Regulatory MiR-148a-ACVR1/BMP Circuit Defines a Cancer Stem Cell-Like Aggressive Subtype of Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and the third most common cancer in Asia. HCC has heterogeneous etiologic and molecular profiles and a varied response to therapeutics. The high recurrence rate and curtailed survival in this cancer are attributed to its resistance to therapy. The ultimate goal is to develop a more effective personalized therapeutic strategy for HCC, but the first step is to develop a system for classifying the disease on the basis of molecular biomarkers. To that end, we performed mRNA and microRNA (miRNA) expression profiling in 100 HCC tissues. Clustering analysis of informative genes identified two robust subtypes, which were validated by an independent dataset. The subtype characterized by a cancer stem cell-like signature was clinically aggressive and associated with poor survival. Integrated analysis of miRNA and mRNA expression in this subtype showed that miR-148a was expressed at a significantly lower level in these tumors than in the other subtype. MiR-148a has been shown to directly suppress the expression of activin A receptor type 1 (ACVR1), a key receptor in the signaling pathway of the bone morphogenetic proteins (BMPs), which regulate many stem cell markers as well as the clinically important cytokine interleukin-8 (IL-8). Increased expression of ACVR1 and its downstream genes EPCAM, CD24, CD90, and IL-8 was associated with shorter survival in a larger cohort of 227 HCC cases. Introduction of miR-148a resulted in suppressed tumor phenotypes both in vitro and in vivo. Conclusion: We identified a clinically aggressive stem cell-like subtype of HCC that is characterized by an miR-148a-ACVR1-BMP-Wnt circuit. We propose that miR-148a may serve as a prognostic biomarker and therapeutic target for this subtype of HCC. (HEPATOLOGY 2015;61:574-584)
molecular changes in the subtypes and to identify the key molecular targets for development of new therapeutic strategies, integrated analysis of high-throughput data from multiple platforms is needed.

MicroRNAs (miRNAs) have emerged as key regulators in gene expression networks affecting many biological processes, including cellular proliferation, differentiation, and apoptosis in liver cancer.\(^8,9\) MiRNAs have also proven to be an important class of regulators in molecular subtypes of cancers.\(^10\) Thus far, very few studies have examined expression profiling of both mRNAs and miRNAs in tumor tissues from a large cohort of patients with HCC.

Here we report an integrated analysis of mRNA and miRNA expression profiling in 100 HCCs and a subsequent validation study that identified two distinct molecular subtypes of HCC; from this study, we identified an miRNA that might have therapeutic implications in the aggressive stem cell-like subtype of HCC.

**Materials and Methods**

**Human Subjects (Also See Supporting Methods).** Study subjects were selected from the set of patients who underwent curative surgery for HCC at Tianjin Medical University Cancer Institute and Hospital between 2003 and 2011. Tianjin cohort1 of 100 tumor tissue samples analyzed in this study for mRNA and miRNA expression profiling was randomly selected from the patients who underwent surgery between 2003 and 2009, and Tianjin cohort2 of 197 tissue samples included in the subsequent independent validation study were selected from the patients who underwent surgery between 2004 and 2011. The gene expression data of the validation cohort (n = 225) was published earlier (accession number GSE14520).\(^10\)

**MiRNA and mRNA Expression Profiling.** MiRNA and mRNA expression profiling was conducted by using Affymetrix Human Genome U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA) and GeneChip miRNA arrays (Affymetrix; including 7,815 miRNA probe sets from Sanger miRNA database V11), respectively. All of the procedures, including labeling and hybridization were conducted according to the manufacturer’s protocol and recommendations. A GeneChip scanner 3000 was used to scan the microarray chips and the obtained images were analyzed by the GeneChip Operating Software for gene expression profiling. A GeneChip-compatible program was used for analysis of miRNA expression profiling data.

**Results**

**Two Molecular Subtypes of HCC With Different Prognoses Identified by Consensus Clustering Analysis.** Consensus k-means unsupervised clustering of the 309 genes defined as highly variable in the 100 Tianjin cohort1 HCC samples identified two robust clusters (Supporting Fig. S1), and data were visualized according to the highly variable genes identified as significantly differentially expressed in the two clusters (Fig. 1A). To determine whether the same classification approach would yield the same two subtypes in another group of tumors, we analyzed transcriptome data from an independent set of 225 HCC (accession number GSE14520)\(^11\) by applying the same clustering approach of Tianjin cohort1 (n = 100). This analysis also yielded two distinct clusters, which were visualized using the same genes as used in Fig. 1A (Fig. 1B). The proportions of the two subtypes were similar in the two independent cohorts (Cluster 1, 26% in the Tianjin cohort1 and 33% in the validation set).

Kaplan-Meier survival curve analysis showed that patients in Cluster 1 of Tianjin cohort1 had poorer overall survival than those in Cluster 2 when the survival time was capped at 60 months (Fig. 1C; log rank P = 0.016, hazard ratio [HR]: 2.90, 95% confidence interval [CI]: 1.22-6.92). Similarly, Cluster 1 from the
validation set also showed significantly poorer prognosis (Fig. 1D; log rank \( P = 0.016 \), HR: 1.75, 95% CI: 1.11-2.75). Thus, HCC comprises at least two molecular subtypes with distinct clinical outcomes.

**Functions of the Molecular HCC Subtypes Characterized by Integrated mRNA and miRNA Analysis.** To characterize the biological functions of the two HCC transcriptome subtypes, we first performed a significance analysis of microarrays\(^{12}\) to identify signature genes that were specifically altered in each subtype. In all, we identified 821 genes in Cluster 1 that had a statistically significant 2-fold or greater difference in magnitude (\( P < 0.05 \)) from the same genes in Cluster 2, of which 326 were down-regulated and 495 were up-regulated. The signature genes identified as overexpressed in the poor-survival subtype were significantly enriched in stem cell-related genes identified from published studies (\( P < 1.0 \times 10^{-05} \)),\(^{6,13-15}\) including epithelial cell adhesion molecule (EPCAM), CD133, CD24, KRT19, KIT, and alpha-fetoprotein (AFP) (Fig. 2A). AFP has been used together with EPCAM to isolate cells with stem cell features\(^{15}\) and is a clinical serum biomarker of HCC.\(^{16}\) Other well-known markers of hepatic oval cells such as VIM, CD44, and CD90 were not included in the gene signature list because of the fold cutoff, but they exhibited significantly higher expression in Cluster 1 as well (Fig. S2). A hepatoblast subtype of HCC exhibiting stem cell-like features was identified and was likely driven by dysregulation of the AP-1 network.\(^{6}\) Notably, the genes comprising the AP-1 complex (i.e., FOS, JUNB, and FOSL2) and many of their reported downstream targets were significantly up-regulated in Cluster 1 (\( P < 0.05 \), Fig. S3).
Next we performed gene set enrichment analysis (GSEA) of the genome-wide expression profiles to identify overrepresented gene sets. Our data identified six curated stem cell gene sets that were significantly enriched in Cluster 1, four of which were up-regulated and two were down-regulated (Fig. S4A). A leading edge analysis indicated limited overlap among these enriched sets (Fig. S4B), likely because they were derived from different cancer types. Strikingly, the down-regulated genes (YAMASHITA_LIVER_CANCER_STEM_CELL_DN) identified from the EPCAM(+) AFP(+) HCC subtype of liver cancer, which had features of hepatic stem/progenitor cells, were most significantly enriched among the genes that were consistently down-regulated in Cluster 1, with an enrichment score of $-0.79$ (nominal $P$-value < 0.0001). IL-8 and SOX4 were highly expressed in Cluster 1, with a difference in magnitude of 2-fold or greater and statistical significance ($P < 0.0001$). High levels of IL-8 and SOX4 were associated with poorer overall survival than low levels.

In addition to the stem cell markers already mentioned (Fig. 2A; Fig. S2), interleukin-8 (IL-8) and SOX4 were included in the gene signature for these HCCs, and their mRNA expression levels were significantly higher in Cluster 1 (Fig. 2C). SOX4 is known to be involved in neural progenitor cells and IL-8 is a cytokine involved in cancer metastasis. The expression levels of these two genes in the tumors were significantly correlated with overall patient survival (Fig. 2D).

Correlation of molecular subtype with clinicopathological features of the HCCs (Table S2) showed that the subtypes were not significantly associated with tumor stage or alcohol/smoking history, even though the stem cell-like subtype tended to be more invasive than the metabolic subtype. Interestingly, the patients with the stem cell-like subtype were significantly younger ($P = 0.006$, Table S2) and more likely to be female ($P = 0.018$).
MiR-148a-ACVR1 Circuitry Revealed by Integrated Analysis. MicroRNA expression profiling of the same 100-sample set (Tianjin cohort1) provided an opportunity for integrated analysis to identify the miRNA-mRNA regulatory networks that may underlie the stem cell gene enrichment in the stem cell-like subtype. Using supervised analysis, we identified 10 miRNAs that were differentially expressed between the two transcriptome subtypes (P < 0.0001, Fig. 3A); among these, miR-148a exhibited the greatest difference and was down-regulated in the stem cell-like subtype (P = 4.2E-7, Fig. 3A). The second most significantly differentially expressed miRNA, miR-181b, was overexpressed in the stem cell-like subtype, consistent with its known association with stem cell features. Integrated analysis of the down-regulated miRNAs and the signature gene expression data showed that miR-148a was significantly and negatively correlated with most of the overexpressed genes in the signatures (Fig. S6A), as compared with the other four significantly down-regulated miRNAs (Fig. S6B). Moreover, miR-148a expression was significantly correlated with patient overall survival (Fig. 3D). These results suggest that loss of miR-148a expression might be a key mechanism for up-regulation of stem cell signature genes in the poor-survival HCC subtype.

To further evaluate the relationship between expression of miR-148a and survival in HCC, we performed quantitative reverse-transcription polymerase chain reaction (qRT-PCR) in 297 HCC tumor tissues (the 100 HCCs represented by the microarray [Tianjin cohort1] and 197 from the independent HCC cohort [Tianjin cohort2]). The baseline and clinical characteristics of both sets were similar (Table S3). In Tianjin cohort1, miR-148a expression in the stem cell-like subtype was significantly lower than in the metabolic subtype (P < 0.0001), which is consistent with the microarray data (Fig. S7). There was no significant
association between miR-148a expression and baseline/clinical characteristic except tumor size ($P = 0.0147$) in the 297 HCC tumor tissues (Table S4).

Of the 297 HCCs in the two Tianjin cohorts, those with low miR-148a expression were likely to be associated with significantly shorter overall survival ($P = 0.011$, log-rank test) but not in women ($n = 44$, $P = 0.613$, log-rank test). Interestingly, the associations of miR-148a with overall survival and recurrence-free survival was not significant in the entire group ($n = 297$, $P = 0.393$, log-rank test) but was marginally significant in men ($n = 253$, $P = 0.063$, log-rank test). Expression of miR-148a was calculated by use of tertile cutoffs in all patients. Red lines represent low expression, blue lines represent middle expression, and black lines represent high expression.

![Graphs showing survival probability](image)

**Fig. 4.** Validation study of miR-148a expression by qRT-PCR. (A-C) The expression of miR-148a was significantly associated with overall survival in the group of patients taken as a whole ($n = 297$, $P = 0.040$, log-rank test). The association was significant in men ($n = 253$, $P = 0.011$, log-rank test) but not in women ($n = 44$, $P = 0.613$, log-rank test). (D-F) The association between miR-148a expression and recurrence-free survival was not significant in the entire group ($n = 297$, $P = 0.393$, log-rank test) or in women ($n = 44$, $P = 0.500$, log-rank test) but was marginally significant in men ($n = 253$, $P = 0.063$, log-rank test). Expression of miR-148a was calculated by use of tertile cutoffs in all patients. Red lines represent low expression, blue lines represent middle expression, and black lines represent high expression.

When we matched the 326 overexpressed genes in the stem cell-like subtype to the miR-148a conserved target genes predicted from TargetScan, only 11 genes (~3.3%) were computationally predicted to bind directly with miR-148a (Table S6), suggesting that an indirect mechanism, such as pathway regulation mediated through intermediate key node, may be at play. Activin A receptor type 1 (ACVR1) expression was significantly negatively correlated with miR-148a and was significantly differentially expressed in Cluster 1 and Cluster 2 (Fig. 3B). ACVR1 is an important receptor of the bone morphogenetic protein (BMP) that is closely involved in regulation of the BMP/Wnt signaling frequently activated in stem cells, and has been reported previously to directly bind with miR-148a through its 3′-untranslated region (UTR). We validated this regulation by using a reporter gene assay in which the luciferase activity was decreased when cotransfection of MHCC97H cells with the pmirGLO-ACVR1 3′UTR-Luc construct and an miR-148a mimic (Fig. 3C). Consistent with this was our finding that many of the direct downstream targets of the Wnt signaling pathway (e.g., EPCAM, IL-8, and FOS) were up-regulated in the stem cell-like subtype (Fig. 3E). Therefore, the miR-148a-ACVR1 circuit appeared to be highly active in the stem cell-like Cluster 1 of HCC.
Cancer Stem Cell Markers Are Prognostic in HCC. The integrated genomic analysis defined a set of miR-148a-ACVR1 circuit genes, including cancer stem cell-regulatory genes and metastasis-related genes that are associated with poor survival in HCC. To determine whether these genes are indeed prognostic markers for HCC at the protein level in a larger cohort of HCC, we performed an immunohistochemical analysis on a tissue microarray representing the 227 HCC patients of the validation set. Immunostaining showed membranous and/or cytoplasmic staining for ACVR1, CD44, EPCAM, CD24, CD90, and KRT19 and nuclear and/or cytoplasmic staining for SOX4. Kaplan-Meier analysis demonstrated that positive staining for ACVR1, CD44, CD24, and CD90 was significantly associated with poor overall survival and recurrence-free survival. The expression of EPCAM and IL-8 was significantly associated with poor overall survival but not with recurrence-free survival. Red lines represent positive for protein markers, and blue lines represent negative for protein markers.

Fig. 5. Expression of hepatic cancer stem cell-related proteins in HCC tissues related to HCC prognosis in validation study. (A,B) Hematoxylin & eosin and hepatic cancer stem cell-related protein immunostaining of stem cell-like subtype HCCs (A) and metabolic subtype HCCs (B) from the 227-case validation dataset. The staining images show high expression of ACVR1, CD44, EPCAM, CD24, CD90, and IL-8 in the stem cell-like subtype HCC tissues (A) and low expression of these proteins in metabolic subtype HCC tissues (left panel: magnification ×40; right panel: magnification ×400). (C,D) Kaplan-Meier analysis of overall survival (C) and recurrence-free survival (D) according to expression of hepatic cancer stem cell-related proteins in the validation set. The expression of ACVR1, CD44, CD24, and CD90 was significantly associated with poor overall survival and recurrence-free survival. The expression of EPCAM and IL-8 was significantly associated with poor overall survival but not with recurrence-free survival. Red lines represent positive for protein markers, and blue lines represent negative for protein markers.
ACVR1 \((P = 0.050)\), CD44 \((P = 0.035)\), CD24 \((P = 0.040)\), and CD90 \((P = 0.019)\) was associated with shorter recurrence-free survival (Fig. 5D). There was no statistically significant association between survival and expression of either SOX4 or KRT19 (Fig. S9B,C). Positive staining for EPCAM and IL-8 was not associated with recurrence-free survival (Fig. 5D).

**Overexpression of MiR-148a Suppresses Liver Cancer Cell Proliferation, Migration, and Invasion In Vitro and Inhibits Subcutaneous Growth of Liver Cancer Cells In Vivo.** To determine the effect of miR-148a overexpression on liver cancer cell properties, we treated liver cancer cells with miR-148a mimic or inhibitor. We transfected MHCC97H cells with miR-148a mimic or negative control; we found that
transfection of miR-148a mimic led to inhibited cell proliferation, migration, and invasion, and there was the opposite effect when SMMC7721 cells were treated with miR-148a inhibitor or negative control (for details, see Supporting Results) (Fig. 6A-D). We next examined the effect of overexpression of miR-148a on tumor growth in BALB/C nude mice. MHCC97H cells (5.0 × 10^6 cells) were subcutaneously injected into both flanks of each mouse. Each mouse was treated with miR-148a mimic (1 nm/mouse) or vehicle (negative control), and tumor volumes were measured twice a week. We found that tumor growth was significantly inhibited in the miR-148a mimic-treated group compared to the control group (P = 0.007; Fig. 6E). MiR-148a expression was significantly higher in the miR-148a mimic-treated group than in the control group (P = 0.007; Fig. 6F).

**Discussion**

Starting with gene expression profiling from 100 human HCC samples, we identified a clinically aggressive subtype of HCC with a cancer stem cell signature, a finding supported by the discovery of a similar cluster in an independent cohort with the same gene signature. Simultaneous profiling of the transcriptome and miRNAs gave us further insight into the key regulatory relationships in the miRNA-mRNA network that underlies the aggressive nature of this cancer stem cell-like subtype. Through integrated computational analysis coupled with experimental validation, we identified a key tumor-suppressing miRNA, miR-148a, that is attenuated in the cancer stem cell-like HCC subtype. Loss of miR-148a resulted in consistent up-regulation of a critical substrate, ACVR1, a key receptor of BMP7 and an important regulator of the BMP/Wnt signaling pathway, which is critical for cancer stem cells. BMP7 may also repress the expression of IL-8, a major cancer-related cytokine that promotes cancer growth and metastasis.

Recently, it was reported that stem/progenitor cell markers such as EPCAM, JAG1, and Sox9 were enriched in severe nonalcoholic fatty liver disease (NAFLD) compared with mild NAFLD. These findings suggest that stem cell-related biological processes might be initiated long before the neoplasms emerge and that these markers may serve as biomarkers for HCC risk prediction and early diagnosis.

The molecular subtyping of HCC has been reported in the literature but often without sufficient attention to the underlying mechanisms and therapeutic implications. The limitations of past studies were attributable mainly to the lack of multiplatform data and integrated analysis. In this study, we performed integrated analysis of gene expression and miRNA expression profiles produced from the same set of 100 HCC cases. We further experimentally interrogated a key miRNA node that is responsible for regulating this stem cell-like subtype and provided evidence suggesting that miR-148a is a potential therapeutic tool for patients whose HCC is of this stem cell-like subtype. This is a clinically relevant discovery because a number of published studies have shown that HCCs that possess cancer stem cell components carry a greater risk of chemoresistance, radioresistance, and recurrence than other HCCs.

Pathway analysis showed that the hepatic fibrosis/hepatic stellate cell activation-related pathway was significantly highly expressed in the stem cell-like subtype. Hepatic stellate cells are progenitor cells that have differentiation potential and can express stem cell markers, such as CD133, Notch1, and Notch3. Activated hepatic stellate cells have been shown to induce the occurrence and development of liver fibrosis, and Wnt signaling pathways are up-regulated and implicated in the process.

A number of cancer stem cell factors have been studied in depth, including EPCAM, CD24, and CD90. These three proteins are targets for Wnt signaling and, together with ACVR1, were shown to be associated with shorter survival in our validation studies of a larger cohort of HCC cases in a tissue microarray. Given that the liver tissue has an intrinsic capacity to renew damaged tissue, it is conceivable that abnormal activation of this process can move beyond tissue damage repair and into the tumorigenic program. Indeed, cirrhosis and hepatitis B and C viral infections, which both damage normal liver tissues, are the most common etiologic factors for HCC.

Because miRNAs are relatively stable and are naturally secreted and taken up by cells, they are considered a promising new class of therapeutic tool for cancer treatment. Recent studies have been found that altered miRNA expression in liver cancer stem cell subsets compared with noncancer stem cell subsets. We showed that expression of miR-148a was significantly lower in the stem cell-like subtype than in the metabolic subtype and was negatively correlated with ACVR1 expression. MiR-148a regulates ACVR1 protein expression by directly targeting the 3’-untranslated region of its mRNA. ACVR1 is a type I receptor of BMPs and belongs to the transforming growth factor-beta superfamily. Mutation of ACVR1 has been reported in fibrodysplasia ossificans progressiva.
Genetic variants of ACVRI were associated with breast cancer and an anti-Müllerian hormone level in women with polycystic ovary syndrome. BMP signaling by way of ACVRI in osteoblasts reduces canonical Wnt signaling by suppressing of Wnt inhibitors SOST and DKK1. Because ACVRI activate Wnt signaling, which plays an essential role in cancer stem cells, there has been extensive effort to develop an inhibitor for ACVRI. However, no ACVRI inhibitor has become available. The current study suggests that introduction of miR-148a might be an alternative approach.

The gene encoding miR-148a located at 7p15.2, a locus that can be silenced by hypermethylation. MiR-148a was also reported to interact with DNMT1 (DNA methyltransferase 1) in gastric cancer. Recent studies have reported that miR-148a promotes myogenic differentiation, contributes to DNA hypomethylation in lupus, suppresses the BMP signaling pathway in fibroblast ossificans progressiva, and suppresses tumor cell invasion and metastasis in gastric cancer. MiR-148a may play a central role in HBx- and DKK1. Because ACVRI activate Wnt signaling, its relationship with HCC survival. The role of miR-148a in cancer stem cells and BMP/Wnt signaling, and metastasis through ACVRI/BMP/Wnt signaling, could indeed suppress tumorigenesis and progression.

In summary, we identified a clinically aggressive stem cell-like subtype of HCC and found that expression of miR-148a, which is related to cancer growth and metastasis through ACVR1/BMP/Wnt signaling, was low in this subtype. Thus, this molecular classification study and integrated analysis of the miRNA-mRNA circuit in HCC may lead to a novel therapeutic strategy that improves the prognosis of an aggressive subtype of HCC.

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References


Author names in bold designate shared co-first authorship.

Supporting Information

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