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Research Article

MicroRNAs as Regulators of Neo-Angiogenesis in Hepatocellular Carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is a highly vascularized neoplasm. In the tumor niche, abundant angiogenesis is fundamental in providing nutrients for tumor growth and represents the first escape route for metastatic cells. Active angiogenesis, together with metastasis, are responsible for the reduction of recurrence-free survival of HCC.

MicroRNAs (miRNAs) are small non-coding RNAs that have recently drawn attention in molecular targeted therapy or as diagnostic and prognostic biomarkers. MiRNA expression in HCC has been widely studied in the last decade. Some miRNAs have been found to be up- or down-regulated, besides association with apoptosis, metastasis progression and drug resistance have been found. This review article aims to summarize the angiogenetic process in tumor diseases and to update on what has been found in the vast world of HCC-related-miRNAs and, eventually, to report the latest finding on several miRNAs involved in HCC angiogenesis. We searched the state of the arts for the 12 miRNAs found to be involved with angiogenesis in HCC (miR-29b, miR-126-3p, miR-144-3p, miR-146a, miR-195, miR-199a-3p, miR-210-3p, miR-338-3p, mir-491, mir-497, mir-638, mir-1301) and reported their main molecular targets and their overall effect in the sprouting of new vessels.

Introduction

MiRNAs are a subclass of non-coding RNAs, ~22-nucleotides long, firstly discovered in *C. elegans* [1,2], they participate in the post-transcriptional gene regulation by inhibiting the translation or determining the degradation of the target messenger RNA (mRNA) (Figure 1) [3]. MiRNAs are found in most eukaryotes, including humans, and they are present in all cell types and tissues. Even if miRNAs account for a small portion of the human genome (1-5%), they regulate around 30% of protein-coding genes.

It has been known that miRNA dysfunction is associated with apoptosis resistance and uncontrolled cellular proliferation. Emerging evidence also suggests that miRNAs dysregulation may be central in tumor angiogenesis and metastasis [4].

Hepatocellular carcinoma (HCC) represents the most frequent hepatic malignant neoplasm and is one of the leading causes of cancer-related deaths worldwide [5], with approximately 800,000 new cases and 745,500 death occurring each year [6]. Most of the new cases (85%) occur in Asia and sub-Saharan Africa, while the

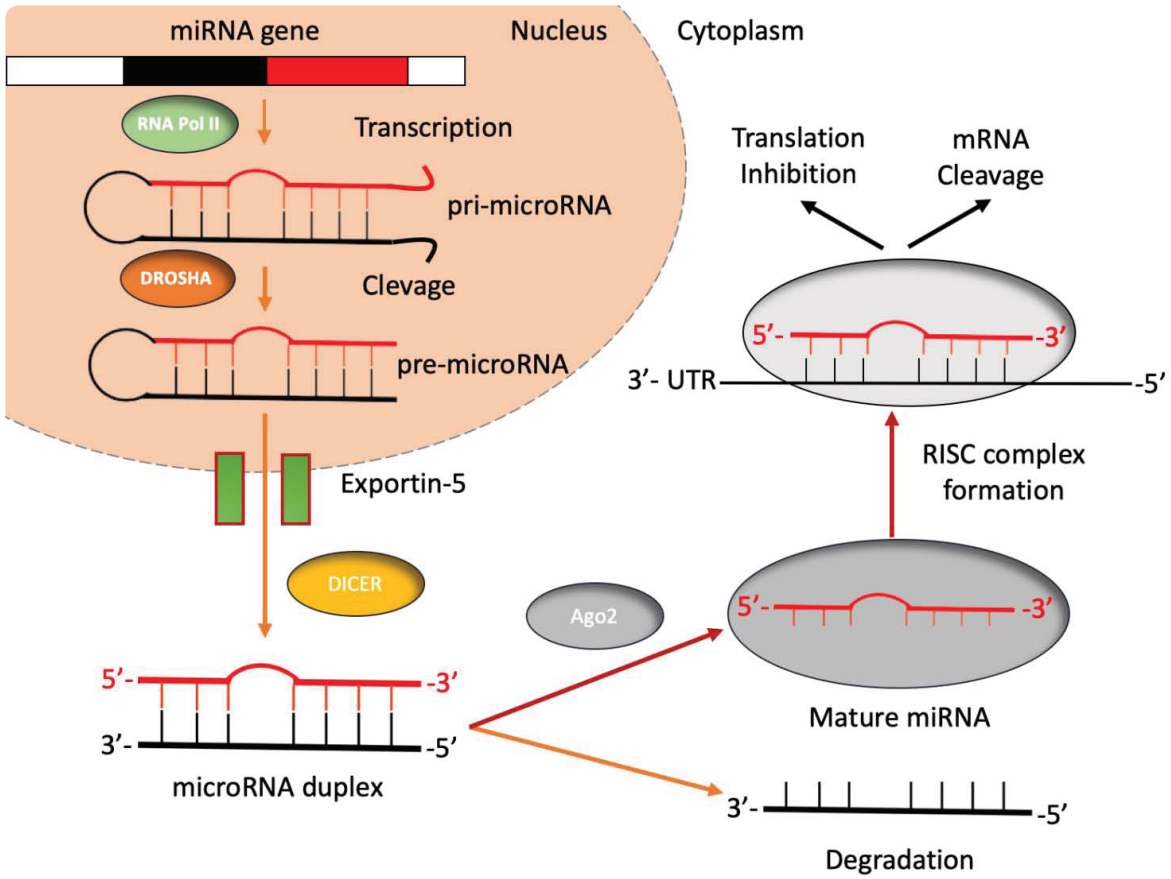

Figure 1: The biogenesis of miRNAs. MiRNA genes are transcribed by RNA polymerase II into a pri-miRNA, that may contain one to six miRNA precursors. The double strand RNA structure is recognized by a nuclear protein called Pasha (DGCR8) which binds the enzyme Drosha to release the pre-microRNA. Pre-miRNA are exported from the nucleus to the cytoplasm using the shuttle of Exportin-5. Once in the cytoplasm, the pre-miRNA is cleaved by the RNase III Dicer which interacts with the 3'-end and cuts the loop joining the 3'- and 5'- arms, producing a yet-not-final miRNA duplex (about 22 nucleotides in length). One strand of the mature miRNA is usually degraded, whereas one strand will become a mature miRNA which will be bound to an RNA-mediated silencing complex (RISC). In this complex, the mature miRNA targets the 3'-UTR region of its target mRNA to regulate its translation.

remaining 15% occur in western and European countries.

HCC is a highly vascularized neoplasm with frequent intrahepatic metastasis. In the tumor niche, abundant angiogenesis is fundamental in providing nutrients for tumor growth and represents the first escape route for metastatic cells. Active angiogenesis, together with metastasis, are responsible for the reduction of recurrence-free survival of HCC [7].

Although HCC treatment options have improved over the last decade, the survival rate of patients remains low. Hence, it is urgent to explore new therapies and to detect new and more accurate markers for early diagnosis, treatment, and prognosis in HCC. Nucleic acid-based therapies such as microRNAs (miRNAs) may have promising therapeutic potential for HCC treatment.

This review article aims to briefly overview the angiogenetic process in tumor and to update on the current knowledge of HCC-related-miRNAs and, eventually, to report the latest finding on miRNAs involved in angiogenesis in HCC.

Tumor Angiogenesis

Tumor angiogenesis is divided into three macro-stages: avascular, vascular and metastatic. During the avascular stage, the tumor mass (1-2 mm) gains nutrients by passive diffusion [8]. Thus, neo-angiogenesis plays an essential role in the growth of uncontrolled tumors. Tumor angiogenesis is a process that mimics the physiological one. New blood vessels are generated by proliferation, migration and subsequent differentiation of endothelial cells using pre-existing vascular architectures [9]. Parallel to the tubulogenesis of neo-vasculature, a process of demolition destroys the limits of the old vessels. This process involves degradation of vascular basement membrane and extracellular matrix.

In the beginning, both pro- and anti-angiogenic factors are counterbalanced. However, in response to the hypoxic stimulus, neoplastic cells undergo into an angiogenic switch. The production of pro-angiogenic mediators such as vascular endothelial growth factor (VEGF) and proteolytic enzymes [10] is increased with a side-by-side decline in anti-angiogenic factors such as angiostatin [11],

endostatin [12] and angiopoietin 2. VEGF is one of the most effective cytokines in the angiogenic process [13]. It is a family of structurally related molecules, which consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PGF (placental growth factor). The primary mediator in the angiogenetic process is VEGF-A (usually referred to as VEGF) [14,15].

VEGF is widely-expressed in most human cancers, and its increased expression is often associated with a less favorable prognosis. Induction of VEGF expression can be caused by a multitude of environmental factors such as hypoxia (HIF-1α), inflammatory cytokines (e.g., IL-6), low pH, growth factors, sex hormones, and chemokines (e.g., stromal-cell-derived factor 1).

VEGF interacts with cells essentially through VEGF Receptor 2 (VEGFR-2), which is expressed at a high level by endothelial cells undergoing angiogenesis. On the other hand, the role of VEGF Receptor 1 (VEGFR-1) regarding VEGF-mediated angiogenesis remains unknown. The VEGFR-1 binds to VEGF with approximately ten times the affinity of VEGFR-2, but its post-signaling effects are extremely weak [16].

The binding of VEGF to its receptor leads to a cascade of different signaling pathways. This binding results in the up-regulation of genes involved in cellular proliferation and migration of endothelial cells, promoting their survival and increasing vascular permeability. When VEGF binds to VEGFR-2, the receptor dimerizes, and activates the PLCγ-PKC-Raf kinase-MEK-MAPK pathway with the consequent initiation of DNA synthesis and cell growth driving the cell machinery to proliferation. At the same time, the activation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway leads to increased endothelial-cell overall survival. The activation of the src-pathway leads to the modification of actin cytoskeleton and the induction of cell migration [17]. These are the most relevant pathway involved in VEGF signal transmission. Several secondary intracellular interactions may occur simultaneously [18].

Regarding the role of the miRNAs in tumor angiogenesis, it has been demonstrated that several miRNAs can influence VEGF-A action. Some of them have been experimentally proven to directly target the 3'-UTR region of VEGF-A mRNA in different types of tumor. Further, several miRNAs have been identified as direct regulators of both VEGF-A and VEGF-C [19]. Besides, miRNAs can modulate VEGF expression by targeting VEGF inducers such as the HIF, PI3K/Akt mTOR and IGF1R pathways [20].

MiRNA and Hepatocellular Carcinoma

MiRNAs control several key-regulatory pathways in cells. Their aberrant expression may contribute to cancer tumorigenesis and progression. In particular, several miRNAs have been found to be up- or downregulated in HCC [47]. HCC usually originates from a cirrhotic liver, which may be the result of different liver injuries: each etiology causes a different miRNA expression profile in HCC cells [48]. For example, miR-217 could promote ethanol-induced fat storing in hepatocytes [49] and miR-126 was found to be downregulated in alcohol-induced cirrhosis (and HCC consequently) [50].

Considering the potential of miRNAs, several scientists are assessing their utility in HCC diagnosis, prognosis, and treatment.

Since laboratory biomarkers (such as AFP and des-γ-carboxy-prothrombin) have proven their lack of reliability, miRNAs have been investigated as possible diagnostic markers. More than twenty miRNAs can differentiate a healthy liver from one with chronic hepatitis or cirrhosis [51,52].

Altered miRNAs expression may lead to aberrant programmed cell-death. In HCC tissues, reduced expression of miR-101-3p and increased expression of miR-224-5p and miR-438-5p were associated with resistance to apoptosis. At the same time, apoptotic resistance was correlated with reduced expression in blood of miR-101-3p, miR-16-5p, miR-195-5p, miR203a-3p and miR-221-3p [53-56]. The measurement of these miRNAs in blood serum may help in assessing HCC prognosis.

Macroscopically, several miRNAs have been recognized to precisely stage patients with HCC [57]. In addition, miRNAs expression profile of primary HCC may predict early venous metastatic route [58].

Aberrantly expresses miRNAs signature in HCC make them potential diagnostic and prognostic tools, and may help in providing information on how the disease will progress or metastasize.

MiRNAs Involved in HCC Angiogenesis

Several miRNAs have been discovered involved in HCC angiogenesis. In the following section, we will report the most recent findings in miRNA-related angiogenesis in hepatocellular carcinoma.

Xiong et al. [59] discovered that miR-29b is down-regulated in HCC tissues and it was correlated with a worse recurrence-free survival period. In a sequent study they verified that miR-29b has the capability of suppressing HCC tube formation of endothelial cells and extracellular matrix invasion.

A human HCC clinical study discovered that miR-126-3p can predict the recurrence rate after liver transplantation and the reduction of metastasis rate in HCC [60]. An *in vitro* study also showed the importance of both gain and loss of function in miR-126-3p, while *in vivo* assay, miR-126-3p had an inverse correlation with vessel marker CD34. Further, tumor masses of miR-126-3p overexpressing cells had a smaller diameter of vessels, which may be related to the low-rate formation of new micro-vessels. It was demonstrated that the overexpression of miR-126-3p significantly inhibited PI3KR2/P-Akt pathway.MiR-144-3p has also been found to influence the PI3K proto-oncogenic pathway by the suppression of SG3K activity, thus modulating angiogenesis [61]. Two other miRNAs have been found to both influence the PI3K pathway and the VEGF expression from liver cancer cells: miR-199a-3p which suppressed tumor angiogenesis by directly reducing VEGF-A secretion from cancer cells and suppressing the expression of VEGFR1 and VEGFR2 on endothelial cells [62]; whereas miR-497 directly suppressed VEGF-A expression by binding to the 3'-UTR of VEGF-A mRNA. [63,64].

Several miRNAs have been proven to interact with VEGF and VEGF-R. For example, miR-195 directly inhibits expression of VEGF and other factors (e.g., VAV2 and CDC42) with pro-metastatic activity. MiR-195 down-regulation resulted in increased VEGF concentrations in the tumor niche, thus allowing more VEGF molecules to bind with their receptor to proceed with signal transmission [26]. MiR-451 suppresses VEGF production in HCC cells by targeting IL-6R-STAT3 signaling, as well as inhibiting the VEGFR2 signaling *in vitro* [65]. IL-6/STAT-3 signaling is also modulated by SMAD3 which is the target of miR-491, whose overexpression leads to a reduction

in VEGF secretion [66]. MiR-1301 reduced tumor angiogenesis by down-regulation of VEGF-A expression [67]. MiR-638 was able to suppress HCC angiogenesis by reducing VEGF expression; however, the exact mechanisms remain unknown [31].

Other miRNAs influenced VEGF expression through one of its stimulants: HIF-1α. MiR-210-3p has been found to be upregulated in HCC and to be associated with increased microvessel density (MVD). This miRNA is induced by low oxygen levels and modulates angiogenesis via HIF-1α/3α regulatory feedback circuit [68]. The overexpression of miR-338-3p has been correlated with decreased VEGF, GLUT-1 and MDR1, which are all regulated by HIF-1α [69]. At the same time, suppression of miR-338-3p showed increased VEGF expression in HCC cells [70].

Zhu et al. [71] demonstrated that miR-146a enhances the angiogenic activity of endothelial cells, by promoting the expression of platelet-derived growth factor receptor α (PDGFRA) and the involvement of BCRA-1 gene. MiR-146a was found to inhibit HCC invasion and metastasis through the upregulation of APC and the downregulation of VEGF [72].

Challenges in miRNA Delivery

The therapeutic application of miRNAs involves two main strategies. Administration of miRNA antagonists downregulates the gain of function in an oncogenic miRNA [73]. The miRNA antagonists are oligonucleotides targeting the endogenous miRNA and blocking them into a configuration unable to be processed by RISC, resulting in an accelerated degradation. Another strategy involves the replacement of a tumor suppressor miRNA to restore the loss of function through the so-called miRNA mimics [74].

One of the significant challenges in the use of miRNA as therapeutics is represented by the successful delivery of miRNA to the target tumor tissue. This may not be effective due to the leaky endothelial barrier [75] and the complexity of the extracellular matrix surrounding the tumors [76]. Another challenge that remains is to preserve the stability and integrity of miRNAs in circulation. Naked miRNAs are degraded within seconds after entering the bloodstream because of the presence of a serum RNase A-type nuclease in the circulating blood [77]. Besides, naked miRNAs are rapidly cleared via renal excretion [78].

However, much work has been done in exploiting and evaluating the characteristics of tumor microenvironment to improve miRNA delivery. It was shown that effective *in vivo* gene-silencing had been accomplished by intratumoral injection or local administration without carriers [79]. In systemic route delivery, several significant progress has also been demonstrated. For example, miRNAs can be tailor-modified to be resistant to degradation by blood RNase [80], they can be enveloped by "smart" nanoparticle coats whose retention could be tissue-specific [81], or they can be delivered using viral vectors [82].

MiRNA in HCC Treatment

The recent success of a human trial using miravirsen, a miR-122 inhibitor, in the treatment of HCV infection has witnessed significant interest in miRNA therapeutics [83]. Up to now, several clinical trials involving miRNA analogous or antagonist are under evaluation [84]. Unfortunately, none of the proposed drugs are focused on HCC. However, clinical trials for miR-145, miR-451, miR-195, and miR-107 have been already established for vascular disease targeting. Other pre-clinical phase trials against HCC are under evaluation in murine models. In healthy liver tissue, miR-26a exhibits high expression, and it is downregulated in both human and murine HCC tumors. MiR-26a directly target cyclins D2 and E2 inducing G_1 arrest of liver cancer cells. In the experiments performed by Kota et al. the administration of miR-26a, through adenovirus vectors, resulted in drastic inhibition of cancer cell proliferation with induction of tumor-specific apoptosis [85].

Other evidence proved the anti-cancer effects of miRNA-antagonists. Callegari et al. [86] created a murine model of miR-221 overexpression leading to spontaneous liver multiple tumors. Three intravenous doses of anti-miR-221 oligonucleotide (AMO) separated by a 15-days interval induced a reduction in the size of liver tumor nodules in treated mice. In addition, intratumoral subministration of miR-520e oligonucleotides repressed HCC growth [87].

No direct evidence linking those miRNA mimics/inhibitors to angiogenesis are reported in the aforementioned studies, however, considering the pathways targeted by those miRNAs further investigations are needed to clarify the role of these candidate miRNA therapeutics on tumor angiogenesis.

Discussion

HCC is the fifth most common cancer in men, and the ninth in women worldwide [88]. The median survival following the diagnosis is poor, ranging from four to twenty months [89]. Potentially therapeutic approaches (e.g., liver transplantation or ablation) can result in 5-year overall survival > 70% but apply to less than 30% of patients with HCC [90]. Currently, viable treatment for those patients who are in an intermediate or advanced cancer is limited and is considered as a palliative.

Over the last decade antiangiogenic molecules that inhibit VEGF or its receptor have been approved for HCC treatment, for example, sorafenib for advanced-stage HCC [91] or chemotherapy in combination with bevacizumab for metastatic colorectal cancer [92]. Sorafenib, a multi-kinase inhibitor, is currently the only systemic drug approved for use as a first-line treatment in advanced HCC by the FDA. According to Llovet et al. [91] patients in the sorafenib group had a median survival higher than the placebo group (10.7 v.s. 7.9 months respectively). Besides, sorafenib is a safe and well-tolerated drug, the most common adverse effects include diarrhea, fatigue, hand-foot skin reaction, and hypertension. Most of these effects are considered mild and manageable; however cardiovascular events may be fatal [93].

The utilization of anti-VEGF/R molecule has seemed to be fundamental following transcatheter arterial chemoembolization (TACE). TACE occludes inner vessel within the tumor mass, in the meanwhile tumor progression may occur due to neovascularization at the edge of the tumor, where the procedure can increase the angiogenesis rate secondary to hypoxia [94]. Hypoxia-induced factors upregulate VEGF inducing a surge of VEGF. After embolization VEGF rises up to 160% of the baseline on day 1, reverting back to 130% on day 2 and 120% on day three [95]. For this reason, there has been considerable interest in combining TACE with sorafenib [96], weighing that high serum levels of VEGF had been associated with poor prognosis after TACE in HCC [97].

Oral regorafenib is as a second-line drug and is the first systemic agent approved for treatment of patients who had diseases progression during sorafenib treatment [98]. As well as sorafenib, regorafenib is a multiple kinase inhibitor, targeting kinases involved in angiogenesis (VEGFR) and oncogenesis (KIT, RET, c-RAF). In the RESORCE trial, administration of regorafenib resulted in overall survival of 10.6 months (v.s. 7.8 months in the placebo group) and doubled progression-free survival and time to progression (3.1 and 3.2 months respectively) in comparison to the placebo group [99]. Adverse effects include hand-foot skin reaction [100], hypertension [101], stomatitis [102], diarrhea, hyperbilirubinemia and fatigue [103,104].

That being said, even if sorafenib and regorafenib appear to be important tools in the hands of the clinicians, the overall survival is not comparable to more therapeutic approaches such the ones applied to early-diagnosed HCC. Thus, there is still a constant need for the development of novel therapeutic tools. The ability of miRNAs to regulate fundamental cellular processes by concurrently intervening in multiple pathways illustrates their potential role. MiRNA-based therapy holds great hope as highly specific, target-delivery for cancer treatment. Nevertheless, to reach superior sensitivity and specificity, and accelerate their adoption in the every-day clinical scenario, there is still much work to do. For example, it is compelling to better define the tangled network of interactions between miRNAs and the human genome and its products. Besides, a full assessment of their toxicological activity needs to be performed (nucleic acid administration can trigger the immune system promoting immuno- and neuro-toxicity) [105,106].

MiRNAs hold a great responsibility in the future of target therapy.

Table 1: miRNA and its associated target/pathway. In the table are reported miRNAs that interfere with VEGF-A, VEGF-C, HIF, PI3K/Akt, mTOR, and IGF1R.

Target	miRNAs
3'-UTR of VEGF-A	MiR-20 [21], miR-29b[22], miR-93 [23], miR-126[24], miR-190 [25], miR-195[26], miR-200[27], miR-203 [28], miR-497 [29], miR-503 [30]and miR-638[31]
3'-UTR of VEGF-C	MiR-27b [32]and miR-128[33]
HIF Pathway	miR-22[34], miR-107[35], miR-519c [36], miR-145 [37]
PI3K/Akt Pathway	miR-26a[38], miR-145 [39]
mTOR Pathway	miR-18a[40], miR-128[41], miR-145 [42] miR-218 [43]
IGF1R Pathway	miR-126 [44], miR-181b [45], miR-148a and miR-152 [46]

Exciting results have been obtained in murine models of HCC and in human trials of HCV infection. Considering the importance of angiogenesis in HCC, we believe that miRNAs involved in neo-vasculature formation will lead the investigation in the discovery of new anti-angiogenetic drugs that might result in better therapeutic outcomes.

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