MicroRNAs Horizon in Retinoblastoma

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Abstract- In the retinoblastoma research, it is of great interest to identify molecular markers associated with the genetics of tumorigenesis. microRNAs (miRNAs) are small non-coding RNA molecules that play a regulatory role in many crucial cellular pathways such as differentiation, cell cycle progression, and apoptosis. A body of evidences showed dysregulation of miRNAs in tumor biology and many diseases. They potentially play a significant role in tumorigenesis processes and have been the subject of research in many types of cancers including retinal tumorigenesis. miRNA expression profiling was found to be associated with tumor development, progression and treatment. These associations demonstrate the putative applications of miRNAs in monitoring of different aspect of tumors consisting diagnostic, prognostic and therapeutic. Herein, we review the current literature concerning to the study of miRNA target recognition, function to tumorigenesis and treatment in retinoblastoma. Identification the specific miRNA biomarkers associated with retinoblastoma cancer may help to establish new therapeutic approaches for salvage affected eyes in patients.

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Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules with approximately 22 nucleotide in length. The initial evidence about the miRNAs function was published by Lee *et al.* in 1993 (1). They demonstrated the lin-4 involvement in regulatory the temporal progression of cell differentiation in the nematode *Caenorhabditis elegans*. At the present, numerous published studies linked miRNAs to gene regulatory networks in cancerous cells that result a new class of biological regulators with preserved function in tumor. The miRNA-mediated gene regulation is very complex and is involved into the different aspects of tumor cell function including cell cycle regulation, growth, differentiation, apoptosis, and stress response (2). Hence, identification miRNA expression profiling has been the interest subject in tumor cell biology studies. The first report in linkage miRNAs to cancer demonstrated that the loss of heterozygosity regions (LOH) in chromosome 13 is associated to the deletion and down-expression of miR-15 and miR-16 in more than 50% of chronic lymphocytic leukemia (CLL) patients (3). miRNAs are able to have tumor suppress or oncogenic entity when they are up or down regulated in tumorigenesis process. For example; miR-15a, 16–1, 17–5p, 143, 145, and let-7 were identified as a tumor suppressor miRNA while miR-18a, 19a-b, 20a, 21, 92, 155, and 372 were known as oncomiRs (4). The tumorigenesis role of miRNAs has been recently revealed in many types of cancers consisting leukemias, breast, lung, colon and retinoblastoma (5). In this review, we summarize the biogenesis of a miRNA and its regulatory function in cells. We focus, then, on evidence suggestive of miRNA dysregulation in retinoblastoma and its possible therapeutic role in this cancer.

MicroRNA biogenesis

Studies have demonstrated that genes encoding for miRNAs can be located in an intergenic region and/or in the intronic region of a protein-coding gene. Intronic

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miRNAs have two forms of transcriptional regulation, either co-transcribed together with the protein-coding gene or transcribed with their own promoters (6). Therefore, the expression levels of those miRNAs can be independent the expression levels of the host gene. Hence, miRNAs has been known to be independent regulators of gene transcription in eukaryotic biology. Dysregulation of miRNA has been concerned to etiology of many diseases, as well as tumor formation. miRNAs act as negative regulators, thereby, up-regulated miRNAs resulted in the down-regulated target mRNAs, and vice versa. Based on their downstream targets, miRNAs are classified as a novel group of oncogenes or tumor suppressor genes in tumor development.

The mature microRNAs (miRNAs) are single-stranded RNA molecules of 20-23-nucleotide (nt) length are generated from primary transcripts (pri-miRNAs) by two enzymatic cleavage steps into nucleus and cytoplasm, consecutively (Figure 1). Pri-miRNAs are long primary transcripts (~1kb) characterized by hair pain loop and synthesized by RNA polymerase II in nucleus (7). The non-loop end structures are removed by the RNAse III Drosha and processed to form 60–70 bp long pre-miRNA (8). RNAase III Drosha (DGCR8) is composed of a double-stranded RNA-binding protein (dsRBP) and RNASEN. Pre-miRNA is exported from the nucleus to the cytoplasm by a nucleus membrane transporter named Exportin (9). In cytoplasm, the loop end of pre-miRNA is excised by DICER1 to yield a RNA duplex intermediate that is subsequently unwound to release mature miRNA and miRNA∗ sequences (2,10).

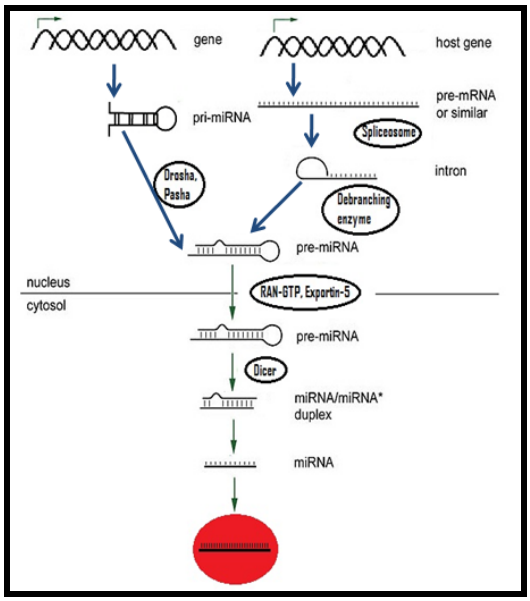

Figure 1. An illustration of miRNA biogenesis.

The miRNA* sequences are mostly released and degraded. While the mature miRNAs are able to regulate gene expression at post-transcriptional level in two different ways, the degradation and translational repression of the target mRNAs (11). To this end, mature miRNAs are loaded into the RNA-induced silencing complex (RISC) and lead the degradation mRNA target or inhibition translation when they are partially matched with their mRNA targets. RISC is a ribonucleoprotein complex containing Argonaute proteins, the two miRNA strands and several additional factors including the trans-activator RNA binding protein (TRBP) (10).

The same miRNA can have multiple mRNA targets, and the same mRNA can be regulated by various miRNAs (12). There is a conserved heptametrical sequence in the 5' end of miRNAs that is named the seed sequence and plays an essential role in target recognition and post-transcriptional suppression (13-15). The miRNA seed complementarity is needed for miRNA target recognition in cell culture systems (16,17). In addition to it has been recently reported that miRNAs can have decoy activity for translational repression in a RISC-independent manner via their seed sequence (18). This activity is known the other way in miRNAs to regulate gene expression at the transcriptional level that is directly bind to the DNA (19-21). The miRNA decoy mechanism can be taken into consideration when developing miRNA-based therapies, consisting the ways to overcomes resistance to current chemotherapy drugs and also design new anticancer drugs in clinical trials.

On the other side, impaired miRNA processing and/or its inhibition biogenesis pathway were also found to be related to tumorigenesis and cancer development (22). For instance, the decrease DICER1 in mono-allelic loss cell resulting in impaired miRNA has been demonstrated to accelerate retinoblastoma formation in *in vivo* model (23).

MicroRNAs in retinoblastoma

Retinoblastoma is a childhood intraocular cancer that is caused by a mutation in the RB gene located on chromosome 13 of humans. The RB gene is known with tumor suppressor activities. The active RB products play a role in control cell division in S phase by suppressing E2F function, an essential nuclear transcription factor. In the hypophosphorylated state, pRb is active and presents its role as a tumor suppressor gene. In the early G1 phase, hypophosphorylated form of pRB interacts with E2F to repress its transcriptional activity, however, its hyperphosphorylated form in late G1 phase release *E2F*

allowing transcription of genes necessary for progression to the S phase (24,25). In cancerous cells, the mutant RB gene product is often phosphorylated, hence, are not able to control of cell proliferation, survival, and differentiation. It is well known that miRNAs play a crucial role in tumor suppressor networks such as *RB* gene. Nemours studies demonstrated that miRNAs directly targets *RB* gene in cancer cells. For example, miR-449 represses *RB* phosphorylation to regulate cell growth in prostate cancer (26). *RB1* has also been known to be a tumor suppressor gene that one of its functions is to prevent apoptosis (27,28). Knockdown of *RB*-induced apoptosis can be canceled by overexpression of miR-17-92 in lung cancer cells (29). Mir-17-92 has oncogenic activity whose overexpression has been observed in multiple tumor types (30). In addition to miR-192 targets the RB1 gene to inhibit cell proliferation and induce cell apoptosis in lung cancer cells (31). MiR-34a and miR-15a/16 are also downregulated or deleted to control cell cycle progression in a RB-dependent manner in non-small cell lung cancer (32).

Inactivation both pRB and p53 pathways are observed in the most human tumors; however, p53 mutation has not been reported in retinal tumors (33). Despite the lack of p53 mutation, p53 pathway inactivation is occurred in RB tumors. These two pathways are inactivated by genetic mutation in RB1 and P53 or in genes that regulate these pathways such as MDM2 and MDMX. After RB1 loss, tumor surveillance is mediated by activation Arf, MDM2, MDMX, and p53 (33). A recently study reported miR-24-mediated repression of Arf is likely able to block activation of p53 tumor surveillance in retinoblastoma in response to RB1 loss (34).

Mir-34a is a cell proliferation inhibitor that down-regulates transcription factor E2F and up-regulates p53, thereby it has tumor suppressor activity. Dalgard and *et al*. (35) by using semi-quantitative RT-PCR and real-time qPCR elevated levels of miR-34a in two RB cell lines (Y79 and Weri-Rb1) and two primary RB samples. The miR-34a affect on p53-induced apoptosis, senescence, and cell cycle arrest by repressing the expression of several oncogenes such as SIRT1 (36), CD44 (37), antiapoptosis factor BCL2 (38) and MYC (39). The subtle functional role of miR-34a in the p53 pathway remains to be fully understood in RB tumor. It is thought, the study of miRNA in RB tumors elucidates the mechanisms underlying cancers development and their tumorigenesis. To date, the relationship between the expression of miRNAs and RB has been reported in several studies. An array results indicated upregulation of multiple miRNAs including miR-494, 513-1, 513-2, 518c, 129-1, 29-2, 198, 492, 498, 320, 503, 373 and let-7e in RB tumors compared to normal tissues (40). Mitra and *et al.* (41) developed Y79 cells with PLGA-gelatin microparticles to test the efficacy of antiproliferation activity of drugs. They revealed up-regulation of different oncomiRs such as miR-18a, 19b, 106a, and 198 while tumor suppressor miRNA including let-7 family, miR 15a, 16-1, and 34a were downregulated in RB tumor tissues. Ectopic expression of Let-7 downregulates oncogenes such as Ras, Myc, and HMGA2 and inhibits proliferation and tumorigenesis (42).

The studies demonstrated that microenvironment factors such as hypoxia are able to effect on miRNAs expression in tumor cells. Hypoxia was known to be a major feature of RB tumors and is associated with poor prognosis and chemoresistance (43). In addition to it may be a potential therapeutic target of advanced RB (44). Miss-expression of hypoxia-inducible factor (HIF) was found to be correlated to hypoxia conditions (45,46). The HIF is able to effect on expression of particular miRNAs that are named hypoxia-regulated microRNAs (HRM) and related to the survival of cancerous cells in low-oxygen status. So far, a study reported the effect of hypoxia on miRNA expression in RB cell line HXO-RB44 (43). They identified, using the microarray method, a cluster of HRMs including miR-181b, 125a-3p, 30c-2, 497 and 491-3p in these cells. Among them miR-181b was observed with the most different expression in hypoxia area.

microRNA and chemotherapy

miRNAs are involved in the most cell processes consisting viability, proliferation, cell cycle regulation, and apoptosis. Moreover, they are amenable to therapeutic manipulation. Hence they can be potentially suitable therapeutic targets in a broad range of diseases. Several miRNAs have demonstrated therapeutic potential in cell based experiments, supporting the point that the inhibitors or promoters of miRNAs could lead to identification of new agents in chemotherapy. For instance, overexpression of miR-17-92 was found to reduce excessive DNA damage in RB inactivation of small-cell lung cancer cells (29). It suggests that this miRNA being appropriate therapeutic target candidate in chemotherapy with DNA-damaging anticancer drugs (29). One of the earliest studies compared normal and advance stage tumorigenic RB tissues and identified 19 miRNAs that were differentially expressed in tumors

(47). It was the first step to identify miRNAs that was related with RB progression and could serve as potential therapeutic targets for RB.

A recent study demonstrated treating RB cells by miR-181b inhibitor was able to prevent their proliferation (43). Both miR-181b and miR-21 have also been known as best prognostic biomarker in S-1/Oxaliplatin based chemotherapy of Gastric cancer patients (48). TGFβ-mediated high expression of miR-181b showed to stimulate hepatocarcinogenesis in an animal model in which mice fed CADD diet by suppressing tissue inhibitor of metalloprotease 3 (TIMP3) a miR-181b target (49). While metallopeptidases 2 and 9 activity is promoted by this miRNA that result increased growth, clonogenic survival, migration, invasion and their resistance to the anticancer drug doxorubicin (49). Furthermore, miR-181b was presented with the most different expression in RB cells with hypoxia condition (43). Therefore, it may have potential in tumor development, progression, and metastasis in RB.

Additionally, extracellular miRNAs that exist in the circulation and bodily fluids have been known as biomarkers for cancer diagnosis and therapeutic, due to their inherent stability in a number of different biological tissues and fluids. Detection biomarkers in blood and other bodily fluids provide a less aggressive approach to diagnostic cancer. Since miRNAs are normally resistant to degradation by RNases, they can be isolated from the serum, saliva, semen, vaginal secretions, urine, stool, and sputum (50-54).

This aspect has been addressed in several types of cancers such as B cell lymphoma, lung, colorectal, ovarian, and breast cancers (55-60). Lawrie *et al.* (55) initially showed miR-141 in serum could be biomarkers to distinguish prostate patients and controls. In addition to raised miR-31 and reduced miR-125a and miR-200a in both saliva and serum, they have been identified as biomarkers to detect oral squamous cell carcinoma patients (60,61). To date, there is no such data in relate to RB therapy.

It is also of great interest to identify miRNAs with distinct profile expression in cancer stem cells (CSC). This aspect of miRNA-based therapeutic is studied in regenerative medicine area. CSCs are a sub-population of cells in tumors that strongly are resistance to chemotherapy and likely cause metastasis and relapse after treatment. It is thought; the expression miRNAs profiles in these cells can be targeted and manipulated into consideration when developing miRNA-based therapeutics. For example, the inhibition miR-200 family play important role during CSC formations. On the other side, the Suz12 that represses Cdh1 in CSC growth is targeted by miR-200b. A study showed the miR-200b-Suz12-cadherin pathway for CSC growth and invasive ability in breast cancer (63). An animal model study demonstrated decrease CAMTA1 that can function as a tumor suppressor lead to reduce neurosphere formation and tumor growth (64). MiR-9 is highly expressed in glioblastoma stem cells can regulate the levels of CAMTA tumor-suppressor (64). The miR-10b regulates tumorigenesis in neurofibromatosis type 1 by targeting neurofibromin and RAS signaling (65). Furthermore, the CCCTC-binding factor (CTCF) that regulate several miRNAs such as miR-125b1, miR-375 and the miR-290 cluster has been considered to the attention in cancer therapy and regenerative medicine (66).

Screening microRNAs

Analysis of miRNA is not as easy as other nucleic acids since multiple issues impact the miRNA screening including miRNA length, distinction between pre, pri, and mature miRNAs, variable Tm of primers or probes, RNA ligase sequence bias, high degrees of homology in miRNA families and high rates of miRNA discovery. Combinations of these factors must be considered in designing miRNA detection experiments. Numerous reviews discussed the current methods and their advantages to detect miRNAs. Hence, we will not discuss this subject in details.

The extraction method chosen for small RNA preparation is an important aspect of any RNA analysis, although it is often overlooked in RNA analysis. It can have a downstream effect on the results obtained from a study. This issue has been reviewed by Nelson and their colleagues in 2009 (67). Reverse-transcription quantitative real-time PCR (RT-qPCR), in *situ* hybridization (ISH), and microarray have currently become the frequent techniques for miRNA quantification. ISH assay has been known to be a proper technique to determine miRNA expression in different cell types in complex organs or heterogeneous tumors. Furthermore, sequence-based methods which rely on the next generation sequencing machines have recently been known to be a fast and accurate method to detect unknown miRNAs (68).

Conclusions and future perspective

These documents suggest that dysregulation of miRNAs to be a main step in the development of retinoblastoma. Broad experimental verification in the

suitability of miRNA therapeutics for clinical purposes is currently requiring, emphasizing the need to interpret these predictions in diagnosis and treatment of this type of cancer. Manipulating miRNA expression and their regulators could be a novel approach for targeting cancer therapy and regenerative medicine. We believe that the further experiments into the role of miRNA in RB cancer not only yield valuable data on new potential target for diagnosis and therapy, but it can identify the possible underlying mechanisms in suppressing RB gene in other cancers.

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