Editorial

MicroRNAs are key regulators of hepatocellular carcinoma (HCC) cell dissemination—what we learned from microRNA-494

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Hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death worldwide, and it is well accepted that the poor outcome of HCC patients among others is caused by metastasis and tumor cell dissemination. Early tumor recurrence due to intrahepatic micro-metastases predominantly occurs in early phases of hepatocarcinogenesis (often within the first 2 years after treatment), whereas new primary lesions are observed after longer periods (1). Importantly, metastasis is mainly detectable within the diseased liver itself, with new tumors invading into the portal vein (2).

Many aspects of liver tumor cell migration and invasion are well understood. The cellular mechanisms necessary for the initiation and maintenance of a mobile phenotype includes for example cessation of cell polarity (which is of special importance for highly polarized hepatocytes), cytoskeletal reorganization, re-connection with the microenvironment, and activation of pro-migratory intracellular molecules. However, the upstream regulatory mechanisms leading to a highly invasive cellular phenotype are not completely understood. In the recent study Kuang-Hsiang Chuang and colleagues illustrated that the dysregulation of a single microRNA (miRNA), miR-494, supports HCC invasiveness through the epigenetic regulation of a miRNA network (3).

miRNAs are single-stranded non-coding RNAs, 19–25 nucleotides long, obtained from endogenous hairpin transcripts. They negatively regulate gene expression by binding to target mRNAs, usually in the 3'UTR, which subsequently leads to target degradation or translational repression. A single miRNA may target more than 100 transcripts, and it is estimated that more than 60% of human protein-coding genes are modulated by miRNAs, pointing at them as master modulators of gene expression (4). Indeed, many aspects of tumor cell biology including stemness and epithelial-to-mesenchymal transition (EMT) are modulated by miRNAs (5). Dysregulation of miRNA expression in cancer, as it occurs with other genes, may be due to genetic alterations, modification of epigenetic patterns, transcriptional control, and post-transcriptional regulation (e.g., alteration of the miRNA processing machinery). It is a general characteristic of cancer cells that the expression of most miRNAs decreases in tumor tissue compared to normal tissue. However, some miRNAs are increased in malignantly transformed cells and facilitate oncogenic properties (4).

Few miRNAs such as miR-122 account for >80% of around 300 miRNAs expressed in healthy liver tissue (6). miRNA dysregulation is already detectable in early stages of liver tumorigenesis and a number of studies illustrated the relevance of aberrant miRNAs on different aspects of hepatocarcinogenesis and HCC cell biology. For example, a specific set of miRNAs has been demonstrated to regulate lipid synthesis, fatty acid oxidation, as well as lipoprotein production and therefore is involved in the development of metabolic syndrome (7) and non-alcoholic fatty liver disease (NAFLD) (8). In addition, hepatitis B and hepatitis C viruses (HBV and HCV), which are major risk factors for the development of HCC, not only alter the miRNA profile in HCC cells and patients but also exploit the host miRNAs to improve viral replication and tumor-supporting mechanisms (9,10). Importantly, up- or down-regulation of individual miRNAs and miRNA signatures have been used to further classify HCCs according to specific biological or clinical parameters (11). For example, a signature consisting of 20 miRNAs discriminated between HCC

with and without venous metastasis (12). The presence of this signature correlated with poor outcome and tumor relapse, illustrating that a set of miRNAs and a network of probably hundreds of target transcripts may define a migratory phenotype. In addition, several individual miRNAs have been reported to be involved in HCC cell dissemination. For example, reduced expression of miR-122, which regulates hepatocyte differentiation, increased the metastatic potential of HCC cells. In addition, miR-34a showed tumor suppressor effects by reducing cell migration and invasion via targeting the hepatocyte growth factor (HGF) receptor c-MET. In contrast, miR-21 represented an oncogenic miRNA in HCC that induce cell growth, invasion, and metastasis by inhibiting PTEN gene activity (13).

The study by Kuang-Hsiang Chuang and colleagues identified miR-494 as an oncogenic miRNA in liver cancer, which predominantly mediates its pro-migratory effects through the reduction of an enzyme regulating epigenetic tags, followed by the inhibition of several invasion-suppressor miRNAs (3). Thus, the dysregulation of miR-494 might function as an initial kick-off for a cascade driving HCC cell dissemination. Based on miRNA profiling of HCC patients with and without vascular invasion combined with data derived from HCC cell lines (with low or high invasive capacity) the authors identified a set of significantly up- or down-regulated miRNAs. Highly expressed miR-494 in tumor nodules correlated with poor patient survival. *In vitro*, miR-494 supported migration as well as invasion associated with the induction of EMT-related genes. Interestingly, miR-494 overexpression induced the hypermethylation of proximal CpG-islands—and therefore reduction—of miRNAs that are known suppressors of cell invasiveness. The authors hypothesized that miR-494 affected these miRNAs via inhibition of promoter demethylation. By using different bioinformatic tools, the TET family of methylcytosine dioxygenases was identified as possible miR-494 target. This family of enzymes converts 5-methylcytosine (5mC) to 5'-hydroxymethylcytosine (5hmC) and therefore initiates the removal of the epigenetic tag. Indeed, miR-494 negatively regulated three TET isoforms (TET1-3) and diminished global 5hmC levels. Genetic experiments revealed that the inhibition of TET1 resembles the effects observed after miR-494 overexpression. Importantly, in a rescue experiment, the authors showed that reduced cell mobility after miR-494 inhibition was partly compensated after simultaneous TET1 knockdown. Indeed, HCCs with high miR-494 amounts (associated with vascular invasion) showed increased levels of EMT markers, and reduced 5hmC abundance and TET1 expression. Lastly, reduction of miR-494 amounts diminished the ability of HCC cells to form lung metastases in an orthotopic HCC xenograft model.

Besides the recent study by Chuang *et al.*, other publications described the oncogenic role of miR-494 in HCC. For example, miR-494 was reported to induce proliferation, migration, and invasion, as well as Sorafenib resistance, by targeting the phosphatase PTEN (14). Moreover, miR-494 increased HCC cell proliferation and G1/S cell cycle transition through targeting the tumor suppressor gene mutated in colorectal cancers (*MCC*), and its inhibition decreased transformation in both human HCC cell lines and *de novo* tumor formation (15). miR-494 has also been discussed as biomarker since circulating miR-494 in sera distinguished cirrhotic patients with and without HCC (16). However, the study of Chuang *et al.* for the first time suggested that miR-494 facilitates its pro-migratory properties through modulation of TET1 and probably inactivation of further tumor suppressive miRNAs.

Interestingly, the role of miR-494 is not consistent across different tumor types. miR-494 overexpression found in HCC has also been reported for non-small cell lung cancer (NSCLC) (17), acute myeloblastic leukemia, and retinoblastoma (18). In contrast, miR-494 expression was reduced in many other tumor entities including breast cancer (19), ovarian cancer, prostate cancer, gastrointestinal stromal tumor, pancreatic cancer, and cholangiocarcinoma (18). These data clearly indicate that the tumor-suppressive or oncogenic function of miR-494 is tissue and cell type dependent. This dual role of miRNAs is frequently reported in the literature and reflects the pleiotropic character of miRNAs, which preferentially target distinct mRNA sets according to the genomic background and microenvironment. Reported targets of miR-494 include the oncogene c-MYC in ovarian, gastric (20), and pancreatic cancers (21) as well as the tumor suppressor genes PTEN and MCC in HCC. The molecular mechanism described by Chuang *et al.* might also be relevant for those cancer types where miR-494 acts as an oncogene (e.g., NSCLC); however, this mechanism is probably inactivated or functionally compensated in tumors where miR-494 showed tumor suppressor properties.

Another interesting aspect is that miR-494 belongs to a miRNA megacluster on chromosome 14q32 that has been reported to play an important role in cancer. In HCC, overexpression of this cluster has been associated with

cell stemness and poor survival rates (22). Likewise, the expression of miRNAs located on this cluster was reported to drive aggressiveness in lung adenocarcinoma (23). These results are in agreement with the overexpression of miR-494 in the same tumor type. On the contrary, the 14q32 miRNA cluster has been shown to be repressed in other human cancers, such as glioblastoma, ovarian cancer, breast invasive carcinoma, kidney renal clear cell carcinoma, stomach adenocarcinoma, prostate adenocarcinoma, and bladder urothelial cancer (24). These findings not only support the dichotomous character of miR-494 but also indicate that other 14q32 cluster miRNAs differentially affect tumor cell properties in different cancer types.

A recent study by Lim *et al.* showed an induction of miR-494 as part of the miRNA cluster 12qF1 (mouse orthologue of human 14q32) in three transgenic liver cancer models driven by c-MYC, RAS, and c-MYC+RAS oncogenes. This correlation was confirmed in human HCC samples (15), suggesting that c-MYC and RAS represent potential upstream modulators of miR-494 and the human miRNA cluster 14q32 in general. Moreover, an independent study demonstrated that miR-494 was downregulated after the inhibition of ERK1/2 nuclear activity in 293A cells (17), further supporting the involvement of the Ras/ERK pathway in the modulation of miR-494 expression.

Besides miR-494, only few studies have demonstrated the modulation of DNA methylation levels by miRNAs through the repression of distinct miRNA target genes. For example, the miR-29 family promotes DNA demethylation and consequent reactivation of tumor-suppressor genes by inhibiting the *de novo* DNA methyltransferases DNMT3A and DNMT3B (4). Common epigenetic changes occurring in cancer cells include global hypomethylation but also hypermethylation of tumor suppressor genes (4). The study on miR-494 now suggested that dysregulation of specific miRNAs may act as initial events that drive tumor-supporting imbalance of other miRNAs depending on an epigenetic mechanism.

The overall 5-year survival rate of HCC is still very low, partly due to the unsatisfactory power of conventional HCC biomarkers (e.g., DPC, AFP, and AFP-L3), which are often unable to distinguish between cancer and inflammatory diseases such as chronic hepatitis or liver cirrhosis (22). Unlike currently used biomarkers, miRNAs have a high specificity in cancer detection and classification. In addition to the examples mentioned above, a seven miRNA signature is suitable to differentiate HCC patients from healthy volunteers, patients with cirrhosis, and patients with chronic HBV infection (13). Since highly stable miRNAs can be accurately detected in a wide variety of body fluids (4) even under extreme conditions (13), they represent ideal non-invasive biomarkers that can help physicians with patient evaluation and therapy. Importantly, differential miRNA expression in serum has been detected even at early cancer stages (5). However, there is poor consensus regarding circulating miRNA profiles in patients with HCC (16). The major reasons for this limitation are probably differences in the miRNA isolation protocols, cohort specifications, varying technical detection platforms, and to certain extent tumor heterogeneity in human HCCs. Therefore, additional studies are needed to achieve consistent results on potential miRNA biomarkers. The study of Chuang *et al.* indicates that miR-494 levels might be used to identify highly aggressive HCCs. Moreover, it is possible to analyze the methylation status of the downstream tumor-suppressive miRNAs repressed by miR-494 as indicator of HCC aggressiveness. Indeed, DNA methylation levels have been previously proposed to be useful as markers for cancer prognosis (25).

Besides their potential role as biomarkers, miRNAs represent novel targets for therapeutic intervention, which may include the administration of drugs that modulate upstream regulators of miRNA expression, inhibition of oncogenic miRNAs or reintroduction of tumor-suppressive miRNAs (13). According to the results of Chuang *et al.*, the therapeutic inhibition of one individual miRNA might lead to the consequent upregulation of a set of tumor-suppressive miRNAs, which would inhibit tumor development. Since miRNAs are master coordinators of multiple cellular pathways, it is assumed that miRNA-directed therapies will be less liable to the development of resistance.

One of the main challenges of miRNA-based therapy is to reach the required drug levels in the tumor. However, chemical modifications of the therapeutic miRNA joined with the fact that the liver has a unique affinity for small nucleic acids (15), allowed to demonstrate the efficacy of a miRNA-based therapy in primates infected with HCV. In this study, locked nucleic acid (LNA)-modified anti-miR-122 was administered to chronically infected chimpanzees. Results demonstrated that the treatment induced a long-term suppression of HC-viremia with no evidence of unwanted effects (4). This example shows that miRNA-based therapies might be applicable in the near future. Nevertheless, as shown before, miRNAs may exert both oncogenic and tumor suppressor activities depending on the tissue/cancer type. Therefore, the selection of the

miRNA/s to be targeted or replaced in each specific tumor type must be carefully defined.

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Footnote

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