

## REVIEW ARTICLE

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# MicroRNAs in liver malignancies. Basic science applied in surgery

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## Summary

Liver malignancies represent one of the major public health problems worldwide because of late diagnosis and failure of current treatments to offer a curative option for many of the patients. MicroRNAs (miRs) are small non-coding RNA molecules that are known to regulate the gene expression at a post-transcriptional level through complementary base pairing with thousands of messenger (m)RNAs. Recent data has shown the involvement of miRs in the pathogenesis of many human cancers, including those of the liver, with huge possible impact in the clinic, mainly due to the

identification of non-coding RNAs as biomarkers that can often be detected in the systemic circulation.

In the current review, we present the importance of miRs in liver cancers by discussing their role in the pathobiology of these diseases, apart from their role as diagnostic and prognostic markers for liver malignancies.

**Key words:** hepatic malignancy, microRNA involvement, state-of-the-art management

## Current management of liver malignancies

Liver malignancies represent the 5<sup>th</sup> most common cause of cancer-related deaths in the world, the most common histological type being hepatocellular carcinoma (HCC) [1]. This disease basically has three curative options according to the guidelines of both the European Association for the Study of the Liver (EASL) and American Association for the Study of Liver Diseases (AASLD): liver transplantation, liver resection and radiofrequency thermal ablation [2]. Hepatectomy is recommended for a patient diagnosed with a single HCC nodule and completely preserved liver function and no portal hypertension, while the

ones with tumors larger than 5 cm are considered to be at high risk of vascular invasion. These cases could be operated but are very risky, especially in the cirrhotic liver and leaves mainly palliative chemotherapy or new experimental clinical therapies as best option of management. One of the few exceptions may, nevertheless, be the case in which the diagnosis is a tumor of the right liver lobe and the surgeon will perform a right hepatectomy by anterior approach, as described by Lay et al. more than one decade ago [3]. Using this approach, the technique avoids aggressive tumor manipulation before the large vessel ligation and allows minimal intraoperative hemorrhage, apart from an improved survival as a direct result of the reduction of circulating tumor cells [4].

But very often the diagnosis shows multinodular disease, a contraindication to liver resection or transplant beyond the Milan criteria of less than three nodules, each less than 3 cm [5,6]. Also, in the case of portal hypertension, surgical resection is contraindicated as a result of the Barcelona group experience, that reported this condition to have negative prognosis along with total serum bilirubin value of less than 1 ml/dL. In such cases, the 5-year survival after hepatectomy is 74%, 50% and 25% for patients with zero, one and both negative predictors, respectively [7,8]. During the natural history of HCC that appears and grows in a cirrhotic background, the invasion of portal pedicles or of the main portal trunk is frequently diagnosed and is beyond surgical management due to a high risk of postoperative liver decompensation [9].

Patients that are at risk for future development of HCC are enrolled in surveillance programs consisting of abdominal ultrasonography twice a year and according to EASL guidelines, in the case of underlying cirrhosis, a detected nodule that is less than 1 cm will be followed by regular ultrasonography every 4 months in the first year and every 6 months in the following years [10-12]. In the case of nodules between 1 and 2 cm, management includes a biopsy-proven diagnosis by an expert pathologist, followed by a second opinion in case of inconclusive findings. A lesion larger than 2 cm can be considered to be cancer based only on the typical features of one imaging method.

The diagnosis of liver cancer is based on both non-invasive criteria and pathological examination, in accordance to the recommendations of the International Consensus Panel. The lesions may vary from small hypercellular lesions, named dysplastic nodules, to adenomatous hyperplasia before a diagnosis of malignancy is established. Usually coinciding with chronic inflammation, cancer varies between micro-invasive carcinoma in which the portal tracts within the nodule are preserved, to more aggressive 'nodule-in-nodule-type HCCs [13]. Immunostaining includes markers for GPC3, HSP70, and glutamine synthetase in order to make a differential diagnosis between a high grade dysplastic nodule and an early HCC. When the diagnosis is still debated, the pathologist turns to gene expression. One of the most important papers related to this topic is a oligonucleotide array by Chuma et al. [14], in which 12,600 genes were analyzed and the most highly

upregulated was heat shock protein 70 (HSP70). Consequently, this marker could be used to distinguish between benign from malignant liver nodules, along with the serum markers glypican 3 and glutamine synthetase. The last one is an oncofetal protein expressed mostly in the fetal liver, inactive in the normal hepatic adult tissue and then re-activated in HCC [15-17]. The evolution from a high grade dysplastic nodule to a liver carcinoma described an induction of arterial blood supply, as well as stromal and venous invasion. This characteristic can explain the importance of staining for markers such as Ki-67 and Ep-CAM to assess the progenitor cell features or anti-CD34 to assess neoangiogenesis.

After diagnosis, the clinical management in the Western world is based on the Barcelona Clinic Liver Cancer Group (BCLC) staging. The standard-of-care for BCLC stage A patients is local ablation either with radiofrequency or ethanol injection and for the those diagnosed with BCLC stage B disease (multinodular tumors with minimal clinical symptoms and no vascular invasion or extrahepatic spread) chemoembolization represents the standard-of-care. The advanced cases are treated often with drugs such as sorafenib for a Child-Pugh A class [11,18-21].

In the case of liver metastases, synchronous lesions occur in about 10% of colorectal cancers, whereas metachronous in about 15% [22]. The best prospect of improving prognosis is represented by liver resection to remove the metastases, with the Japanese Society of Cancer of the Colon and Rectum (JSCCR) Guidelines indicating surgery if the primary has been or can be controlled, if there are no extrahepatic metastases and in the case when the function of the remaining liver is adequate. These criteria make the surgery available for only about 40% of Japanese patients and half of this percentage for European or American patients because of frequent hepatic lymph node metastases, as well as extrahepatic cancer spread. According to Kato et al. [23], after having investigated 585 cases of colorectal liver metastases, the 3-year survival rate was 52.8% and the 5-year survival 39.2%. When surgery is no longer an option, protocols suggest therapy with 5-FU and leucovorin, as well as the addition of irinotecan or oxaliplatin (FOLFIRI and FOLFOX), giving a median survival of just over 20 months. Recent clinical trials have also introduced the addition of targeted antibodies such as bevacizumab, cetuximab or panitumumab [24-27].

## MicroRNA expression profiles in primary liver cancers

One study among the first to establish a link between miRs and HCC was published by Murakami et al. [28] 7 years ago who found 3 miRs overexpressed in HCC in comparison with non-cancer tissues, as well as 5 miRs underexpressed. As chronic hepatitis B (HBV) or C (HCV) infection is a major cause of carcinogenesis, some interest was re-focused on investigating specific miRs downregulation in HBV or HCV-related cancers, with Ura et al. demonstrating that there is a difference between the two groups of chronic infection [29]. One such case is miR-122, that is liver-specific and accelerates ribosome binding to HCV RNA, which in turn stimulates viral translation [30] and leads to a repression of miR-122 in HCC. This could, nevertheless, be a compensatory mechanism to allow the resistance to HCV replication in HCC cells [31]. Also, an *in vitro* HCV infection of a primary culture of hepatocytes helped prove that the upregulation of miR-141 in HCV genotype 1a infected cells will target the tumor suppressor DLC-1 [32,33]. DCL-1 is known to be frequently deleted on most digestive cancers, including HCC, and as HCV replication proved to be pretty dependent on miR-141 induction, its targeted depletion of the DLC-1 protein demonstrated that the antagomir-mediated knockdown of miR-141 will inhibit HCV replication.

miRs are also important in mediating interferon (IFN)-mediated antiviral defense. IFN mediates the inhibition of HCV replication and in this way it induces the synthesis of miRs that have complementary sequences of the HCV genome. One case is IFN- $\beta$ , that induces the expression of various cell miRs, some with predicted sequence targets within the HCV genomic RNA [34]. This agent also interferes with miR differential expression after its therapy as miR-122 is downregulated because is required for the interaction with HCV 5'-UTR and promotes viral RNA accumulation [31,35]. The miR species miR-7, miR-196b, miR-433 and miR-511 are known to affect viral polymerase, as well as the S gene of HBV, while miR-205 affects the X gene of HBV, making miR-based assay a useful tool in evaluating HBV-induced hepatitis [36]. This is also the case of miR-345, that appears to target the HBV pre-C gene the downregulation of which facilitates the protein expression of HBV pre-C, a precursor of HBe antigen [37].

Chronic alcoholism, a major cause of hepatocarcinogenesis, is linked to the downregulation

of miR-126 in comparison with non-alcohol damaged liver tissue, linking it to the alcohol-induced development of HCC in the cirrhotic liver [38]. Another potentially applicable miR is miR-181, upregulated in alpha-fetoprotein (AFP) positive cells and normally expressed in the embryonic liver, as well as in hepatic stem cells. Its inverse correlation with mature hepatocyte-specific genes proves that it serves to maintain stemness during hepatocarcinogenesis. miR-21 is quite unique in gastrointestinal oncology because it is upregulated in most digestive cancers [39,40], and in an *in vitro* model that used Cre and Tet-off technology to obtain mice that conditionally expressed miR-21. This experiment was further confirmed in an *in vivo* model of miR addiction which has proven that overexpression of miR-21 will lead to pre-B malignant lymphoid phenotype. miR-21 inactivation resulted in regression of the tumor in mice [41]. In the side-population (SP) cancer cells, the expression level of miR-21 is usually found upregulated and it proves a constitutive role in the maintenance of chemoresistance in the case of SP cells [42]. AFP+ cells that are also EpCAM+ have cancer stem-like progenitor features, with a unique miR signature that partially coincides with carcinogenesis. miR-181 family members are highly expressed in aggressive HCCs, as well as in isolated hepatic cancer stem cells and promote the stemness of HCC by targeting CDX2, as well as GATA6 (hepatic transcriptional regulators of differentiation) or NLK, an inhibitor of the WNT/ $\beta$ -catenin pathway [43].

Both normal stem cells and cancer cells share common features such as surface markers or the molecular machinery, as well as being defined by potential for multilineage differentiation and self-renewal [44-46]. The common signaling pathways, such as Hedgehog, Notch, Wnt/ $\beta$ -catenin, HMGA2, Bcl2, Bmi-1 or c-MET, are involved in survival, self-renewal or differentiation in colorectal cancer (CRC) [47-49]. These molecular loops closely relate to miRs in terms of initiation or progression of cancer, as well as cell proliferation or cell death [50-52]. miR-34 is regulated by p53 and plays a role as tumor suppressor-like p53 in p53+ cancer cells. The target genes Notch and Bcl-2 regulate the self-renewal and survival of cancer stem cells (CSC) and make miR-24 activation to determine the inhibition of the clonal proliferation of the stem-like cancers. Along with the activation of caspase-3, miR-24 induces cell apoptosis [53,54], apart from increasing the resistance to chemotherapy [55]. miR-30 was recently sug-

gested to be one of the most important miRs that regulate stem-like features of cancer cells. Using a miR microarray assay, Yu et al. [56] reported that miR-30 is decreased in stem-like cells when they compared them with the more differentiated ones and definitely proved that overexpression of miR-30 inhibits the cancer cell's potential of tumorigenesis, anoikis resistance and metastasis [57]. In an endeavor to investigate miR involvement in CSCs, Ma et al. [58] performed a quantitative PCR miR analysis of CD133+ from HCC tissue samples and compared them with a hepatoma cell line, thus revealing 8 candidate miRs that are differentially regulated. miR-130b correlated best with CD133 expression and its introduction into CD133- cells resulted in enhanced proliferation and resistance to polychemotherapy. PicTar and Target Scan miR-130b target prediction then revealed 289 potential downstream targets and when combined with a microarray analysis of miR-130b transfected cells this research group found 3 probable targets – including the tumor suppressor gene TP53INP1. An *in vitro* luciferase assay using the TP53INP1 3'-UTR validated this transcript as a possible target of miR-130b. These data strongly support that miR-130 has a strong correlation with the stem-like phenotype and has a potential role in future therapy. Kim et al. [59] proved that various subtypes of miRs may act in a lineage-specific way both during hepatocyte differentiation and during embryonic cell differentiation. This statement is confirmed by high grade HCCs with poor prognosis and a stem-like miR profile that includes the miR-371-3 cluster. Its upregulation in embryonic stem cells correlates with downregulation during normal liver differentiation, as is also the case of let-7 and miR-181 that are downregulated in normal liver tissue and upregulated in liver malignancies [58].

Gramantieri et al. [60] used a microarray-based comparison of miR differential expression between cirrhotic and HCC tissues and identified 35 miRs, including some of them involved in other human malignancies. The transfection of miR-122 into HEP3B hepatoma cells decreases cyclin G1 expression, as well as other analyses such as Western blotting revealed an inverse correlation between cyclin G1 protein and miR-122 expression when comparing the normal with the cirrhotic tissue. This means that a decreased miR-122a could allow overexpression of genes involved in the cell cycle progression with an increased risk of malignant transformation.

ADAM 17 (a disintegrin and metalloprotease

17) has already been proven to be a negatively regulated target of miR-122 after having been shown to regulate cell invasion potential of two different HCC cell lines (SK-Hep1 and Mahlavu). MiR-122 expression or RNA interference-mediated suppression of ADAM17 in Mahlavu cells before the injection into nude mice intensified the reduction of the tumor masses and proved that restoration of physiological miR targeting in hepatocytes could decrease the oncogenic properties of hepatoma cells. Other evidence that miR-122 is a functional actor in HCC initiation and progression is its function as a tumor suppressor in two different ways: by inhibiting hepatocyte cell growth after targeting cyclin G1 and by promoting apoptosis after targeting Bcl-w, apart from playing a substantial role in the stimulation of HCV RNA translation [60-62].

Oposing miR-122 is miR-221, that acts as an oncomiR in hepatocytes. miR expression profiling in 104 HCC and other 90 adjacent cirrhotic liver tissue samples found that 12 miRs were closely linked to the progression to HCC [63]. Both miR 221 and 222 were transfected into HepG2 cells and led to increased cell proliferation. A decreased proliferation was noticed when cells were treated with an antagomir directed against miR-221 and the injection of miR-221 overexpressing immortalized liver progenitor cells into previously irradiated mice has led to decreased tumor latency when compared with other control immortalized progenitors.

In a study from NIH [64], Budhu et al. examined 492 CRCs and normal tissues from surgical resection pieces from no less than 241 patients and found 20 miRs signatures that included miR-122a which was predictive for HCC venous invasion in comparison with non-metastatic cancer. miR-31 was described as a regulator of metastasis not only in liver malignancies but also in a wide variety of cancers, as it controls both the metastasis-related genes and the genes that regulate cell proliferation, cell cycle and apoptosis [65], as is also the case of miR-492, reported to be upregulated in metastatic hepatoblastoma [66].

Apart from their application in detecting early cancer dissemination, miR alteration status could be used in predicting the response to cancer chemotherapy. Using bio-informatics miR-199a-3p was demonstrated to be downregulated in a series of cancers that included liver malignancies. Mammalian target of rapamycin (mTOR), a known regulator of cell proliferation, was identified as a target of miR-199a-3p with an expression

inversely correlated to the expression of mTOR [67]. The clinical application could rest in the idea that a restoration of this miR's expression in the cancer cell may lead to cell cycle arrest, decreased invasion and a higher sensitivity to doxorubicin.

Just like in the case of HCC, miRs are also dysregulated in cholangiocarcinoma (CCA). By using both surgically resected HCCs and CCAs, Karakatsanis et al. [68] analyzed the expression levels of miRs with the purpose to find some that could be used in the clinic as biomarkers. Their results showed that miR-21, miR-31, miR-122, and miR-221 are upregulated in the case of HCC when compared with tissue samples of healthy liver, as already described before. MiR-223, miR-21 and miR-31 were upregulated in the case of intrahepatic cholangiocarcinoma (ICC) in comparison with non-cancerous tissues, whereas miR-122, miR-145, miR-146 A, miR-200c, miR-221 and miR-222 were found to be downregulated, corresponding to the results of Chen et al., Kawahigashi et al., and Selaru et al. [69-71]. Overexpression of miR-141 was reported by Meng et al. in CCAs [72], the inhibition of which had the potential to increase the expression of the key regulator of the circadian rhythm CLOCK, and thus suppress CCAs. Experiments on CCA cell lines proved that miR-29b is suppressed in the KMCH line, as well as approximately in one third on all CCA tissue specimens [73]. An enforced expression of miR-29b had the ability to reduce the expression of antiapoptotic proteins of the Bcl-2 family, such as Mcl-1, as well as sensitizing the CCA cell to tumor necrosis factor (TNF)-related apoptosis-inducing ligand cytotoxicity. As a result, the suppression of miR-29b expression in ICC could allow the expression of Mcl-1 and suppress the acquired resistance of the cancer cell's to therapy.

It is worth mentioning the experiments conducted by Razumilava et al. [74], who found some members of the miR-25 cluster to be upregulated in the CA cell lines KMCH and Mz-ChA-1. This very miR-25 is known as an antiapoptotic non-coding RNA via protection against TRAIL-induced apoptosis. This group also identified a new target of miR-25 in the extrinsic pathway DR4. An antagonism of miR-25 has the ability to increase DR-4 protein expression and sensitize a cancer cell to programmed cell death, revealing a possible functional role of this RNA species. In experiments done by the team of Thorgeirsson et al. [75] at NIH in Bethesda, a conducted transcriptomic profiling of 23 ICCs and combined HCC-ICC specimens was done using a Affymetrix mRNA and

Nanostring miR microarrays to search a unique gene signature possible linked to tumor subtype and patient prognosis. An analysis of the ICC-specific mRNA and miR profile found a common signaling pathway that linked miR-200c to EMT and preferentially activated the ICC stem-like expression status. An inactivation of miR-200c could result in a reduction of EMT, as well as reduced cell migration and invasion. The data was confirmed by the fact that miR-200c and the hepatic progenitor-specific marker NCAM1 expression were negatively correlated with their expression levels. The stem-like status is of major importance as activated or inactivated by IL-6, correspondingly to the signals received from outside the niche. An enforced IL-6 overexpression in human CCA cell lines significantly increased let-7a expression, as reported by Meng et al. [76]. A target of this miR is the neurofibromatosis 2 (NF-2) gene, a negative regulator of STAT3. As a direct consequence, overexpression of IL-6 in CCA causes upregulation of let-7a and decrease in NF2 expression, causing the removal of the negative regulation of STAT-3. The roles of miRs in primary liver malignancies are shown in Tables 1 and 2.

### MicroRNA expression profiles in liver metastases from colorectal cancer

When investigating liver metastases, CRC cells play key roles in angiogenesis, EMT and degradation of the extracellular matrix via upregulation or downregulation of a wide panel of genes in the border between cancer and the surrounding stroma [77,78]. The first study that proved that miR expression is different in the center of the tumor in comparison with the invasive front of CRC liver metastases was done by Kahlert et al. [79]. In this paper, microarray data from pooled tissues showed mostly downregulation of miR at the tumor invasive front, with more than half of these miRs (miR-143, miR-145 and let-7) already proven by previous studies to be tumor suppressors. Wang et al. [80] showed that miR-143 and miR-145 are decreased in CRCs when compared with the normal mucosa, just before Chen et al. [81] showed that miR-143 can suppress the proliferation of a cancer cell *in vitro* through inhibition of KRAS translation. Ng et al. [82] also proved a tumor suppressor role of miR-143 via the regulation of DNA methyltransferase 3A in CRCs. A comparison of miR-21 and miR-143 expression in colorectal samples and their corresponding liver metastases was investigated by Kulda et al. [83],

**Table 1.** miRs and their relationship with premalignant conditions of hepatocellular carcinoma

Type of miR [Ref]	Level	Target gene/protein	Outcome	Notes
miR-122 (HCV infection) [88]	Not mentioned	-	accelerates ribosome binding to HCV RNA	- stimulates viral translation and leads to repression of miR-122 in HCC - promotes viral RNA accumulation
miR-141(HCV genotype 1a infected cells) [29]	Up-regulated	the tumor-suppressor DLC-1	inhibit HCV replication	None
miR-7, miR-196b, miR-433 and miR-511(HBV infection) [36, 131]	Not mentioned	viral polymerase, as well as the S gene of HBV	Not mentioned	None
miR-205 [18]	Not mentioned	the X gene of HBV	Not mentioned	None
miR-345 (HBV infection) [132]	Down-regulated	the HBV pre-C gene	facilitates the expression of HBV pre-C, a precursor of HBe antigen	None
miR-181 family members (aggressive HCC) [43]	Over-expressed	CDX2, GATA6 (hepatic transcriptional regulators of differentiation), NLK (inhibitor of the WNT/ $\beta$ -catenin pathway)	promotes the stemness of HCC	None
miR-34 [53]	Not mentioned	Not mentioned	Not mentioned	- tumor-suppressor-like p53 in p53 cancer cells
miR-24 [62]			- inhibits the clonal proliferation of the stem-like cancers - activates caspase-3 - induces cell apoptosis - increases the resistance to chemotherapy	None
miR-30 [56]	Over-expressed	Not mentioned	- inhibits the cancer cell's potential of tumorigenesis, anoikis resistance and metastasis	None
miR-130b [58]		the tumor-suppressor gene TP53INP1	- enhanced proliferation and resistance to poli-chemotherapy	potential role in future therapy
miR-122 (cirrhosis) [60,61,68]	Over-expressed	- cyclin G1 expression (decreased) - ADAM 17 (a disintegrin and metalloprotease 17) - negatively regulated	Promotes HCC venous invasion	- a decreased miR-122a could allow the over-expression of genes involved in the cell cycle progression with an increased risk of malignant transformation - ot functions as a tumor-suppressor in two different ways: by inhibiting hepatocyte cell growth after targeting cyclin G1 and by the promotion of apoptosis after targeting Bcl-w, apart from playing a big role in the stimulation of HCV RNA translation
miR-221 (cirrhosis, HCC) [63,68]	Over-expressed	Not mentioned	Increases cell proliferation	acts as an oncomiR in hepatocytes

Type of miR [Ref]	Level	Target gene/protein	Outcome	Notes
miR-199a-3p [67]	Down-regulated	Mammalian target of rapamycin (mTOR) – regulator of cell proliferation	Increases cell proliferation	restoration of this miR's expression in the cancer cell may lead to cell cycle arrest, decreased invasion and a higher sensitivity to doxorubicin.
miR-21, miR-31, miR-122, miR-221(HCC) [40,72,83]	Up-regulated	Not mentioned	Not mentioned	Could be used as biomarkers

**Table 2.** miRs and their relationship with cholangiocarcinoma

Type of miR [Ref]	Level	Target gene/protein	Outcome	Notes
miR-223, miR-21 and miR-31 (ICC) [68]	Up-regulated	Not mentioned	Not mentioned	Not mentioned
miR-122, miR-145, miR-146 A, miR-200c, miR-221 and miR-222 [68,80,85]	Down-regulated	Not mentioned	Not mentioned	Not mentioned
miR-141 (CCA) [101]	Over-expressed	the key regulator of the circadian rhythm CLOCK	Its' inhibition has the potential to increase the expression of CLOCK, and thus suppress CCAs	Not mentioned
miR-29b [73]	Over-expressed	anti-apoptotic proteins: Bcl-2 family (Mcl-1)	Not mentioned	- an enforced expression of miR-29b reduces the expression of anti-apoptotic proteins of the Bcl-2 family such as Mcl-1, as well as sensitizing the cholangiocarcinoma cell to tumor-necrosis factor (TNF)-related apoptosis-inducing ligand cytotoxicity. - the suppression of miR-29b expression in ICC could allow the expression of Mcl-1 and suppress the acquired resistance of the cancer cells to therapy.
miR-25 cluster (CCA cell lines KMCH and Mz-ChA-1) [74]	Up-regulated	the extrinsic pathway DR4		- miR-25 is known as an anti-apoptotic non-coding RNA via protection against TRAIL-induced apoptosis - An antagonism of miR-25 has the ability to increase DR-4 protein expression and stimulate a cancer cell to programmed cell death (possible functional role of this RNA species)

with higher values when comparing the metastatic lesion with the primary colorectal lesions. A differential expression between the primary cancers and the metastases is reported by Vickers et al. [84], who found an increased expression of let-7a and decreased miR-206. *In vitro*, miR-145 can inhibit invasion of CRC cells with increased invasive potential after targeting mucin-1 and c-myc [85,86], while let-7 can suppress the growth potential of cancer cells isolated from CRC [87]. Kahlert et al. [79] reported that dysregulation of miR-19b at the liver invasion front could alter the function of both proangiogenic and antiangiogenic factors, essential features of metastasis in the liver.

miR-122 is a liver-specific miR that is known to modulate extremely important function in hepatic physiology and pathology, such as lipid metabolism [88], hepatitis C virus replication [31], as well as apoptosis [89]. The expression of this miR has been proven to be downregulated during embryology in the liver development [90], as well as in HCC [91]. miR-122 inhibition in liver malignancies is not limited only to HCC, but also extends to metastases of this organ, as proven of Tsai et al. [92]. Lin et al. [93] proved that 28 miRs are dysregulated after investigating CRCs that had disseminated to the liver and compared these samples with metastatic CRCs. miRs 150, 125b-2, 139-p3 and 1179 were overexpressed in widely disseminated disease and proved that aberrant expression is involved in the metastatic process. miR-101, as well as miR-135a and miR-135b, are known to be downregulated in CRC and have an inverse correlation with COX-2 as Strillacci et al. have reported [94]. These last two miRs regulate the expression of adenomatous polyposis (APC) gene when compared with local colon mucosa and hence influence the activation of the  $\beta$ -catenin pathway [95].

By using a miR array to analyze the differential expression profile of portal vein thrombosis and of the corresponding tumor-associated parenchyma, Liu et al. [96] found that miR-135a is upregulated by FOXM1 overexpression and correlated with an enhanced invasion potential of the portal vein tumor thrombus cell line CSQT-2, both *in vivo* and *in vitro*. miR-135 is important in maintaining stem cell pluripotency because of the regulation of sirtuin 1 in mouse embryonic stem cells [97]. miR involvement in the regulation of the stem-like behavior of the CRC cells and their potential to metastasize to the liver was also evaluated by Takahashi et al. [98]. These authors analyzed the relationship of the Wnt-targeted Lgr5 pathway in

CRC and miR-23a and miR-23b. The Lgr5 protein was highly expressed in the peripheral regions of adenomas as well as in the border between the cancer cells and the surrounding tissue. This supports the idea that the accumulation of genome mutations has the power to interfere with the location and polarity of Lgr5 positive stem-like cells during the evolution from adenoma to invasive carcinoma. In addition, Lgr5+ tumors display a mesenchymal phenotype due to high expression of vimentin and low expression of miR-200c, suggesting that Lgr5-positive CRC stem-like cells are the ones responsible for the invasion and metastasis of this malignancy to the liver.

Pizzini et al. reported that when bioptic specimens are obtained, miRs and gene expression profiles differentiate tumor samples from normal colorectal mucosa [99]. Nevertheless, miRs are more specific in identifying metastatic CRC in the liver from primary liver cancers [99].

Results from relevant colorectal carcinogenesis studies, gathered in a review by Sakai et al. [100] show that some miRs are responsible for unfavorable effects; to sustain this statement, examples show that miR-143, miR-145, miR-125b and miR-21 are associated with cell growth and survival, the miR-17-92 cluster, miR-20 and miR-100 are involved in uncontrolled cellular proliferation, the miR-183 cluster and miR-31 determine cell migration, and miR-150 is a potential biomarker of prognosis and therapeutic outcome in CRC; an exhaustive microarray study performed by Qin et al. [101], described 28 miRs that were in an altered state in CRC with metastasis to the liver, in comparison with non-metastatic CRC (miR-150, miR-125b-2, miR-139-3p, miR-19a and miR-1179 were overexpressed while all others were underexpressed); Zhong et al. et al. [102] stated that miR-499-5p had an impact in human CRC cell migration and invasion. miR-139-5p has been described as a member of a signature predictive of the clinical aggressiveness of stage II CRC [103]. To underline further importance of miR as neoplastic evolution factor, Iwaya et al. [104] reported that miR-191 might also be involved in the progression and metastasis of CRC by downregulating TIMP3 and its effect on the proliferation and apoptosis in CRC cells through pathways independent of metalloproteinases. Okamoto et al. [105] have found that if miR-122 is overexpressed with a subsequent suppression of CAT1 in primary tumors, the risk of developing CRC liver metastasis is increased. Ewing et al. [106] found that if miR-144 is downregulated, and further activation



**Table 3.** miRs and their relationship with liver metastasis from colorectal cancer

Type of miR [Ref]	Level	Target gene/protein	Outcome	Notes
miR-143, miR-145, miR-125b and miR-21 [68]	Not mentioned	Not mentioned	cell growth and survival	None
miR-17-92 cluster, miR-20 and miR-100 [93]	Not mentioned	Not mentioned	uncontrolled cellular proliferation	None
miR-183 cluster and miR-31 [31,65]	Not mentioned	Not mentioned	determine cell migration	None
miR-150 [22]	Not mentioned	Not mentioned	Not mentioned	potential biomarker of prognosis and therapeutic outcome in CRC
miR-150*, miR-125b-2*, miR-139-3p, miR-19a and miR-1179 (metastatic CRC) [69,70]	Over-expressed	Not mentioned	Not mentioned	None
miR-499-5p [108]	Not mentioned	Not mentioned	human CRC cell migration and invasion	None
miR-139-5p [111]	Not mentioned	Not mentioned	Not mentioned	Member of a signature predictive of the clinical aggressiveness of stage II CRC
miR-191 [101]	Not mentioned	TIMP3 (down-regulated)	progression and metastasis of CRC	TIMP3 down-regulated impacts proliferation and apoptosis in CRC cells through pathways independent of metalloproteinases
miR-122 [103]	Over-expressed	CAT1 (suppressed in primary tumors)	Increased risk of developing CRC liver metastasis	None
miR-144 [104]	Down-regulated	mTOR pathway	poor prognosis of CRC patients	None
miR-493 [105,116]		IGF1R and MKK7 expression suppressed in early stages of the disease	formation of liver metastasis of CRC cells - induces the cell death of metastatic CRC	Acts as an 'antimetastatic' factor if over-expressed
miR-30a [117,118]	Down-regulated, under-expressed	- Beclin 1 mRNA in tumor cells - negatively regulated - PIK3CD expression at mRNA and protein levels - down-regulated	decreased autophagic activity	the miR-30a-induced inhibition of migration and invasion could be rescued by overexpression of PIK3CD
miR-106a, -200b, -106b, -200c, -141, -320a and -320b [123]	Over-expressed (both in primary tumors and liver metastasis)	Not mentioned	Not mentioned	only miR-320a and miR-200c -downregulated
miR-320a (in liver metastatic CRC cells) [107]	Over-expressed	Not mentioned	provides loss of cell migration and invasiveness	none

on the mTOR pathway follows, poor prognosis of CRC patients can be expected. On the other hand, the expression of other species of miRs counteracts as 'protective' against invasiveness in CRC; a notable example is that of miR-493, the expres-

sion of which in CRC blocks the formation of liver metastasis of CRC cells, or induces cell death of metastatic CRC cells by suppressing the IGF1R and MKK7 expression in the early stages of disease [107,108]; another antimetastatic situation

was found by Zhong et al. [102], showing that the miR30a was downregulated and underexpressed in metastatic CRC cells vs non-metastatic ones, by previously described effect of negatively regulating Beclin 1 mRNA in tumor cells, thus decreasing autophagic activity [109,110], and by downregulation of PIK3CD expression at mRNA and protein levels [102], thus miR-30a-induced inhibition of migration and invasion could be rescued by overexpression of PIK3CD. In a large study, Zhang et al. [107] identified seven subspecies of miRs (miR-106a, -200b, -106b, -200c, -141, -320a and -320b) that were overexpressed both in the primary tumors and CRC liver metastasis, but only miR-320a and miR-200c were downregulated at a significant rate; overexpression of miR-320a in liver metastatic colon cancer cells provides loss of their migration and invasiveness capability; thus, the pathway of silencing this miR in non-metastatic colon cancer cells provided invasiveness, without implications over the proliferation of malignant cells.

To this day, no general consent has been postulated concerning whether the miR downregulation or upregulation are responsible with the invasiveness and metastatic capacity of CRC cells [99,111-114]. Biopsy or piece-meal fragments of primary CRCs may not express the same type or characteristics of miRs as does the primary tumor as a whole. Since a vast number of mRNAs are regulated by each miR, it is thought that two or even more genes that appear in different molecular pathways, may be modified in their outcome of expression and, bearing in mind the tissue specificity of miR activity, a strict demarcation of cancer-associated miRs into onco- or tumor suppressor miRs may be an oversimplification [99]. A clear identification of the number of up- or down-regulated miRs is not necessarily important in predicting the effect of miRs on cell invasiveness and metastatic capacity, where gene expression is in fact a co-determinant [99]. The role of miRs in liver metastases from CRCs is shown in Table 3.

### **MicroRNAs as viable biomarkers in the clinic**

Currently, the serum levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) represent the main biomarkers of liver injury in a wide variety of diseases [115], but their assessment has very important limitations. The first one is that an elevation in ASAT levels may reflect non-liver injury such as muscle damage and complicate the differential diagnosis. The

second is that in various clinical scenarios such as acetaminophen toxicity, their serum elevations could occur after a critical therapeutic window and third – the serum transaminase concentrations don't really discriminate very efficiently between the etiology of the hepatocyte damage. All these problems could be solved very easily and in an elegant manner by new discoveries in genetics and molecular biology, with various miRs having the potential to replace the classic biomarkers found on any hospital spreadsheet in the world.

Modern cancer classification is aimed to establish adequate prognosis in order to select the best therapeutic option available and help researchers to design clinical trials with comparable criteria because an inadequate prediction could cause an unnecessary harm to patients or could significantly increase the healthcare costs. miRs are very stable in blood and other bodily fluids and their expression pattern seem to be very tissue-specific, making them ideal candidates for non-invasive cancer evaluation. Based on this hypothesis, circulating miRs have been reported in a wide variety of malignancies, from lymphomas, CRCs or breast cancers to liver malignancies or lung cancer [116-124]. Liver cancers are usually diagnosed too late and afterwards the biomarkers have an acceptable sensitivity and specificity, but in order to improve the therapeutic ratio of these patients, an excellent biomarker should alert the clinician about the possibility of a malignancy even before it can be detected via conventional imaging techniques.

One of the scientists that tried to solve this question is Qu and his team who investigated the usefulness of measuring circulating miRs [125], either alone or in combination with classic HCC markers, in an attempt to improve early detection. This group found that miR-16 combined with AFP had a greater sensitivity in comparison with HCC markers used alone. Additional miR-16 measurement as a second-line assay identified a lot of HCC cases that exhibited false-negative results in all three conventional markers. Even if AFP is commonly used all over the world as a serum tumor marker for the screening of HCC, AASLD strongly recommends against its use as a sole marker for screening unless ultrasonography is available [126], supporting the acute need for efficient markers. Even if the measurement of certain miRs as tumor markers has only recently been reported, this approach is extremely attractive because non-coding RNAs are very stable in human blood, plasma, serum or even formalin-fixed tissues

[127], apart from their high tissue specificity [128]. In patients diagnosed with colorectal or liver cancers, adequate prognoses are important for an effective therapy because biochemical markers such as CEA or CA-19-9 are very non-specific. Shibuya et al. [129] studied the association between miR-21 expression and the clinical/pathology evolution and found that the upregulation of this miR is associated with venous invasion, liver metastases and even advanced Dukes' stages. The data was later confirmed by Scheter et al. [130], who showed that a high miR-21 expression is linked to poor survival. Some miR species are involved in the liver development [131,132] and have dynamic changes correlated with cancer initiation and progression. The study of Yamamoto et al. [133] supports this idea, showing that miR-500 is an oncofetal miR overexpressed in both the fetal liver and HCC. Aguello et al. [134] also showed that another miR is upregulated when compared with non-cancerous cirrhotic tissues and can be used to make the differential diagnosis between early HCC and high grade dysplasia, independently of

HBV or HCV presence.

Lu et al. [112] demonstrated how just a relatively small number of miRs can reflect both the tissue of origin and the natural history of a cancer, providing a very accurate classification. Jiang et al. [135] also showed that the expression of more than 200 miRs in HCC or in benign tumors can propose a 19-miR-based molecular signature that is significantly associated with the outcome of disease.

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