its reduced gene expression causes poor prognosis whereas a point mutation in 3′-UTR of Let-7 complementary site of the k-Ras mRNA is associated with increased risk for the disease and shortened post-operative survival. Thus, the genetic interaction of Ras with Let-7 results into their reciprocal expression. Other oncogenes negatively controlled by Let-7 are MYC, HMGA2, and cell-cycle regulators including CDC25A, CDK6, and Cyclin D2. This property can be utilized for the therapeutics to treat lung cancer as it is found to be majorly involved in the onset and progression of lung cancer than any other malignancy (Jakopovic et al., 2013).

2.1.4. miR-34 family

miR-34 family, consisting of miR-34a and miR-34b/c, is a direct transactivation target of p53, which is deleted or down-regulated in various cancers including pancreatic and colon cancer. Being highly conserved these miRNAs are homologous and have redundant and tissue specific functions wherein miR-34a is highly expressed in brain while miR-34b/c is dominantly expressed in lungs (Hermeking, 2010). Up-regulated miR-34 is found to inhibit cancer growth by inactivating NOTCH, c-MET and BCL2 genes involved in cell cycle regulation and apoptosis, while its loss results into chemo-resistance of cancer cells by triggering apoptosis or cell cycle arrest in G1 phase or senescence. In prostate, breast, and ovarian cancers miR-34 gets hyper-methylated with p53 mutations suggesting that their down-regulation co-exists in cancer cells leading to tumor suppression. Although its restoration in p53 deficient pancreatic cancer cell lines showed significant inhibition of tumorsphere growth and tumor formation in association with BCL2 inactivation. Thus miR-34 family is responsible for restoring at least in part of tumor suppressor function of p53 in cancer cells (Qing et al., 2009; Chieh et al., 2014).

2.1.5. miR-206

Aberrant expression of miR-206 is responsible for breast and gastric cancer. Comparative quantitative analysis of its level in breast cancer cell lines and normal breast tissues shows a lower expression leading to cancer development, suggesting its role as a tumor suppressor. It blocks G1/S transition and suppresses cell proliferation by degrading Cyclin D2. Down-regulated miR-206 functions as a tumor suppressor with the inhibition of ERα in $ER\alpha^{+}$ (Estrogen receptor alpha-positive) human breast cancer by binding to its specific sites in the 3′-UTR and blocking cell proliferation leading to terminal cell differentiation (Zhou et al., 2013). Similarly, in skeletal and cardiac muscles, expression of miR-206 blocks p180 subunit of DNA polymerase α and myogenic transcription factors, IS1-3 and MYOR, resulting into differentiation (Goljanek-Whysall et al., 2012). Hence, down-regulation of miR-206 would enforce the cell to become de-differentiated as in cancerous conditions. Additional studies also show that restoring metastatic cells with miR-206 decreases their metastatic potential but it is not involved in cellular apoptosis (Elizabeth and Ashish, 2010).

2.2. Oncomirs as oncogenes

2.2.1. miR-17-92 cluster

miR-17-92 cluster works in co-operation with oncogene MYC, a basic helix-loop-helix transcription factor controlling both cell proliferation and apoptosis to induce tumors. Their association is majorly linked to the growth of B-cell malignancies. The cluster, encoded by a non-protein coding RNA gene, C13orf25 (Chromosome 12 open reading frame 25), located within the 13q31 locus, consists of seven miRNAs: miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1 (Liao et al., 2014). Over-expression of miRNA cluster with a retroviral system in MYC over-expressing HSCs (hematopoietic stem cells) is reported to worsen the condition increasing the chances of lethality (Chung et al., 2011). However, onset and progression of cancer, in cells expressing a single miRNA of the cluster or normal MYC gene, is not much aggressive. One of the major targets of MYC,-miR-17-92 cluster is the E2F1 transcription factor, involved in cell cycle progression and survival. This interaction forms a positive feedback loop. The binding of MYC to the first intron of C13orf25 suggests that it regulates primary transcript of miR-17-92 activating E2F1 by miR-17-5p and miR-20a leading to cell survival and loss of apoptosis. However, in some cancers such as hepatocellular carcinoma these miRNAs act as negative regulators of E2F1, breaking the positive loop, one of the reasons for this could be multiple target genes in different cell types (Ranji et al., 2013).

2.2.2. miR-21

miR-21 is another miRNA which gets over-expressed in various types of cancer. The use of Cre and Tet-off technologies, generating conditionally expressing miR-21 mice, shows that the over-expression of miR-21 resulted in a pre-B malignant lymphoid-like phenotype and knocking down of this miRNA lead to tumor regression (Medina et al., 2010). miR-21 silencing in glioblastoma cells activates Caspases leading to increased apoptosis, suggesting that aberrant expression of miR-21 blocks critical apoptosis-related genes resulting into the cellular transformation (Chen et al., 2008). In another study, down-regulation of miR-21 using lentiviral vectors shows impeded tumor growth in pancreatic adenocarcinoma derived cell lines by apoptosis through mitochondrial pathway (Flavie et al., 2013).

2.2.3. miR-155

miR-155 are encoded within the phylogenetically conserved region of BIC gene, an integration site for proviral DNA in avian leukosis virus-induced lymphomas, which is associated with up-regulated c-MYC gene and brings about the progression of lymphomas and leukemia. Residing within the only conserved region of this gene, miR-155 is suspected to be involved in cancers in association with MYC gene. Quantification of the levels of BIC and miR-155 in B-cell lymphomas reveals that their over-expression leads to oncogenesis. miR-155 can target certain tumor suppressor genes by repressing their translation (Shi et al., 2014). It is majorly involved in breast cancer by targeting the VHL (Von-Hippel Lindau) tumor suppressor thereby up-regulating HIF (Hypoxia Inducing Factors) transcription factors resulting into sustained angiogenesis (Czyzyk and Zhang, 2014). In malignancies related to hematopoietic cells, miR-155 is suggested to be responsible for prolonged inflamma-

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tion leading to cancer, as its level changes dynamically during cell differentiation and immune response (Tili et al., 2009).

3. miRNAs in cancer diagnostics

Cancer, a major cause of mortality worldwide, requires better techniques and treatments for which miRNAs may prove to be incredibly beneficial. Many cancer biomarkers are available for diagnostics; however, they fail to delineate benign and malignant tumors and other benign diseases such as cirrhosis, inflammatory bowel disorder, due to their elevation in these ailments. Some of the commonly used markers are CEA (carcinoembryonic antigen) for colon cancer, AFP (alpha-fetoprotein), an associated marker for hepatocellular carcinoma, and PSA (prostate specific antigen), a protein normally present at low levels in the blood of adult men having higher association with prostate cancer. Nevertheless these markers have low sensitivity towards early stage tumor detection and delineating aggressive tumors from the indolent ones. The role of miRNA in maintaining cellular homeostasis has itself proved to be a major cause behind cancer as a result of their deregulation. Hence in cancer diagnosis, expression profile of specific cancer associated circulating miRNAs released into the body fluids by the necrotic/apoptotic cells or tumors can be easily analyzed in both the normal and cancerous cells (Dharanija et al., 2013). Circulating miRNAs in body fluids such as plasma, serum, urine, and saliva can show higher diagnostic potential as biomarkers in cancer due to their association with argonaute protein or high density lipoprotein which provide resistance to extreme pH and temperature conditions and RNase activity. Moreover, they are easy and inexpensive to detect using quantitative polymerase chain reaction. A “gold standard” method recently utilized TaqMan miRNA cards with a target specific, stem-loop, reverse transcription primer which overcomes the problem of short length of mature miRNA, which is difficult to quantify during diagnosis (Cherie and Eric, 2007). Additional advantage of oncomirs is their ability to differentiate molecularly different tumors and higher stability than mRNAs, both in vitro and in vivo, which could be utilized for diagnosis purposes.

For the purpose of diagnostics, northern-blotting, real time-PCR and miRNA microarrays have proved to be useful. A unique microarray technique for miRNA expression analysis that simultaneously analyzes more than 1500 mature miRNAs and their corresponding precursors from 474 human and 373 mouse miRNA genes is developed on CodeLink platform for high throughput and accurate profiling (Wozniak et al., 2015; Liu et al., 2008). In CodeLink platform to identify over-expression of tissue specific miRNAs, slides coated with a cross-linked long-chain hydrophilic polymer facilitating easy hybridization of cDNA with lower background along with amine-reactive groups having 3-D chemistry are used. A single miRNA regulating multiple mRNAs can discriminate between the tumor types and stages owing to their involvement in stem cell maintenance and cell differentiation during development. Circulating miRNAs such as miR-21 in serum can be used as biomarker for B-cell lymphoma due to its association with relapse free survival in patients; miR-141 can be used in prostate cancer and miR-92 which is found to have increased level in colorectal cancer than in gastric cancer and inflammatory bowel disease, as well as in normal subjects and hence can be used as a potential biomarker to detect colorectal cancer (Nobuyoshi et al., 2010). Invention of the methods and compositions for generating miRNA profiles for therapeutic, diagnostic, and prognostic applications in cancer has been patented in 2011 under USPTO (United States Patent and Trademark Office). Some of the miRNAs used as biomarkers in different forms of cancer and their impact on the development of therapeutics are discussed in the following section of this review.

3.1. Colorectal cancer

Colorectal cancer is the third most common cancer in US. It starts as a polyp in the colon or rectum, which can further transform into malignant cancer. Recently, miR-126 is elucidated to be responsible for sustained angiogenesis during tumor progression, wherein, its expression is found to be correlated with the expression of VEGF-A (vascular endothelial growth factor-A). This class of miRNAs is thus being used as a prognostic marker for colorectal cancer to determine the sensitivity or response towards a particular drug. In addition to miR-126, miR-31 is also known to modulate the response to chemotherapeutic drugs. miR-31 acts as an oncomer and the incorporation of anti-miR-31 inhibitor is found to enhance the drug response and inhibit cell proliferation. However, miR-192, miR-215 and miR-148a act as tumor suppressors and hence their low expression in colorectal cancer tissues is used as biomarker (Verena et al., 2014).

3.2. Thyroid cancer

One of the most lethal classes of anaplastic thyroid cancer, is found to occur with the deregulation by various miRNAs. An overexpression of EGFR (Epidermal Growth Factor Receptor) leads to the down-regulation of miR-200 family. As a result it activates EMT (Epithelial-to-Mesenchymal Transition) by inducing TGFβ signaling, leading to the formation of an aggressive tumor (Zhang et al., 2012). Additionally, down-regulation of let-7 and miR-30 family of microRNAs enhances the metastatic potential of cancer cells. However, a set of other miRNAs such as miR-17-92 cluster, miR-146, miR-221 and miR-222 are found to be elevated resulting in an aggressive anaplastic thyroid cancer by triggering the transcription factors and proteins for cell proliferation and blocking the cell cycle checkpoint factors. Hence, the level of expression of these biomarkers can be used to determine the thyroid cancer risk and progression (Fuziwara and Kimura, 2014).

3.3. Cervical cancer

The fourth most common cause of cancer-related deaths, originating from the cervix in women has associated up-regulated expression of let-7b, let-7c, miR-23b, miR-143 and miR-196b while miR-21 gets down-regulated in cervical cancer (Granados López and López, 2014). To diagnose the cancer and determine the risk of failure of individualized therapy, these miRNAs play an important role. However, recently miR-9 and miR-200a are found to play an important regulatory role in cervical cancer and predict patient survival. Suppression of miR-200a results in high metastatic potential of cancer cells due to the over-expression of genes for cell motility, however, down-regulation of miR-9 leads to an increased rate of tumor cell metabolism. Additionally, miR-34a and miR-125b interact with HPV (Human Papilloma Virus) proteins and hence consequently, a persistent infection occurs with cervical cancer progression (Ribeiro and Sousa, 2014).

3.4. Soft tissue sarcomas

The circulating miRNAs in human blood and serum are important candidates for biomarkers in sarcomas. The down-regulation of miR-143 and up-regulation of miR-155 is reported in the development of liposarcoma due to increased cell proliferation (Fujiwara

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et al., 2014). Another type of soft tissue sarcomas is peripheral nerve sheath tumor where miRNA profiles showed miR-29c, miR-30c, miR-139-5p, miR-195, miR-151-5p were down-regulated causing blocking of cell invasion and metastasis (Presneau et al., 2013). miR-17-92 cluster is also involved in a type of sarcoma–angiosarcoma, wherein it suppresses the activity of THBS1 (thrombospondin-1), a protein involved in platelet aggregation and angiogenesis (Italiano et al., 2012).

3.5. Breast cancer

Breast cancer is the second major cause of cancer-related deaths in women worldwide. It is a heterogeneous disease with various subtypes depending on the expression of hormone receptors and tumor grade. Amongst its subtypes, the triple negative breast cancer has the worst outcome with currently no approved therapy (Carrie et al., 2015). Clinical studies with miRNAs show an increased overall survival rate with down-regulated miR-205 and up-regulated miR-21, which is associated with a decreased relapse rate. These studies can be used in conjunction with the patient's miRNA expression analysis reports to further infer the disease onset and prognosis (Markou et al., 2014). Secreted miRNAs can play an eminent role in cancer progression and this is further supported by the study showing that miR-451 and miR-1246 are released by the malignant cells. Hence, by culture methods these can be used for the diagnosis and prognosis of the disease. Additionally, lymph node and ductal carcinomas in breast cancer can be further differentiated by utilizing miR-10b and miR-373, circulating in the plasma of lymph nodes, as biomarkers for lymph node cancer. Other circulating miRNAs which are found to be dysregulated in breast cancer and hence have the potential to be used as a biomarker include miR-215, miR-299-5p and miR-411, which are expressed at lower levels and miR-195 which is highly expressed (Goh et al., 2015).

3.6. Lung cancer

It is the most common cause of cancer deaths worldwide. Circulating miRNAs such as miR-125a-5p, miR-145 and miR-146a can be used as biomarkers for non-small cell lung cancer. These miRNAs mainly act as tumor suppressor; however, their serum level varies for different tumor types. The different expression in different cancer can help differentiating the type of cancer. miR-145 targets the c-Myc gene which further inhibits progression of lung cancer. However, miR-125a-5p is up-regulated supporting the growth of cancer (Wang et al., 2015). Additionally, four miRNAs including miR-21, miR-126, miR-486-5p, and miR-210 have been detected with higher diagnostic efficiency for all stage non-small cell lung cancers (Shen et al., 2011).

3.7. Prostate cancer

Prostate cancer is a highly heterogeneous disease affecting men and due to the lack of clear understanding of the contributing factors, it is a challenge to find suitable diagnostic tools and therapies. Most of the miRNAs are down-regulated in prostate cancer. However, a subset including miR-182 and miR-375 in blood is reported to be over-expressed and thus used to analyze their diagnostic importance. miR-375 level directly associates with higher lymph node metastasis and hence can be crucial in depicting the progression of prostate cancer. Additionally, it can play dual role acting differently in different prostate cancer types i.e. androgen responsive and androgen independent by acting upon a panel of genes (Pinheiro et al., 2015). Deregulation of a large number of miRNAs is responsible for the stage and severity of the disease which is demonstrated by a recent study showing miR-141 to be elevated in higher grade of cancer. On the other hand, tumor suppressor miRNAs such as let-7a, miR-24, miR-93, miR-106b, miR-130b and miR-146a in serum from the prostate-derived cells are in lower levels in higher grade prostate cancer (Mihelich et al., 2015).

3.8. Pancreatic cancer

The cancer of pancreas in the lower abdomen is one of the most deadly and difficult cancers to manage. Increased level of miR-192 in serum is a potential biomarker for pancreatic cancer with high sensitivity. Elevated miR-18a is another miRNA in pancreatic cancer diagnostics, wherein its level is reduced after tumor resectioning. As reported, miR-27a-3p in combination with serum marker CA19-9 can be 85.3% sensitive in diagnosing pancreatic cancer (Halkova et al., 2015). Moreover, miR-186 and miR-224 can help in predicting the lymph node metastasis and survival of the patients. An increased expression of miR-186 predicts poor prognosis, which helps in the differentiation of pancreatic cancer from the others types of cancer, owing to its contrasting functions and regulation in other tumors with specific pathogenesis. In pancreatic cancer the NR5A2 gene, responsible for damage recovery of the damaged pancreatitis cells, is repressed by miR-186, thus inhibiting its tumor suppressive function leading to carcinogenesis. With respect to miR-326, its down-regulation results into an increased venous invasion. However, additional studies are required for the detection of affected downstream pathways targeted by this miRNA to establish its regulatory effect (Zhang et al., 2015).

4. miRNA delivery systems in cancer therapeutics

Owing to the function of miRNAs to be used as tumor suppressors and oncomers, they have a great potential to target the pathways involved in cancer development and metastasis. In cancer therapeutics, miRNAs can be used for degrading the anti-apoptotic genes or can be silenced to up-regulate the tumor suppressor genes. For example the restoration of miR-34 inhibited tumor formation while antagonizing miR-21 using anti-sense oligonucleotides causes pro-apoptotic response. The anti-sense oligonucleotides used to correct the interaction between miRNA and mRNA needs to be modified chemically for stability and cellular uptake. miRNAs can be targeted in therapeutics by two mechanisms: either by the use of synthetic anti-miR sequences with partial or full complementary reverse sequence for a particular up-regulated miRNA or by employing miRNA mimics for substituting the down-regulated miRNAs (Ben-Shushan et al., 2014). Although, theoretically these alternative approaches sound efficient, these have some associated limitations in practical treatment. For the synthesis of miRNA mimics, requirement of recognition of the guide strand by the cell must be accomplished, however, it is limited by the allowed chemical modifications. Additionally the delivered miRNAs, lacking tissue specificity might be up-taken by other tissues thus leading to adverse reactions. Nonetheless, the use of adeno-associated vectors and tissue-specific promoters can overcome this problem. Furthermore, the anti-miR use requires it to be cell permeable, bioavailable, highly specific and stable with high affinity. To attain these properties certain modifications are done to the sequences. One such mechanism is adding of 2′-*O*-methyl-group (OMe)-modified oligonucleotides and LNA (locked nucleic acid)-modified oligonucleotides. The conventional virus-mediated and lipid-based delivery system can be used for introduction of miRNAs. However, the practicality of these approaches has been limited by the cytotoxicity and associated immunogenic responses respectively (Zhang et al., 2013). A novel approach for such treatment is the use of nanoparticles for delivering the anti-miRNAs and miRNAs for specific proteins. A poly L-lysine complex is developed which releases trace amounts of anti-miRNA slowly for

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weeks into cancer cells with very high efficacy and no associated toxicity. Another successful approach for silencing oncogenes with synthetic miRNAs has been applied to target over-expressed HER-2 (Human Epidermal Growth Factor-2) protein in breast cancer. miRNA sponges, transiently expressing miRNA inhibitors, can also be used to regain the function of tumor suppressor genes repressed by miRNAs. However, one limitation of these therapeutic approaches is the partial knock-down of oncomirs by anti-sense oligonucleotides (Stefanie et al., 2008).

5. Circulating miRNAs in therapeutics

Through facilitation of cell–cell communication, circulating miRNAs are suggested to serve as potential therapeutic tools. Based upon over-expression or knocking down of these small molecules, several progressive researches are currently going on for employing them in cancer therapeutics (Zhang et al., 2013). The treatment of colorectal cancer by targeting miR-135b which is abnormally up-regulated in diseased state, causing evasion of apoptosis by down-regulating TGFβR2 (Transforming Growth Factor B Receptor 2), DAPK1 (Death-Associated Protein Kinase 1), APC (Adenomatous Polyposis Coli), FIH (Factor Inhibiting HIF) along with the activation of APC/β-catenin and SRC-PI3K pathways. In vivo study with anti-miRNA has reported positive results against colon cancer in mice. Additionally, miR-26 re-expression in hepatocellular carcinoma inhibits the cell cycle regulators cyclin D2 and cyclin E2 with increase in apoptosis in mouse model (Wen and Yue, 2014).

Targeting miRNAs can also be beneficial in enhancing chemotherapeutic response to a drug. The same is shown by a study with knocked out miR-200b and miR-21, which increased the response to Gemcitabine in cholangiocarcinoma (Mostert et al., 2011). Another study showed an improved response to tyrosine kinase inhibitors in breast cancer with the introduction of miR-205, which by targeting HER3 receptor inhibits the downstream signaling pathway of Akt. The study analyzes the potential of miR-205 to be used as tumor suppressor gene with suppression of clonogenic potential of cells and enhanced response for the drugs Gefitinib and Lapatinib (Iorio et al., 2009). Hence, circulating miRNAs expression or level in blood can be useful in determining the outcome and prognosis after a therapy.

6. Recent patented research on miRNAs

Oncomirs, emerging as novel biomarkers for diagnosis, prognosis and treatment of cancer by employing molecular biology techniques are being extensively patented worldwide, which corresponds to a great research being done in this field. The patenting era for miRNAs began in early 2000s and achieved a peak after 2008, showing the intense research being conducted in this field. Amongst the United States (US), Europe (EP) and WIPO patents, US leads in the number of patented applications followed by Europe. This is majorly because of the patenting procedures followed by the two patent offices. USPTO has the first miRNA patent issued in the year 2006 while for EP it came in 2008. After the year 2006, all the three offices together contribute to a vast number of accepted and filed patent applications. As per the data, sharp increase in the number of issued patents from 2009 to 2011–2012 was observed with three times more patents being published (Wu, 2011). This graph of increasing miRNA research is yet to reach its highest peak. Currently there are several patent applications filed and granted approval on the methods using miRNAs for cancer diagnostics and therapeutics. Extensively characterized miR-15, miR-16, miR-21, miR-34, let-7, miR-206, miR-155, miR-17-92 cluster discussed in this review alone have over thousands of patents for their use as cancer biomarkers. However, a wide range of other cancer associated miRNAs are also patented. For instance, the expression analysis of miR-15 along with several other microRNAs including miR-1, miR-10, miR-20, miR-25, miR-30 in detection of ovarian cancer by employing a method to compare the expression profile of these pre-determined set of miRNAs with a reference expression profile, has been applied for patent in 2012. In addition, method to detect miR-15 expression along with other miRNAs in colorectal cancer has also been filed for patent. A method and composition for cancer diagnosis and therapy utilizing miR-15 and miR-16 got patented in 2013. Invention regarding compositions and methods for detection of biogenesis of let-7 mediated by lin28 binding has filed for patent in 2012. Several other miRNAs also associated with cancer are increasingly being patented on their use in tumor immunotherapy, treatment of auto-immune inflammation during cancer, prediction of cancer prognosis, differentiating early and late tumors and their promoter driven targeted therapies (Febit Holding GmbH, 2012; Thomas Jefferson University, 2013a; Asuragen, Inc, 2009; Whitney, 2015; Thomas Jefferson University, 2013b; MiRNA Therapeutics, Inc, 2010; Asuragen, Inc, 2010; Reneuron limited, 2015; Children's Medical Center Corp., 2012; Yale University, 2013; Hu et al., 2008; Regulus Therapeutics Inc., 2015; Mira Therapeutics, Inc, 2013; Andreas et al., 2009a; Rosetta Inpharmatics Llc et al., 2008; The Ohio State University research Foundation, 2013; Charite-Universitatsmedizin Berlin, 2013; Veridex Llc et al., 2009; Council of Scientific and Industrial Research, 2015; Thomas Jefferson University, 2013c; Ramot At Tel Aviv University, Ltd, 2013; Andreas et al., 2009b; The Ohio State University, 2013; California Institute of Technology, 2012; The Ohio State University research Foundation and Carlo, 2013; Cold Spring Harbor Laboratory, University of North California, 2014; The University of Pittsburgh-of the commonwealth system of higher education, 2010; Hospital Clinic De Barcelona, 2013). The extensive patenting field of miRNAs in cancer itself shows the valuable contribution in research involving cancer diagnostics, therapeutics and prognostics. The data on some of the recent patents applied or granted on miRNAs are included in Table 1.

7. Future prospectives in cancer therapeutics

Cancer being a very complex and multi-factorial disease leaves us with many unexplored questions. Curing cancer to its root requires intensive treatment, but still complications develop owing to the side effects of the therapy due to deficient interruption of multi-signaling oncogenic pathways and drug-induced adverse effects. Therefore, new treatment strategies are required to be developed to improve the clinical outcome and quality of life of patients. Employment of miRNAs for targeting the oncogenes and the downstream signaling proteins can prove to be a breakthrough in cancer treatment. We are still at the edge of understanding the role of miRNAs in various types of cancers. Nevertheless, the current existing information about miRNAs in cancer has lead to the advancement in the field of cancer aetiology, diagnostics, therapeutics, prognosis and other alternative translational applications involving miRNAs and their associated molecules and pathways leading to the accurate prediction of overall survival for cancer patients. miRNAs have emerged as a major player in gene regulation and analysis of expression profiling of these small molecules can help in delineating cancerous and normal tissues. Most of these oncomirs are found to have altered function or expression in cancer which disturbs the normal functioning of other genes associated with cancer.

Researchers have used strategies such as knocking down over-expressed miRNAs in cancer and re-expressing those which are down-regulated to treat cancer in cancerous cell lines and mice. However, major challenges for the use of miRNA in therapeu-

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Table 1
Recent patents applied/granted on oncomirs associated with different types of cancers.

miRNA	Recent patents	Inventors/applicant	Patents available	Function	Cancer association	References
mir-15	(i) EP 2531611 Title:miRNA in the diagnosis of ovarian cancer Publication date:12/12/2012 (ii) US 8557515 Title: compositions and methods for cancer diagnosis and therapy Publication date: 10/15/2013 (iii) US 20090131356 Title: miR-15, miR-26, miR-31, mir-145, mir-147, mir-188, miR-215, miR-216, miR-331, mmu- miR-292-3P regulated genes and pathways as targets for therapeutic intervention Publication date: 05/21/2009	Inventors: Andreas Keller, Matthias Scheffler, Markus Beier Applicant:Febit Holding GmbH Inventors: Carlo Croce, George Calin Assignee: Thomas Jefferson University Inventors:Andreas G. Bader, Lubna Patrawala, Mike W. Byrom, Charles D. Johnson, David Brown Assignee: Asuragen, Inc.	727	TS	B-cell leukemia, bladder cancer, prostate cancer, Ovarian cancer	(Febit Holding GmbH, 2012; Thomas Jefferson University, 2013a; Asuragen, Inc, 2009)
mir-16	(i)US 20150080243 Title: methodsand compositions for detecting cancer based on miRNA expression profiles Publication date: 19/03/2015 (ii) US 8557515 Title:compositions and methods for cancer diagnosis and therapy Publication date: 10/15/2013 (iii) US 20100179213 Title:methods and compositions involving miRNAs in cancer stem cells Publication date: 07/15/2010 (iv) WO 2009154835 Title:compositions and methods related to miR-16 and therapy of prostate cancer Publication date: 04/29/2010	Inventors:Whitney D.H., Luo J. Applicant: Whitney D.H., Allegro Diagnostics Corp., Jun Luo Inventors: Carlo Croce, George Calin Assignee: Thomas Jefferson University Inventors: Lubna Patrawala, Dean G. Tang, Kevin Kelnar, Jason Wiggins, Stephanie Volz, Jeffrey Shelton, Can Liu, Andreas G. Bader, David Brown Assignee:Mirna Therapeutics, Inc., The Board of Regents of the University of Texas System Inventors: Fumitaka Takeshita, David Brown, Takahiro Ochiya Applicant: Asuragen, Inc.	1478	TS	Prostate cancer, multiple myeloma, B-cell leukemia	(Whitney, 2015; Thomas Jefferson University, 2013b; MiRNA Therapeutics, Inc, 2010; Asuragen, Inc, 2010)
Let-7	(i)WO 2015052526 Title: stem cell microparticles and miRNA Publication date: 16/04/2015 (ii) WO 2012135081 Title: Lin28-mediated control of let-7 biogenesis Publication dDate:10/04/2012 (iii) US 20130089862 Title:genetic lesion associated with cancer Publication date: 04/11/2013 (iv)WO 2008095096 Title: Let-7 microRNA and mimetics thereof as therapeutics for cancer Publication date: 08/07/2008	Inventors: Hicks Caroline (GB), Sinden John (GB), Stevanto Lara (GB), Corteling Randolph Applicant: Reneuron Limited Inventors: Richard Regory, Elena Piskounova, Dimitrios Iliopoulos Applicant: Children's Medical Center Corp., Dana-Farber Cancer Institute Inc. Inventors: Frank J Slack, Joanne B. Weidhaas, Lena J. Chin, Elena Ratner Assignee: Yale University Inventors: Judy Lieberman, Erwei Song, FengyanYu,Xiaogu Hu Applicant: Xiaogu Hu, Immune Disease Inst, Judy Lieberman et al.	2034	TS	Lung cancer, breast, urothelial and cervical cancer	(Reneuron limited, 2015; Children's Medical Center Corp., 2012; Yale University, 2013)
mir-34	(i)US 20150087607 Publication date: 26/03/2015 Title: microRNA compositions and methods (ii) US 8586727 Title: synthetic mimics of miR-34 Publication date:11/19/2013 (iii) US 20090227533	Inventors: Marcuss E.G., Bhat B., Linsley P., Akinc A. Appilicant: Regulus Therapeutics Inc., Alynylam Pharmaceuticals Inventors: Kevin Kelnar, David Brown Assignee: Mira Therapeutics, Inc. Inventors:Andreas G. Bader, Mike Byrom, Charles D. Johnson, David Brown, Lubna Patrawala, Jason F. Wiggins	472	TS	Pancreatic and colon cancer	(Regulus Therapeutics Inc., 2015; Mira Therapeutics, Inc, 2013; Andreas et al., 2009a; Rosetta Inpharmatics Llc et al., 2008)

Recent patents applied/granted on oncomirs associated with different types of cancers.

miRNA	Recent patents	Inventors/applicant	Patents available	Function	Cancer association	References
	Title:miR-34 regulated genes and pathways as targets for therapeutic intervention Publication date: 09/10/2009	Assignee: Andreas G. Bader, Mike Byrom, Charles D., Johnson et al.				
	(iv) WO 2008137867 Title:compositions comprising miR-34 therapeutic agents for treating cancer Publication date: 11/13/2008	Inventors: Michele A. Cleary, Aimee L Jackson, Peter S. Linsley, Julja Burchard, Lee P. Lim, Jill F. Magnus Applicant: Rosetta Inpharmatics Llc, Michele A. Cleary, Aimee L. Jackson et al.				
mir-206	(i) EP 2468898 Title:microRNA-based methods and compositions for the diagnosis and treatment of solid cancers Publication date:03/06/2013	Inventors: Carlo Croce, George Calin, Stefano Volinia Applicant:The Ohio State University research Foundation	865	TS	Breast and gastric cancer	(The Ohio State University research Foundation, 2013; Veridex Llc et al., 2009)
	(ii) US 20130281508 Title:microRNA target site for cell- or tissue-specific inhibition of expression of a transgene Publication date: 10/24/2013	Inventors: Henry Fechner, Geisler Anja Assignee: Charite-Universitatsmedizin Berlin, Technische Universitat Berlin				
	(iii) WO 2009059016 Title: process for predicting the prognosis of squamous cell lung cancer Publication date: 05/07/2009	Inventors: Mitchi Raponi, Lesley Estelle, Dossey				
mir-21	(i) US 20150080244 Title:biomarkers usefulfor detection of types, grades and stages of human breast cancer Publication date:19/03/2015	Inventors:Kumar L.D., Verma V.K., Nair R.A., Prabhakar J., Kattoor J Applicants:Council of Scientific and Industrial Research	1763	OG	Glioblastoma, breast and pancreatic cancer	(Council of Scientific and Industrial Research, 2015; Thomas Jefferson University, 2013c; Ramot At Tel Aviv University, Ltd, 2013; Andreas et al., 2009b)
	(ii) US 13/972759 Title: diagnosis and treatment of cancers with microRNA located in or near cancer-associated chromosomal features Publication date:12/12/2013	Inventors: Carlo M. Croce, Chang-Gong Liu, Cinzia Sevignani Assignee:Thomas Jefferson University				
	(iii) US 20130310446 Title:miR-21 promoter driven targeted cancer therapy Publication date: 11/21/2013	Inventors: Rina Rosin-Arbesfeld, Ella Sklan, Alona Zilberberg, Naama David Assignee: Ramot At Tel Aviv University, Ltd.				
	(iv)US 20090192102 Title: miR-21 regulated genes and pathways as targets for therapeutic intervention Publication date: 07/30/2009	Inventors: Andreas G. Bader, Mike Byrom, Charles D. Johnson, David Brown Assignee: Andreas G. Bader, Mike Byrom, Charles D. Johnson, David Brown				
mir-155	(i) US 8664192 Title:mutator activity induced by microRNA-155 (miR-155) links inflammation and cancer Publication date: 12/14/2013	Inventors: Carlo M. Croce Assignee:The Ohio State University	1347	OG	B-cell lymphomas, Hodgkin's lymphoma, human breast cancer	(The Ohio State University, 2013; California Institute of Technology, 2012; The Ohio State University research Foundation and Carlo, 2013)
	(ii) WO/2012/037043 Title:treatment of autoimmune inflammation using mir-155 Publication date: 03/22/2012	Inventors: David Baltimore, Ryan, M. O'connell, Daniel Kahn Applicant: California Institute of Technology				
	(iii) CA 2650026 Title:Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in mir155 transgenic mice Publication date: 01/22/2013	Inventors: Carlo M. Croce Applicant: The Ohio State University research Foundation, Carlo M.C.				

Recent patents applied/granted on oncomirs associated with different types of cancers.

miRNA	Recent patents	Inventors/applicant	Patents available	Function	Cancer association	References
mir 17-92 cluster	(i) EP 1880027 Title:composition and methods for cancer diagnosis utilizing the miR-17-92 cluster Publication date:02/19/2014 (ii) US 20100322909 Title:Th1-associated microRNAs and their use for tumor immunotherapy Publication date:12/23/2010 (iii) WO 2013093635 Title: plasma microRNAs for the detection of early colorectal cancer Publication date: 07/27/2013	Inventors: Gregory J. Hannon, Scott Hammond, Lin H.E., Summer Goodson, Michael J. Thomson Applicant:Cold Spring Harbor Laboratory, University of North California Inventors:Hideho Okada, Gary Kohanbash, Kotaro Sasaki Assignee: The University of Pittsburgh-of the commonwealth system of higher education Inventors: I. Cos Meriyxell Gironella, Salvatella Juan Lozano, Garangou Antoni Castells, Maria Dolores Giraldez Applicant: Hospital Clinic De Barcelona, Centro De Investigacion Biomedica En Red De Enfermedades Hepaticas Y Digestivas	142	OG/TS	Hepatocellular carcinoma,B-cell lymphomas	(Cold Spring Harbor Laboratory, University of North California, 2014; The University of Pittsburgh-of the commonwealth system of higher education, 2010; Hospital Clinic De Barcelona, 2013)

OG: oncogenes; TS: tumor suppressors.

tics is its tissue-specific delivery and multiple target genes, as a result its off-target effects might result into toxicity. Hence, more strategies are required to be designed to normalize or restore the functioning of oncomirs and reduce toxicity. In this regard, nanoparticles based miRNA entrapped delivery devices can be improved which may prove to be highly efficient in cancer therapeutics. These nano-miRNAs should have a site specific action that is capable of delivering the microRNA or anti-microRNA directly to the transformed cells and thus would help in reducing the undesired toxicity to non-target cells. These nanoparticles due to their targeted action would increase the efficacy of miRNA based therapy and also their small size as compared to other deliver systems gives them an edge while evading the immune responses with the ability to cross relatively impermeable membranes. Nanoparticles used for entrapment can be synthesized by emerging green methods which not only ensure the safety and effectiveness of the nanoparticles being created but also provide us with the availability of cheaper and non-toxic nanoparticles. During green synthesis natural products having anti-oxidative, anti-inflammatory and anti-cancerous potential can be utilized for better efficacy and less adverse effects observed in conventional approaches of nanoparticle synthesis as this mini-combination of miRNA and nanoparticles with natural anti-cancerous products will have a synergistic impact on the tumor cells, increasing the efficiency of therapeutic response.

In summary, an increased understanding of the role of miRNAs in molecular pathogenesis of cancer, therapeutics and modification in the existing delivery systems would help in translation of these benchbased discoveries of microRNAs to the hospitals and clinics.

8. Conclusions

miRNAs, an emerging class of gene regulators, are involved in many physiological and pathological processes, including cancer. The characterization of deregulated miRNAs in cellular transformation, benign and malignant states, progression of cancer and as a regulator of multiple biological pathways has immense implications and represents a powerful therapeutic strategy in cancer.

Conflict of interest

Authors declare that they have no conflict of interest.

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