MicroRNAs Involved in Metastasis of Hepatocellular Carcinoma: Target Candidates, Functionality and Efficacy in Animal Models and Prognostic Relevance

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Abstract. Hepatocellular carcinoma (HCC) is responsible for the second-leading cancer-related death toll worldwide. Although sorafenib and levantinib as frontline therapy and regorafenib, cabazantinib and ramicurimab have now been approved for second-line therapy, the therapeutic benefit is in the range of only a few months with respect to prolongation of survival. Aggressiveness of HCC is mediated by metastasis. Intrahepatic metastases and distant metastasis to the lungs, lymph nodes, bones, omentum, adrenal gland and brain have been observed. Therefore, the identification of metastasis-related new targets and treatment modalities is of paramount importance. In this review, we focus on metastasis-related microRNAs (miRs) as therapeutic targets for HCC. We describe miRs which mediate or repress HCC metastasis in mouse xenograft models. We discuss 18 metastasis-promoting miRs and 35 metastasis-inhibiting miRs according to the criteria as outlined. Six of the metastasis-promoting miRs (miR-29a, -219-5p, -331-3p, 425-5p, -487a and -1247-3p) are associated with unfavourable clinical prognosis. Another set of six down-regulated miRs (miR-101, -129-3p, -137, -149, -503, and -630) correlate with a worse clinical prognosis. We discuss the corresponding metastasis-related targets as well as their potential as therapeutic modalities for treatment of HCC-related metastasis. A subset of up-regulated miRs -29a, -219-5p and -425-5p and down-regulated miRs -129-3p and -630 were evaluated in orthotopic metastasis-related models which are suitable to mimic HCC-related metastasis. Those miRNAs may represent prioritized targets emerging from our survey.

Hepatocellular carcinoma (HCC) is the second-leading cause of cancer-related death worldwide (1). In the next couple of years, an annual incidence of one million cases is expected (1). Risk factors for HCC are non-alcohol steatohepatitis, hepatitis B and C virus (HBV/HCV), alcohol and aflatoxin (2). In Asia and Africa, 60% of HCC cases are associated with HBV, 20% are related to HCV (2). Patients with early- and intermediate-stage HCC are treated with locoregional therapies, those with advanced disease receive systemic treatment (3). Sorafenib, a multikinase tyrosine kinase inhibitor was the only approved agent between 2007 and 2016 (4). This agent gives rise to only marginal therapeutic benefit. Recently, therapeutic benefit was shown with multikinase inhibitors levantinib as frontline therapy, and regorafenib, cabazantinib and ramicurimab, a monoclonal antibody directed against vascular endothelial growth factor receptor 2, as second-line therapies (4). In addition, nivolumab, a monoclonal antibody directed against programmed cell death protein 1 (PD1) has been granted accelerated approval by the Food and Drug Administration for treatment of HCC and other checkpoint inhibitors are undergoing phase III clinical trials for this indication (5). Unsupervised clustering has revealed three subtypes of HCC based on transcriptional profiling (6) and immune phenotyping with respect to lymphocyte infiltration has discovered HCC with high, moderate and excluded tumors, pointing to...
opportunities for personalized therapies for HCC (1, 4). Since the increment of therapeutic benefit for most drugs is relatively modest, identification of new targets and treatment modalities is of paramount importance. In this review, we focus on metastasis-related microRNAs (miRs) with in vivo efficacy in corresponding preclinical models of HCC.

**HCC: Metastasis-related aspects.** Several reports describe the study of HCC-related metastasis (7-12). Intrahepatic metastasis is an important route of metastasis of HCC. In this type of tumor, the blood flow is abnormal and the microvessels are extremely leaky, resulting in promotion of metastasis (13). The most frequent sites of distant metastases are the lung, lymph nodes, omentum, adrenal glands and brain (7-12). Bone metastases of HCC are associated with particularly poor prognosis (14). The molecular mechanisms promoting intrahepatic metastases and dissemination to distant organs have only poorly been resolved.

**microRNAs and their role in cancer.** miRs are RNAs composed of 20-22 nucleotides which post-transcriptionally inhibit mRNA targets in several eukaryotic cell lines and tissues (15). They mediate degradation or translational inhibition of miRNAs by binding to complementary nts of corresponding miRNAs (15, 16). More than 1,000 miR-related genes have been identified in the human genome. The vast majority of them are transcribed to RNA polymerase II (17, 18). They are synthesized as primary mRNA precursors, processed in the nucleus by endonucleases such as DROSHA and microprocessor complex subunit Di George syndrome chromosomal region 8 (DGCR8), transported to the cytoplasm and cleaved by RNase III DICER (19). Finally, the mature miR duplex forms the RNA-induced silencing complex (RISC) which recognizes complementary sequences in the 3'-untranslated region (3'-UTR) of the target miRNAs, leading to their degradation or inhibition of their translation (20). A single miR can target multiple miRNAs and vice versa, multiple miRs can target the same mRNA (21). Therefore, inhibition or reconstitution of the function of miRs can interfere with several pathways and cellular networks (21). The relevance of miRs in cancer has been demonstrated first for miR-15a and -16-1 in the pathogenesis of B-cell chronic lymphocytic leukemia (22, 23). The role of miRs in metastasis has been documented in several types of cancer (24-27). In this review, we focus on the role of miRs involved in metastasis of HCC. We have selected miRs with in vivo efficacy in preclinical metastasis-related models of HCC (28, 29).

**Up-regulated miRs Mediating Metastasis**

miRs targeting tumor-suppressor genes. miR130b, -425-5p and -429 (Figures 1 and 2A) target phosphatase and tensin homolog (PTEN) which functions as a tumor suppressor in HCC and many other types of tumors (30, 31). miR-130b (Figure 1) promotes proliferation and invasion of HepG2 and HCCLM3 cells in vitro and increases intrahepatic and lung metastases of these cell lines. Metastasis-mediated by miR-130b is driven by PTEN/AKT/hypoxia-inducible factor-1α (PTEN/AKT/HIF-1α) signaling (32). High levels of miR-130b correlate with poor overall survival of patients with HCC (32). Stem cell antigen-1 (SCA1) and PTEN have been identified as direct targets of miR-425-5p (Figures 1 and 2B) (33). SCA1 localizes to the nucleus and inhibits migration through transcription of integrin β1 and binds to and inhibits myelin and lymphocyte protein by forming a ternary complex with serum response factor (34). miR-425-5p (Figures 1 and 2B) promotes invasion and migration, but not proliferation of HCCLM3 cells in vitro. It further mediates epithelial mesenchymal transition (EMT), local growth of HCCLM3 in the spleen as well as intrahepatic metastasis (35). In addition to PTEN/AKT, integrin β1/focal adhesion kinase (FAK)/SRC (SRC), Ras homology family member A/cell-division control protein homolog 42 (RHOA/CDC42) and tissue inhibitor of metalloproteinases 2/matrix metalloproteinase 9 (TIMP2/MMP9) signaling are affected by miR-425p (35). It is a marker for poor prognosis in patients with HCC and long-term post-operative survival (35) and is up-regulated in HCC versus normal liver tissues according to data derived from The Cancer Genome Atlas (TCGA) (Figure 3).

miR-429 (Figure 1) induces invasion of HCCLM3 and SMM-7721 cells in vitro (35). miR-429 activates phosphoinositol 3-kinase (PI3K)/AKT/glycogen synthase kinase 3β (PI3K/AKT/GSK3β) by inhibition of PTEN signaling, resulting in nuclear translocation of β-catenin (35) (Figure 1). In vivo, liver and lung metastases are enhanced by miR-429 after tail vein injection of HCCLM3 and MHCC97L cells transplanted with miR-429 (35).

miR-135a (Figure 1) promotes invasion of HCC cell lines in vitro and reduction of its expression in CSQT-2 cells inhibits intrahepatic metastasis in vivo (36). Metastasis suppressor 1 (MTSS1) has been identified as a direct target of miR-135a (36). MTSS1 functions as a tumor suppressor in gastric cancer and HCC, and interacts with the actin cytoskeleton (36, 37). Another investigation revealed down-regulation of Krüppel-like factor 4 (KLF4) as a direct target of miR-135a in SK-Hep1 HCC cells. KLF4 is a zinc finger transcription factor which is down-regulated by transforming growth factor β (TGFβ) and functions as a regulator of the cell cycle, proliferation and apoptosis, and as a tumor suppressor in HCC (38, 39). miR-135a mediated increased lung metastasis after tail vein injection of SK-Hep1 cells expressing miR-135a in comparison to the control cell line (40).

miR-362-5p (Figure 1) is up-regulated in HCC, is associated with HCC progression (41) and promotes cell growth, invasion and migration of HL-7702 and SMC-7721 cells in vitro. Subcutaneous implantation of HepG2 cells expressing miR-362-5p results in increased lung metastasis in comparison to
miR-362-5p targets cylindromatosus (CYLD), a deubiquitination enzyme which acts as a tumor suppressor in HCC and other types of cancer (42). CYLD interacts with various signaling pathways including TGFβ, wingless-integrated (WNT)/β-catenin and c-jun N-terminal kinase and inhibits NFκB signaling, resulting in cell proliferation, survival, invasion and metastasis (43).

miR-382-5p (Figure 1) is an essential member of the HBV-regulated miR network. miR-382-5p promotes cell motility in vitro and lung metastasis of transfected HepG2 cells after tail vein injection (44). It targets deleted in liver cancer 1 (DLC1), a tumor-suppressor gene in liver cancer (45). DLC1 contains a Rho GTPase activating protein domain, a sterile alpha motif (SAM) and a star-related lipid transfer domain (START) domain (46). Loss of DLC1 leads to aberrant Rho GTPase function and contributes to abnormal migration and metastatic properties.

miRs interfering with cell signaling and cell cycle. miR-21: It has been observed that colony-forming unit endothelial cells (CFU-ECs) induced chemotaxis through RAS-related C3 botulinum toxin substrate 1 (RAC1) and MMP9 activation mediated by monocyte chemoattractant protein-1 (MCP1) released from CFU-ECs (47). From a mechanistic point of view this phenomenon is due to induction of miR-21 by interaction of MCP1 with C-C chemokine receptor 2 (CCR2) on tumor cells (47-49). miR-21 mediated metastasis by directly targeting Rho GTPase-activating protein 24 (ARHGAP24) and tissue inhibitor of metalloproteinases 3 (TIMP3) (47, 50, 51). Inhibition of ARHGAP24 activated RAC1 and inhibition of TIMP3 activates MMP9, both mediators of metastasis (50, 51). Huh7 HCC cells co-cultured with CFU-ECs formed multiple nodules after orthotopic implantation into the liver of mice and inhibition of miR-21 in Huh7 cells attenuated intrahepatic metastasis (47).

miR-29a (Figure 2A) promoted metastasis by silencing suppressor of cytokine signaling 1 (SOCS1), an inhibitor of metastatic signal transduction (52, 53), due to methylation of its DNA, and subsequent activation of Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling (52). miR-29a directly targets ten eleven translocation enzymes (TET) which convert 5-methylcytosine to 5-hydroxy-
Figure 2. Metastasis-promoting miRs in hepatocellular carcinoma with in vivo activity in metastasis-related models and correlation to poor prognosis in patients. A: miR-29a, miR-219-5p and miR-331-3p; and B: miR-425-5p, miR-487a and miR-1247-3p. 5CH₂OH-C: 5 Hydroxymethyl-cytosine; 5CH₃-C: 5 methyl-cytosine; AKT: serine-threonine AKT; B4GALT3: β-1,4-galactosyltransferase 3; CAF: cancer-associated fibroblast; CDH1: cadherin 1, E-cadherin; G2/M: G2/M or S-phase of cell cycle; GSK-3β: glycogen synthase kinase 3β; IL6,8: interleukin 6,8; INT β1: integrin β1; JAK: Janus kinase; MAPK: mitogen-activated protein kinase; NFκB: nuclear factor κB; PHLPP: PH domain and leucine rich repeat protein phosphatase; PI3K: phosphatidylinositol-3 kinase; PIK3R1: phosphatidylinositol-3 kinase regulatory subunit 1; PTEN: phosphatase and tensin homolog; SCA-1: stem cell antigen-1; SNAIL: transcription factor SNAIL; SPRED2: Sprouty-related and EvH1 domain-containing protein 2; STAT3: signal transducer and activator of transcription 3; TC: tumor cell; TET: ten-eleven translocation methylcytosine-dioxygenase.
methylcytosine, resulting in inhibition of SOCS1 promoter demethylation (52-54). miR-29a promotes proliferation and invasion of HCC cell lines SMMC-7721 and HCCLM3 and increases tumor growth and lung metastasis of orthotopically implanted HCCLM3 and SMMC-7721 HCC cells transfected with mir-29a (52). mir-29a overexpression is correlated with poor clinical outcome in patients with HCC and inhibition of TET-SOCS mediated signaling in HCC tissues.

miR-135b promoted migration and invasion of HCC cell lines SMMC-7721 and Huh7 (55). Orthotopic implantation of SMM-7721 cells transfected with miR-135b revealed that miR-135b induced formation of metastatic nodules in the liver (55). Reversion inducing-cysteine-rich protein with kazal motifs (RECK) and ectopic viral integration site 5 (EV5) were identified as direct targets of miR-135b (55). RECK is a membrane-anchored MMP inhibitor which acts as a key regulator of ECM integrity and angiogenesis (56). Suppression of RECK by miR-135b resulted in activation of MMP2 and MMP9 (55). EV5 is a regulator of cell-cycle progression and in addition functions as an inhibitor of F-actin (55, 57). Down-regulation of EV5 by miR-135b increases invasion and migration of HCC cells (55).

miR-192-5p promoted cell proliferation and metastasis of HCCLM3 cells, targeting the cancer stem cell (CSC) population of this cell line (58). Semaphorin 3A, a suppressor of angiogenesis (59) was identified as a direct target of miR-192-5p (58).

miR-331-3p (Figure 2A) was found to promote proliferation and migration of HCC cell lines HCCLM3, HepG2 and Huh7 (60). In these cell lines, miR-331-3p promotes intrahepatic metastasis and metastasis to the lungs after intrahepatic administration (60). PH domain and leucine repeat protein phosphatase (PHLPP) was identified as a direct target of miR-331-3p (60, 61). Down-regulation of PHLPP activates AKT/GSK-3β/SNAIL metastasis-promoting signaling (60) and mediates entry of HCC cells into the S or G2/M phase of the cell cycle (60). Increased expression of miR-331-3p correlates with poor long-term survival of patients with HCC (60).

miR-487a (Figure 2B) induced migration and wound healing in HCC cell lines such as HCCLM3 and HepG2 (62). An orthotopic mouse model with HCCLM3 cells injected into the liver revealed promotion of growth, intrahepatic metastasis and distant metastasis to the lungs mediated by miR-487a (62). Sprouty-related EVH1 domain containing 2 (SPRED2) and phosphoinosine-3-kinase regulatory subunit 1 (PIK3R1) were identified as direct targets of miR-487a (62). SPRED2 acts as an inhibitor of the mitogen-activated protein kinase (MAPK) cascade (63, 64) and deletion of PIK3R1 has been shown to activate AKT and to inhibit PTEN (65, 66). miR-487a is highly expressed in HCC and its expression correlates with poor postoperative prognosis of patients with HCC (62). Down-regulation of miR-487a in HCC in comparison to normal liver was not confirmed through the TCGA data set (Figure 3).

miRs promoting EMT or affecting the ECM. Exosomes from highly miR-103-expressing HCC cells can interact with endothelial monolayers (67). miR-103 destabilizes tight junctions by targeting vascular endothelial cadherin, β120 catenin and zona occludens 1, leading to destabilization of endothelial integrity (68). miR-103 was also found to promote migration of HCC cells by targeting p120 catenin (67, 69). QGY-7703 cells transfected with miR-103 as xenografts gave rise to high rates of hepatic and pulmonary metastases in comparison to the control cell line (67).

miR-144 was found to be significantly up-regulated in patients with HBV-HCC (70). Transcription factor NFkB up-regulated miR-143, resulting in migration and invasion of HCC cell lines HepG2 and Huh7 (70). Fibronectin-type III domain containing 3B (FNDC3B) has been identified as a direct target of miR-143 (70, 71). FNDC3B was previously described to be down-regulated in tumor cells with high metastatic potential (72). HepG2 cells overexpressing miR-103 orthotopically transplanted into nude mice gave rise to intrahepatic and distant lung metastases in contrast to the control cell line (70).

miR-186 is repressed by metastasis inhibiting runt-related transcription factor 3 (RUNX3) and inhibits E-cadherin (CDH1) expression by binding to the 3'-UTR of its mRNA (73). miR-186 mimics reduced the expression of CDH1 in HCC cell lines HepG2 and Hep3B (73). RUNX3 suppressed migration, invasion and angiogenesis of human renal cell carcinoma (74) and the gene encoding RUNX3 is methylated in various types of cancer (75).

miR-219-5p (Figure 2A) can promote proliferation, cell-cycle transition from G1 into S-phase and increase the anti-apoptotic potential of HepG2 and MHCC-97H cells (76). CDH1 mRNA has been identified as a direct target of miR-219-5p (76). CDH1 inactivation results in loss of cell-cell adhesion which contributes to metastasis in a variety of tumors (77-79). Antagonisms of miR-219-5p transfected into MHCC-97H cells and implanted into the livers of nude mice reduced the volume of tumors and the total number of lung metastasis (76). Up-regulation of miR-219-5p is associated with metastasis and dismal prognosis in patients with HCC (76).

miRs promoting metastasis by interference with sugar-modifying enzymes. It is well documented that altered glycosylation of tumor cells can promote metastasis (80, 81). miR-23 is up-regulated in metastatic mouse HCC cell lines such as Hca-P and Hepa 1-6, and promotes migration in vitro (82). Subcutaneous injection of Hca-P cells transfected with a miR-23 mimic promoted metastasis to the inguinal lymph nodes (82).
Mannoside acetylgalcosaminyl-transferase 3 (MGAT3), an enzyme catalysing transfer of acetyl-glucose in a β1,4 linkage to mannose on N-glycans and thus forming a bisected acetyl-glucose structure, was identified as a direct target of miR-23 (82). MGAT3 has been reported to inhibit cell migration and to modulate cell adhesion, indicating a relationship between aberrant glycosylation and metastasis (83).

Exosomes secreted from HCC cells in the pre-metastatic niche contain miR-1247-3p (Figure 2B) which activates fibroblasts in the neighbourhood to associated fibroblasts promoting cancer progression by secreting pro-inflammatory cytokines such as interleukin (IL) 6 and IL8 (84). miR-1247-3p targets β1,4 galactosyltransferase 3 (B4GALT3) leading to activation of β1 integrin–NFκB signaling in fibroblasts of the lung premetastatic niche of liver cancer (84). B4GALT3 transfers galactose to N-acetylgalucosamine to form N-acetyllactosamine in glycosylated proteins (85). B4GALT3 suppresses integrin β1-mediated phenotypes and metastasis (86, 87). Lung metastasis of subcutaneously implanted HCC cell line SMMC-7721 was promoted by intravenously administered exosomes from CSQT-2 and HCC-LM3 cells (84). In patients with HCC, high expression of miR-1247-3p in exosomes shows a positive correlation with lung metastasis (84). miR-1247-3p was down-regulated in HCC compared to normal liver samples in the available TCGA data set (Figure 3).

**Down-regulated miRs Which Inhibit HCC Metastasis**

**miRs affecting angiogenesis.** Down-regulation of miR-100 and miR-125b (Figure 4A) in HCC tissues is associated with the presence of blood vessels encapsulating tumor cells (VETCs) facilitating their entry into the bloodstream (88). In a mouse orthotopic xenograft model, expression of miR-100 and miR-125b suppressed VETC formation and abrogate VETC-dependent metastasis (88). In xeno- and allografts expressing miR-100 or miR-125b much lower rates of hepatic and pulmonary metastases were observed with human VETC-2 and mouse Hepa 1-6 cells in comparison to control cell lines (88). miR-100 and miR-125b inhibit VETC formation by attenuation angiopoietin 2 (ANGPTN 2) expression (88, 89). For miR-125b, ANGPTN2 has been identified as a direct target; miR-100 targets mammalian target of rapamycin (mTOR) directly, resulting in attenuation of ANGPTN2 expression (88, 90).

**miR-125a (Figure 4A)** is down-regulated in HCC-related tissues and cell lines (91). Ectopic expression of miR-125a inhibited proliferation of HepG2 and HCC-LM3 HCC cells and miR-125a directly targets vascular endothelial growth factor (VEGF) and MMP11, both mediators of metastasis (91-94). Increased expression of VEGF in HCC is associated with high proliferation index and poor encapsulation of tumors (92, 93). HCC-LM3 cells expressing miR-125a showed reduced metastasis to the lungs and liver after tail vein injection (91).

**miR-195 (Figure 4A)** is down-regulated in HCC and is associated with worse prognosis (95). miR-195 inhibited HCC cell line QGY-7703 promoted migration of endothelial cells and suppresses in vitro migration and invasion of QGY-7703 HCC cells (95). In an orthotopic xenograft model, miR-195 suppresses intrahepatic and pulmonary metastasis (95). miR-195 inhibits angiogenesis and metastasis by targeting VEGF, guanine nucleotide exchange factor VAV2 and cell division control protein 42 (CDC42) (95). VAV2 functions as a guanine nucleotide exchange factor and CDC42 is a member of the Rho family of GTPases. Both are involved in regulation of the cell cycle and in attenuating pro-invasive functions of the cytoskeleton (96).

**miR-199a-3p (Figure 4A)** is down-regulated in HCC and inhibits tumor–stroma cross-talk (97). VEGF is a direct target of miR-199a-3p, which inhibits VEGF secretion from tumor cells as well as expression of VEGF receptors 1 and 2 on endothelial cells and thus restricts the cross-talk between tumor and endothelial cells (97). As further direct targets, hepatocyte growth factor (98) and MMP2 (99) have been identified as mediators of metastasis. miR-199-3p inhibits growth and lung metastasis of SNU449 HCC cells subcutaneously implanted into nude mice (97).

**miR-497 (Figure 4A)** is down-regulated in HCC-related cells and tissues (100). Overexpression of miR-497 in Huh7 and PLC-PRF-5 HCC cell lines inhibits their migration, invasion and pro-angiogenic activity (100). miR-497-transfected Huh7 cells gave rise to smaller tumors in comparison to the non-transfected cell line after subcutaneous implantation and reduced the number of lung nodules after tail vein injection (100). Direct inhibition of VEGF by miR-497 inhibits angiogenesis. Astrocyte elevated gene 1 (AEG1) has been identified as another direct target of miR-497 (100). AEG1 promotes tumor cell invasion and metastasis by activation of PI3K/AKT and WNT/β-catenin pathways (100-102).

**miRs interfering with signaling, cell cycle and cytoskeleton function.** Ectopic expression of miR-7 reduced invasion and migration of QGY-7703 cells in vitro (103). Metastatic nodules in the lungs and the liver were repressed after tail vein injection of QGY-7703-mir-7 transfected HCC cells (103). Tumor growth of these cells was inhibited after scapular implantation in comparison to control cells (103). PI3K subunit delta, the major component of PI3K, was identified as a direct target of miR-7 (103). Down-regulation of miR-7 results in activation of AKT-mTOR signaling (104, 105). An inverse correlation between expression of miR-7 and PIK3CD in clinical HCC specimens has been observed (103).

**miR-10a inhibited metastasis of HCC-related QGY-7703 and HepG2 cells to the liver after intrasplenic implantation (106).** EPH tyrosine kinase receptor EPH4 was identified as a direct target of miR-10a (106). Epha4 binds to EPH-
receptor-interacting proteins A and B and mainly affects cell shape and motility by regulating cytoskeletal organization and cell adhesion (106). miR-10a and EPHA4 affect cell adhesion via the β1 signaling pathway (106). miR-10a probably suppresses homing of metastatic HCC cells to distant organs (106).

miR-26a is frequently down-regulated in HCC tissues and targets IL6/STAT3 and MYC/enhancer zeste homolog 2 (EZH2)-mediated metastasis (107, 108). In HCC cell lines HCC-LM3 and MHCC97-H, miR-26a induced G1 arrest and promotes apoptosis (108). In an intrahepatic nude mouse model, growth and metastasis to the lungs of HCC-LM3 and MHCC97-H cells were inhibited by miR-26a. These observations are based on direct inhibition of IL6 mRNA by miR-110 (108). IL6 promotes tumor cell growth and invasion via STAT3 signaling in many types of tumors (109, 110). In a different system miR-26a interfered directly with several targets such as cyclin-dependent kinase 8 (CDK8), p21 activated protein kinase 2 (PAK2) and EZH2 (108). Inhibition of CDK8, a co-activator of WNT signaling, results in reduced expression of c-MYC (110, 111). PAK2 mediates invasion of HCC cells by linking Rho GTPases to cytoskeleton reorganization during the metastatic process (112). EZH2, a member of the polycomb group of proteins, can repress tumor suppressor miRs by H3K27-dependent and -independent mechanisms (113, 114).

TGFβ suppressed expression of miR-34a and is associated with persistent presence of HBV in liver tissue (115). Chemokine (C-C motif) ligand 2 (CCL2) was identified as a direct target of miR-34a (115). TGFβ induces production of CCL2 via suppression of miR-34a (115, 116). CCL2 functions as a recruiter of regulatory T-cells (117). In Hepa1-6 mouse HCC cells, tumor growth and metastasis to mouse liver and abdomen after intrapleural injection in immuno-competent mice was suppressed by overexpression of miR-34a in these cells (115). Growth and metastasis to the lungs of fat pad-implanted 4T1 murine breast cancer cells overexpressing miR-34a was severely inhibited in immuno-competent mice in comparison to control cells (115). An inverse correlation between CCL2 level and miR-34a was found in HBV-HCC tumor samples. These results emphasize the importance of TGFβ–miR-34a–CCL2 signaling in HBV-HCC.

miR-100 inhibits invasion of Hepa 1-6 mouse and human QGY-7703 HCC cells in transwell assays and reduces the incidence of pulmonary metastases of miR-100 transfected and orthotopically implanted Hepa 1-6 cells (118). As direct targets of miR-100, isoprenylcysteine carboxylmethyltransferase (ICMT) and RAC1, both promoters of metastasis, were
identified (118). ICMT functions as a post prenylation processing enzyme which methylates a group of proteins including Rho GTPases and mediates activation of RAC1 (119, 120). RAC1 signaling promotes actin polymerization, subsequent lamellipodia formation and induces PI3K/AKT signaling (121, 122).

miR-101 (Figure 4B) and miR-139 target RHO-associated protein kinase 2 (ROCK2) (123, 124). ROCK2 is a downstream effector of RhoA GTPase and induces formation of stress fibers and focal adhesions by phosphorylating myosin light chains (125). ROCK2 confers motility and invasive capacity to HCC cell lines in vitro and in vivo and is frequently overexpressed in HCC (125, 126). In addition to ROCK2, several additional targets for miR-101, such as induced myeloid leukemia cell differentiation protein, cyclooxygenase 2, stathmin 1, EZH2 and FOS, all involved in proliferation, invasion and metastasis, have been identified (123). In vivo, upon transfection with miR-101, the HCC cell line LM9 exhibited reduced metastatic colonization to the lungs and the liver after tail vein injection (123). miR-101 is frequently down-regulated in HCC patients with distant metastasis and predicts poor prognosis (123). According to data retrieved from TCGA, miR-101 was down-regulated in HCC samples in comparison to normal liver tissues (Figure 3). miR-139 reduced migration, but had no impact on proliferation of SMMC-7721 and BEL-7402 HCC cell lines in vitro and suppressed lung metastasis of miR-139-transfected orthotopically implanted MHCC97-H cells in comparison to control cells (124).

miR-129-3p (Figure 4B) inhibited migration and invasion of HCC cell lines HCCLM3 and MHCC97-H in vitro and reduced intrahepatic and lung metastasis in nude mice (127). Aurora A, a serine-threonine kinase (128), has been identified as a direct target of miR-129-3p (127). miR-129-3p inhibits PI3K/AKT and p38/MAPK signaling (127). Expression of aurora A correlates with lymph node metastasis of HCC, its inhibition leads to reduced cell growth, proliferation and enhanced apoptosis in HCC (129, 130). Methylation-dependent miR-129-3p down-regulation correlates with metastasis and poor prognosis of patients with HCC (127).

miRs-137 and miR-612. miR-137 (Figure 4B) inhibited proliferation and migration of SK-Hep1 and QGY-7703 HCC cells and reduced liver and lung metastasis of HCC xenografts injected into the flanks of mice (131). AKT2 (132) has been identified as a target of miR-137 (131). Overexpression of AKT2 leads to up-regulation of β1 integrins, increased invasion and metastasis in ovarian and breast cancer cells (133) and correlates with poor prognosis in HCC (134). Significant down-regulation of miR-137 in HCC versus normal liver tissues was not demonstrated in TCGA data set (Figure 3). miR-612 also targets AKT2, is down-regulated in patients with metastatic HCC and inhibited metastatic foci of HCCLM3 cells in the liver and the lungs after tail vein injection (135).

miR-149 (Figure 4C) inhibited migration and invasion of HepG2 and MHCC97-H HCC transfectants and in vivo metastasis to the lungs of miR-149-transfected HepG2 cells injected into the caudal veins was reduced (136). Mg2+/Mn2+-dependent protein phosphatase 1F (PPM-1F) was identified as a target of miR-149 (136). PPM-1F belongs to the protein phosphatase 2C family of serine-threonine protein phosphatases and acts as a mediator of motility and adhesion of cancer cells by regulating cytoskeleton remodeling (137, 138). miR-149 is frequently down-regulated in HCC tissues and is associated with poor clinicopathological factors and low postoperative survival rate (136).

miR-150 suppressed proliferation, migration and invasion of MHCC97-H and SMMC-7721 HCC cell lines and in vivo lung metastases of i.v. injected miR-150-expressing MHCC97-H cells was inhibited in comparison to control cells (139). GRB2-associated-binding-protein-1 (GAB1) was identified as a direct target of miR-150 (139). GAB1 functions as a scaffolding adaptor, is involved in tumorigenesis, invasion and metastasis, and can mediate activation of MAPK signaling (140, 141). miR-188 is frequently down-regulated in HCC and in vivo, HCCLM3 cells expressing miR-188 gave rise to smaller tumors and intrahepatic and lung metastases were reduced (142). Fibroblast growth factor 5 was identified as a direct correlation to poor prognosis in patients. miR-101, miR-129-3p and miR-137. C: Further metastasis-inhibitory miRs in hepatocellular carcinoma with in vivo activity in a metastasis-related model and correlation to poor prognosis in patients. miR-149, miR-503 and miR-630. AEG-1: Astrocyte-activated gene 1; AKT: serine-threonine kinase AKT; ANGPTN-2: angiotensin 2; ARFGEF19: brefeldin-inhibited guanine nucleotide exchange protein 1; Aurora A: serine-threonine protein kinase aurora A; CDC42: cell division control protein homolog 42; COX2: cyclo-oxygenase 2; EMT: epithelial–mesenchymal transition; EZH2: enhancer of zeste homolog 2; FOS: transcription factor FOS; HGF: hepatocyte growth factor; INT β1: integrin β1; MCL-1: induced myeloid leukemia cell differentiation protein 1; MLC-P: phosphorylated myosin light chain; MMP2,9: matrix metalloproteinases 2, 9; mTOR: mammalian target of rapamycin; NFxB: nuclear factor xB; P38MAPK: p38 mitogen-activated protein kinase; PAK: p21-activated kinase; PI3K: phosphoinositol-3-kinase; PPM-1F: protein phosphatase 1F; RHO A: RAS homolog gene family, member A; ROCK2: RHO associated coil-coil-containing protein kinase 2; SLUG: transcription factor SLUG; STMN1: stathmin; VAV2: guanine nucleotide exchange factor VAV2; WNT: wingless-integrated.
Weidle et al: MicroRNAs Involved in Metastasis of Hepatocellular Carcinoma (Review)
target of miR-188 which attenuates MAPK signaling (142), and regulates tumor growth and invasion (143).

miR-345 inhibited liver metastasis of HCCLM3 cells after i.v. injection. Interferon regulatory factor (IRF1) was identified as a target of miR-345 (144). IRF1 functions as an oncogene which up-regulates p-mTOR, p-STAT3 and p-AKT and inhibits expression of SLUG, SNAIL and TWIST, modulators of migration and invasion (145). In a parallel approach, YES-associated protein (YAP1), a transcription-regulating oncogene in human cancer, promoting growth and metastasis of HCC cells (146-148), was identified as a target of miR-345. Cell migration and invasion of MHCC97-H cells overexpressing miR-345 as well as lung metastasis of these cells after tail vein injection were inhibited in comparison to control cells (144).

miR-367 inhibited tumor growth, intra-hepatic and lung metastatic foci of HCC cell lines and targets mouse double minute 2 homolog (MDM2) directly (149). Ring-finger containing E3 ligase MDM2 (150) ubiquitinylates androgen receptor, leading to its degradation. Increase of androgen receptor expression promoted expression of FK506 binding protein 5 (FKBPs) and PHLP, resulting in inactivation of p-AKT (149, 151) and concomitant inhibition of metastases.

miR-379 is significantly down-regulated in patients with HCC and inhibits HCC cell line migration in vitro and metastasis in vivo (152). FAK has been identified as a direct target of miR-379 (152). Repression of FAK leads to inhibition of AKT signaling (152). FAK has been identified as a mediator of metastasis of HCC (153).

miR-422 is down-regulated in HCC samples and cell lines and inhibited proliferation and migration of HCC cell lines HCCLM3, MHCC97-H and SMMC-7721 (154). Forkhead box transcription factors FOXG1, FOXQ1 and FOXE1 were identified as direct targets of miR-422 (154). FOXG1 plays a critical role in HCC pathogenesis and metastasis (155). HCCLM3 and MHCC97-H cells transfected with an expression vector for miR-422 gave rise to reduced numbers of hepatic metastases after subcutaneous implantation into immuno-deficient mice in comparison to a corresponding control cell line (154).

Down-regulation of miR-503 (Figure 4C) correlates with increased metastatic potential of HCC cell lines and clinical HCC (156). Subcutaneous implantation of HCCLM3 cells transfected with an expression vector for miR-503 reduced tumor growth and lung metastases in comparison to a corresponding control cell line (156). Brefeldin-inhibited guanine nucleotide exchange factor protein 1 (ARHGEF19), was identified as a direct target of miR-503 (156). ARHGEF19 catalyzes the release of GDP on small GTPases in exchange for GTP and results in their activation (157). Small GTPases play an important role in metastasis of HCC (158).

Expression of miR-885-5p suppressed migration of HCCLM3, SK-Hep1 and HepG2 cells (159). In vivo, in an orthotopic HCCM3-miR-885-5p model, number and size of nodules in the lungs are reduced in comparison to corresponding controls (159). β-Catenin was identified as a direct target of miR-885-5p (159). The WNT signaling pathway plays an important role in pathogenesis and metastasis of HCC (160). Down-regulation of miR-885-5p correlates with poor survival in patients with HCC and miR-885-5p expression is inversely correlated with expression of β-catenin (159).

miRs interfering with EMT. miR-139-3p is down-regulated in HCC and inhibits growth after subcutaneous implantation and formation of pulmonary metastatic foci after tail vein injection of HepG2 cells transfected with miR-139-3p in immuno-deficient mice (161). miR-139-3p targets the annexin A2 receptor (ANXA2R) which is involved in EMT, regulation of adhesion, migration, growth and homing of tumor cells (162). miR-139-3p expression is inversely correlated with ANXA2R expression in human HCC tissues (161).

miR-148a inhibits migration of several HCC cell lines in a transwell assay and represses pulmonary metastases in an orthotopic xenograft model (163). Receptor tyrosine kinase c-MET was identified as a direct target of miR-148a (163). Inhibition of c-MET attenuates AKT signaling and reduces nuclear accumulation of SNAIL, a transcription factor promoting EMT (163, 164). Expression of miR-148a is significantly reduced in HCC tissues in comparison to normal liver-related tissues (163).

miR-187-3p is down-regulated in patients with HCC in comparison to normal liver tissue and inhibits invasion of HCC cells in vitro and metastasis in vivo in mice. Hypoxic conditions are responsible for the reduced levels of miR-187-3p in HCC (165). S100 calcium binding protein 4 (S100A4), the direct target of miR-187-3p, promotes metastasis via EMT (166). Down-regulation of miR-187-3p correlates with adverse clinical features and poor prognosis of patients with HCC (165).

miR-192 is down-regulated in metastatic HCC cell lines and patients and inhibited migration and invasion of HCC-LM3, Huh7 and SK-Hep-1 HCC cells (167). In an orthotopic xenograft model, Huh7 cells transfected with miR-192 gave rise to fewer intrahepatic metastases in comparison to control cells (167). Solute carrier 39 A6 (SLC 39 A6 ) was identified as a target of miR-192 (167). SLC39A6 is a transmembrane zinc transporter that regulates invasion of several types of cancer (168, 169).

miR-200a functions in HCC cells enriched in CSC-like activity and CSC-related phenotype, referred to as side population cells (170). miR-200a inhibits invasion and migration of side population cells derived from MHCC-97H and Huh7 HCC cells (170). Up-regulation of miR-200a in side population cells reduced the number of lung metastatic nodules after injection into the caudal vein of mice and increased their overall survival time (170). Transcription factor zinc finger E-box binding 2 (ZEB2) was identified as
a direct target of miR-200a (170). ZEB2 together with SNAIL, AKT and FOX are drivers of EMT, which is associated with expression of mesenchymal markers (N-cadherin, vimentin) and repression of epithelial markers (CDH1 and zona occludens protein 1) (171, 172).

Liver regeneration and hepatectomy has been reported to be involved in recurrence and metastasis of a residual HCC (173). In a rat model, HCC was initiated by administration of diethylaminoamine and CCL4 (174). A recombinant adenovirus expressing miR-203 injected after 30% hepatectomy inhibited residual HCC invasion and lung metastases (174). miR-203 down-regulates EMT mediator IL1β, SNAIL and TWIST (174). Independent work has shown that miR-203 directly targets SNAIL, ZEB and TWIST transcription factors in nasopharyngeal carcinoma (174).

Ectopic expression of miR-449a suppressed invasion and HCC cell colony formation in vitro of Hep3B, Bel-7402, SMCC-7721, MHCC-LM9, Huh7 and HepG2 cells (175). Restoration of miR-449 expression in Huh7 cells inhibited lung metastases after intrahepatic implantation (175). FOS and c-MET were identified as direct targets of miR-449a (175). FOS and c-MET are overexpressed in HCC and are able to induce EMT (176, 177).

miR-501-3p inhibits migration, proliferation and EMT of HCCLM3 and PLC/PRF/5 HCC cells as shown by gain and loss of function experiments (178). The incidence of lung metastasis of HCCLM3 transfected with miR-501-3p cells was reduced in comparison to control cells in an orthotopic mouse model (178). LIN7A homolog A (LIN7A) was identified as a target of miR-501-3p (178). It was shown that LIN7A mediates the effects of miR-501-3p in vivo (178). LIN7A is a member of the crumbs-complex polarity genes and polarity deficiency has been identified as an essential step of EMT progression and a hallmark of tumor invasion and metastasis (179).

miR-630 (Figure 4C) inhibited migration and EMT in HCC cell lines Bel7402 and HLF (180). miR-630 had no effect on tumor growth of Bel7402 cells after subcutaneous implantation, but its inhibition in Bel7402 cells increased metastatic foci in an orthotopic mouse model (180). miR-630 is repressed by TGFβ and directly targets SLUG, a nuclear transcription factor which increases transcription of CDH1 mRNA by binding to the E-box element of the CDH1 promoter (181). Decreased miR-630 expression is associated with metastasis and poor clinical outcome (181).

miR-1271 inhibits HCC migration, invasion, EMT and the formation of lung metastases (182). miR-1271 targets protein tyrosine phosphatase 4A member 1 (PTP4A1) which inactivates SRC, a tyrosine kinase which promotes metastasis (183). miR-1271 down-regulation correlates with HCC progression (182).

miR-1296 inhibited cell migration, invasion and EMT of HCC cell lines HCCLM3 and Hep3B (184). HCCLM3 cells transfected with miR-1296 gave rise to fewer and smaller foci in the lungs of nude mice after injection into the lateral vein in comparison to control cells (184). Serine/threonine-protein kinase (SRPK1) was identified as a direct target of miR-1296 (184). SRPK1 can activate PI3K/AKT which is crucial for EMT of HCC (185).

miRs inhibiting additional targets. miR-30a acts as an inhibitor of autophagy during starvation in HCC cell lines and attenuates anoikis resistance (186). miR-30a reduced lung metastasis of HCCLM3 cells in nude mice after tail vein injection (186). Pro-autophagic proteins beclin 1 and autophagy-related 5 (ATG5) were identified as direct targets of miR-30a (186). Autophagy plays a role in HCC metastasis through facilitating anoikis resistance and colonization of HCC cells (187). In addition, miR-30a inhibited microvascular invasion and recurrence in HCC tissue samples (186).

miR-122 inhibits in vivo growth and metastasis of Mahlavu cells after intrahepatic administration, partly based on inhibition of angiogenesis (188). A disintegrin and metalloproteinase 17 (ADAM17) has been identified as a critical downstream target (188). ADAM17 facilitates activation of tumor necrosis factor α (TNFα) and epidermal growth factor receptor (EGFR) ligand TGFα, modulates integrin signaling during cell adhesion, and is associated with endothelial cells (189-192).

miR-382 is down-regulated in HCC cell lines and tissues (193). miR-382 suppresses migration and invasion of Huh7 and HepG2 cells and inhibits lung metastasis of these cells after tail vein injection (193). No effect on proliferation, apoptosis and cell-cycle-related parameters has been observed in HCC cell lines (193). Golgi membrane protein 1 (GOLM1) was identified as a direct target of miR-382 (193). GOLM1 has been shown to be involved in EGFR recycling and metastasis in HCC (194).

Therapeutic Aspects

We have summarized metastasis-promoting and -inhibiting miRs as modulators of HCC-related metastasis, focusing on those with documented efficacy in metastasis-related in vivo models. They can be grouped into different functional categories such as tumor-suppressor gene-, angiogenesis-, cell-cycle-, cytoskeleton-, signaling-, EMT- and glycosylation-related. For further ranking with respect to potential as therapeutic targets, correlation between expression in patients with HCC and corresponding prognostic value is important to differentiate/prioritize miRNA as target candidates. Based on criteria as outlined we have identified six metastasis-promoting miRs: miR-29a, miR-219-5p, miR-331-3p, miR-425-5p, miR-487a and miR-1247-3p (Figure 2), and six metastasis-inhibiting miRs: miR-101, miR-129-3p, miR-137, miR-149, miR-303, miR-630 (Figure 4A and B) as potential therapeutic targets to address HCC-related metastasis. For those, in vivo efficacy was evaluated in metastasis-related mouse xenograft models.
making use of orthotopic implantation (n=6), subcutaneous implantation (n=2), intrasplenic implantation (n=1) or tail vein injection (n=3). We did not prioritize miRs with deregulated expression in HCC in comparison to corresponding tissues but instead pending correlation with respect to patients’ outcomes. Considering that orthotopic implantation may most closely simulate HCC-related metastasis, metastasis-promoting miR-29a, miR-219-5p, miR-425-5p and miR-487a, and metastasis-inhibiting miR-129-3p and miR-630 may represent potential therapeutic targets. However, the currently available data do not permit an assignment of these miRs to defined molecular and immunological subtypes of HCC.

Depending on the type of deregulation of the target miRs, the therapeutic options are either inhibition or reconstitution their function. miR antagonists (antagomirs) are single-stranded antisense oligonucleotides (ASOs) which anneal to the mature miR guide strand and induce its degradation or formation of stoichiometric inhibitory duplex. Antagomirs are the most popular miR inhibitors because all optimizations achieved for ASOs can be incorporated into the design of antagomirs (16, 196-198). Another option for inhibition of miRs are miR sponges which contain in-tandem multiple complementary binding sites for the target miR. They are expressed in target cells by corresponding vectors (16, 195, 196).

Therapeutic application of nucleic acid-based entities and small molecule inhibitors of miRs are an emerging field (197). Specificity issues inherent to the latter approach might be circumvented by targeting unique secondary structures. There are two options for replenishment of down-regulated miRs. One option is the design of miR mimetics, synthetically derived RNA duplexes which mimic the function of the corresponding miR. The other option is reconstitution of miR function by inserting miRs into viral vectors and express them in corresponding target cells (198). Since miRs regulate a large set of genes, inhibition or reconstitution of their function has the potential of restoring coordinated functionality. On the other hand, there are many issues which have to be resolved regarding application (and optimization) of miRs and miR-targeting entities as therapeutic agents. Among these are toxicity-related issues due to nonspecific hybridization with non-target miRs, targeting of healthy tissues, case-by-case optimization of pharmacokinetic and pharmacodynamic properties, degradation by nucleases, elimination by the reticulo-endothelial system, kidney filtration, immunogenicity, sequestration into endosomes and fundamental issues of their delivery to cancer tissues (199-205).

Efforts to enhance properties of nucleic acid-based therapeutics have led to multiple chemical modifications in the sugar, base and backbone of RNA nucleotides. These modifications have mainly focused on increasing the affinity to the target RNA sequence, improving the pharmacokinetic properties and reducing immunogenicity. The most widely applied nucleotide modification for ASOs has been the replacement of the phosphodiester backbone with a phosphorothioate linkage. This modification dramatically increases nuclease resistance and ASO binding to serum albumin, which leads to a much improved pharmacokinetic profile and distribution to most peripheral tissues, with distinct accumulation in liver and kidney (206-208).

Besides albumin, phosphorothioate-modified ASOs also bind to extracellular, cell membrane and intracellular proteins, which facilitates their entry into cells and intracellular trafficking. Upon adsorption to membrane proteins, ASOs internalize into cells through multiple pathways, which can lead to ‘productive’ uptake and antisense effects intracellularly or the ASOs become routed and trapped in ‘non-productive’ cellular compartments. Although the requirements for productive intracellular trafficking have not been fully identified and antisense efficacy varies greatly between ASOs as well as cell types and their activation status, phosphorothioate-modified ASOs can reach the cytoplasm and nucleus. Achieving antisense efficacy without the need of an additional delivery agent represent a major advantage to previous nucleic acid-based therapeutics (209-211).

Recently, quantitative fluorescence imaging revealed that locked nucleic acid-modified ASOs traffick to the nucleus within minutes upon injection directly into the cytoplasm. Approximately 10^5 ASOs are required for 50% target knock down, suggesting that a large proportion remains bound to nuclear components and is not available for RNase H1-mediated degradation of the target RNA (212).

Considering this high number of required ASOs, current research is focused on the improvement of their intracellular delivery. Uptake of nucleic acid-based therapeutics into cells can be enhanced by various means. For example, making use of polymers such as high molecular weight polyethyleneimine (PEI) and encapsulation in lipid-based nanoparticles with a positive charge enabling binding to the negatively charged cell surface has improved delivery (204). Stabilization of the delivery vehicles or of the therapeutic agents with polyethylene glycol is also an important improvement (213). Liver specific delivery can be achieved by conjugation of PEI with galactosamine which interacts with asialoprotein receptors on hepatocytes and leads to internalization of the complexes by receptor-mediated endocytosis (203, 204). Further options for hepatocyte-specific delivery are outlined elsewhere (203, 204). Even local administration of miRs into the liver may be an option. Advantageous characteristics of a potential miR-related target under consideration would be simultaneous inhibition of proliferation, migration, invasion and intrahepatic metastasis. Eradication of local and distant metastases by the corresponding miR-related therapeutic agent as a single agent or in combination with other agents should be the ultimate goal of anti-metastatic therapy. Unfortunately, the described preclinical models do not include models which allow targeting of established metastases. Preventive scenarios do not seem to...
be realistic due the necessity of continued application and resulting possible toxicity-related issues.

The field of miR-related agents has witnessed several severe drawbacks in the recent past (214). Clinical studies of Regula Therapeutics with miR-17 and miR-21 inhibitors for treatment of inherited polycystic kidney disease and Alpert syndrome were paused due to toxicity issues (214).

In 2016, clinical evaluation of a miR-122 inhibitor for treatment of HCV infections was put on hold due to jaundice in patients caused by inhibition of a bile duct transporter, despite an excellent efficacy profile (214). Also, in 2016, a clinical study of a synthetic mimic of miR-34 (miRNA Therapeutics) for treatment of multiple types of cancer was halted in phase I due to severe side-effects (214). On the other hand, miRagen is pursuing clinical studies with MRG-106 (cobomersen), a gapmer, in patients with leukemias and other lymphomas without any adverse effects observed during phase I (214). It remains to be seen whether second-generation molecules with an improved toxicity profile will emerge as game changers in this field.

Conflicts of Interest

FB, DS and UB are employees of Roche, UHW was an employee of Roche.

Authors’ Contributions

DS, UB and UHW wrote the article, FB prepared the Figures and performed the bioinformatics analysis.

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