Octamer 4/MicroRNA-1246 Signaling Axis Drives Wnt/β-Catenin Activation in Liver Cancer Stem Cells

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Wnt/β-catenin signaling is activated in CD133 liver cancer stem cells (CSCs), a subset of cells known to be a root of tumor recurrence and therapy resistance in hepatocellular carcinoma (HCC). However, the regulatory mechanism of this pathway in CSCs remains unclear. Here, we show that human microRNA (miRNA), miR-1246, promotes cancer stemness, including self-renewal, drug resistance, tumorigenicity, and metastasis, by activation of the Wnt/β-catenin pathway through suppressing the expression of AXIN2 and glycogen synthase kinase 3β (GSK3β), two key members of the β-catenin destruction complex. Clinically, high endogenous and circulating miR-1246 was identified in HCC clinical samples and correlated with a worse prognosis. Further functional analysis identified octamer 4 (Oct4) to be the direct upstream regulator of miR-1246, which cooperatively drive β-catenin activation in liver CSCs. Conclusion: These findings uncover the noncanonical regulation of Wnt/β-catenin in liver CSCs by the Oct4/miR-1246 signaling axis, and also provide a novel diagnostic marker as well as therapeutic intervention for HCC. (HEPATOLOGY 2016;64:2062-2076).

Liver cancer remains one of the most prevalent and deadliest cancer types in the world. Hepatocellular carcinoma (HCC) accounts for over 75% of all liver cancer cases. Tumor recurrence and therapy resistance are common and represent major obstacles to the improvement of patient survival. Even after surgery, the prognosis of HCC remains unsatisfactory, with a 5-year postrecurrence rate at >70%. Elucidation of the mechanisms underlying recurrence and therapy resistance is fundamental for the development of new therapeutic treatments for HCC.

Intratumoral heterogeneity and the presence of cancer stem cells (CSCs) play important roles in tumor relapse, metastasis, drug resistance, and other malignant phenotypes of human cancer.1 The development of CSCs and maintenance of their “stemness” are associated with aberrations of several molecular cascades involving signaling triggered by octamer 4 (Oct4), Wnt/β-catenin, Hedgehog, and Notch; but how these self-renewal pathways are linked to contributing to promoting cancer stemness remains understudied. Among these pathways, hyperactivation of Wnt-mediated signaling has been identified as one of the most frequent events occurring in CSCs of a variety of tumor types.2 Activated Wnt/β-catenin signaling was reported to be essential for the self-renewal capacity and drug-resistant properties of human acute leukemic stem cells, leading to poor patient survival.3

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Abbreviations: AFP, alpha fetoprotein; AUC, area under curve; CADM1, cell adhesion molecule 1; CCND1, CYCLIN D1; ChIP, chromatin immunoprecipitation; CI, confidence interval; CSC, cancer stem cell; EV, empty vector; GSK3β, glycogen synthase kinase 3β; H&E, hematoxylin-eosin; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; ISH, in situ hybridization; miRNA, microRNA; MMP7, matrix metalloproteinase 7; mRNA, messenger RNA; NOD-SCID, nude/obese diabetic/severe combined immunodeficiency; Oct, octamer 4; ROC, receiver-operating characteristic; UTR, untranslated region; WT, wild type; ZIP, scramble hairpin control.

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A high level of Wnt activity in colon cancer cells stimulated by microenvironmental factors functionally designates the CSC population.\(^{(4)}\) Conversely, ablation of the \(\beta\)-catenin gene results in loss of CSCs and complete tumor regression in cutaneous cancer.\(^{(5)}\) In HCC, we have also previously reported \(\beta\)-catenin to be preferentially overexpressed in functionally defined liver CSC subsets marked phenotypically by CD133.\(^{(6,7)}\) This is consistent with past reports where Wnt/\(\beta\)-catenin signaling has been shown to play pivotal roles in regulating stem cell and tumorigenic properties in HCC and its activation to be preferentially deregulated in liver CSCs.\(^{(8)}\) Inhibition of the Wnt pathway has also been shown to be effective in eliminating CSC-like features.\(^{(9)}\) However, mechanisms of Wnt/\(\beta\)-catenin pathway deregulation in liver CSCs is not fully understood.

Intrinsic multilevel regulation of the Wnt signaling pathway has been well recognized and well studied. The pathway is initiated when Wnt ligands bind to its receptor(s), namely, Frizzled and low-density lipoprotein receptor-related protein-5 or -6. Consequently, cytoplasmic Dishevelled is phosphorylated and thus enabled to dissociate \(\beta\)-catenin from the “destruction complex” composed of Axin, adenomatous polyposis coli, and glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)). The stabilized \(\beta\)-catenin, which accumulates in the cytoplasm, is activated to translocate into the cell nucleus, where it forms a \(\beta\)-catenin-lymphoid enhancer factor/T-cell factor transcriptional complex and induces transcription of downstream genes implicated in carcinogenesis.\(^{(10)}\) In the absence of Wnt ligand stimulation, however, \(\beta\)-catenin is sequestered in the “destruction complex,” leading to \(\beta\)-catenin degradation by the ubiquitin–proteasome mechanism and ultimate inactivation of \(\beta\)-catenin signaling. Moreover, the initiation process of Wnt ligand binding to receptors can be directly blocked by extracellular antagonistic proteins, including secreted Frizzled-related proteins, Wnt inhibitory factor-1 and Dickkopf family members.\(^{(10)}\) Another layer of regulation would be mutation in \(\beta\)-catenin leading to its constitutive activation. Additionally, cross-talk between \(\beta\)-catenin signaling and other pathways, such as the phosphoinositide 3-kinase/protein kinase B pathway, has also been indicated in various cancers.\(^{(10)}\) Although Wnt signaling is tightly controlled at various levels, its constitutively activated form has been found in various human cancer types and, in particular, representing over 60% of HCC tumors.\(^{(11)}\) However, unlike colon cancer, mutations in \(\beta\)-catenin appear to be uncommon in HCC,\(^{(12)}\) suggesting that undefined epigenetic mechanisms might be involved in maintaining constitutive activation of the Wnt/\(\beta\)-catenin signaling cascade in HCC cells. Understanding how these negative regulatory effectors on the Wnt/\(\beta\)-catenin signaling pathway are concomitantly deregulated in HCC, and, in particular, its roots in liver CSCs, is biologically and clinically important for future development of anticancer stemness strategies.

Here, we report that a human miRNA, miR-1246, activates the \(\beta\)-catenin pathway by suppressing the expression of AXIN2 and GSK3\(\beta\), two members of the \(\beta\)-catenin destruction complex, thus leading to nuclear accumulation and activation of \(\beta\)-catenin and, consequently, promoting cancer and stem-cell-like phenotypes in CD133\(^{+}\) liver CSCs. Clinically, endogenous and secretory miR-1246 is closely associated with adverse patient survival and likely to represent a novel diagnostic and prognostic biomarker for HCC. miR-1246 overexpression in HCC is driven by direct promoter binding of another important self-renewal molecule, Oct4, which is also commonly overexpressed in CD133\(^{+}\) liver CSCs. Together, the Oct4/miR-1246 signaling axis drives Wnt/\(\beta\)-catenin activation in
this subset of HCC cells. Importantly, miR-1246 overexpression events are inversely correlated with the presence of β-catenin mutations in exon 3 and represent mutually exclusive events in liver CSCs and HCC. Collectively, we demonstrate that Oct4/miR-1246 overexpression substantially activates and sustains Wnt signaling in CD133+ liver CSCs and HCC by simultaneously suppressing multiple Wnt inhibitors of the β-catenin destruction complex (AXIN2 and GSK3β), providing a new layer of molecular mechanism by which Oct4 and Wnt/β-catenin self-renewal pathways are linked by miR-1246.

Materials and Methods

PATIENT SAMPLES

Primary human HCC and adjacent nontumor liver tissue samples were obtained from 114 patients undergoing hepatectomy at the Sun Yat-Sen University Cancer Centre (Guangzhou, China). Venous blood samples were collected from 61 HCC patients and 24 normal control individuals at the Queen Mary Hospital in Hong Kong. For β-catenin mutation studies, freshly resected HCC and adjacent nontumor liver tissue samples were obtained from 14 patients undergoing hepatectomy at Queen Mary Hospital. Tissue samples were collected from patients who had not received any previous local or systemic treatment before operation. Venous blood samples were collected before any therapeutic procedures were performed. Use of human samples was approved by the committee for ethical review of research involving human subjects at the Sun Yat-Sen University Cancer Centre and Queen Mary Hospital.

ANIMAL STUDIES

The study protocol was approved by, and performed in accord with, the Committee of the Use of Live Animals in Teaching and Research at The University of Hong Kong. Tumorigenicity at limited dilution and serial transplantation were determined by subcutaneous injection into the flank of 4- to 5-week-old BALB/c nude mice. Six weeks postimplantation, mice were administered with 100 mg/kg of D-luciferin by peritoneal injection 5 minutes before bioluminescent imaging (IVIS 100 Imaging System; Xenogen, Perkin Elmer, Waltham, MA, USA). Livers and lungs were harvested for ex vivo imaging and histological analysis.

STATISTICAL ANALYSIS

Data were analyzed by SPSS (version 21.0) or GraphPad Prism software (version 6.0) and shown as mean± SD, unless otherwise specified. Differences between groups were analyzed by unpaired Student t test for continuous variables. Correlation between expression and clinicopathological variables were analyzed by chi-square test. Association between miR-1246 expression and survival was analyzed by the Kaplan–Meier method with logrank test. Multivariate analyses were performed by Cox regression model. For β-catenin mutation studies, patients were divided into two groups according to whether they exhibited a high (> the median) or low (≤ the median) miR-1246 expression level. Statistical significance was defined as P ≤ 0.05. Asterisks (*, **, and ***) stand for P < 0.05, P < 0.005, and P < 0.001, respectively.

Results

Wnt/β-CATENIN SIGNALING IS PREFERENTIALLY ACTIVATED IN CD133+ LIVER CSCs

Previous work from our laboratory identified a functionally defined CSC subpopulation in HCC marked by CD133. Importantly, this CD133+ liver subset was identified as a root for treatment resistance and tumor recurrence in HCC. Our past data also found transcriptional β-catenin to be preferentially expressed in the CD133+ liver CSC subset as well as in hepatospheres enriched for CD133 and cancer stemness properties as compared to CD133- non-CSCs and adherent differentiated counterparts. Here, we further demonstrated the Wnt/β-catenin pathway to be hyperactivated in CD133+ liver CSCs at the proteomic expression and activity level. Immunofluorescence staining assays showed substantial increase and nuclear accumulation of β-catenin in CD133+ cells isolated from Huh7 and PLC8024 HCC cells (Fig. 1A). Consistently, increased transactivating activity of β-catenin in CD133+ liver
CSCs was likewise detected by TOP/FOP luciferase assays (Fig. 1B). However, the mechanism of Wnt/β-catenin pathway deregulation in CD133 liver CSCs, and how it interacts with other self-renewal pathways that are also commonly deregulated in CD133 liver CSCs to promote HCC stemness, is not understood.

miR-1246 IS PREFERENTIALLY OVEREXPRESSION IN CD133 LIVER CSCS AND TARGETS TWO KEY PLAYERS OF THE β-CATENIN DEGRADATION COMPLEX TO REGULATE Wnt SIGNALING IN HCC

To screen for miRNAs that are deregulated in the CD133-expressing CSC subset, we first comparatively analyzed the miRNA profiles of CD133 expressing and CD133 absent PLC8024 HCC cells by small RNA sequencing (Supporting Table S5). Of the 17 miRNAs that were found significantly deregulated, as defined by a fold change >2 and P value <0.00005 (Supporting Table S5), we then went on to do a subsequent screen for validation in a series of sorted CD133 liver CSC subsets isolated from PLC8024, Huh7, and HepG2 HCC cells. miR-1246 emerged as one of the most significantly deregulated miRNAs (3.2-fold over-expressed in CD133 expressing vs. CD133 repressed cells with a P value of 5.27E-16) that could also be commonly validated to be up-regulated in a series of sorted CD133 liver CSC subsets isolated from PLC8024, Huh7, and HepG2 HCC cells, as compared with their CD133 non-CSC counterparts (Fig. 1C). Consistently, miR-1246 was also found to be preferentially expressed in CD133-expressing human liver cancer cells (Hep3B, Huh7, PLC8024, and...
HepG2) as compared to CD133 absent liver cancer or immortalized liver cells (BEL7402, LO2, SK-HEP-1, HLE, and QGY7703; Fig. 1D). miR-1246 also came forward as an obvious candidate of interest given that in silico prediction by bioinformatics tools (TargetScan and miRanda) identified two tumor-suppressor genes associated with Wnt/β-catenin degradation complex, namely, AXIN2 and GSK3β, that could potentially be suppressed by miR-1246. One putative binding region at the 3’UTR (untranslated region) of AXIN2 (313-319) and two independent binding regions at the 3’UTR of GSK3β (510-516 and 2905-2910) were found to bear significant complementarity against miR-1246 (Fig. 2A, left). These 3’UTR elements of
AXIN2, GSK3β, and miR-1246 are extremely conserved, as shown by their identical sequences across different species, suggesting a functional role (data not shown). To validate whether AXIN2 and GSK3β are bona-fide targets of miR-1246, fragments of the respective 3’UTRs encompassing the potential binding regions were cloned downstream of the luciferase reporter plasmid to detect the direct inhibitory binding of miR-1246 to the 3’UTRs. Compared with miR control experiments, luciferase activity was markedly reduced by approximately 25% in the cells cotransfected with miR-1246 and the 3’UTR fragments in the sense orientation. As a reflection of specificity, this inhibitory effect was abolished when antisense AXIN2 and GSK3β 3’UTR fragment constructs were used in place of the sense constructs (Fig. 2A, right). Immunofluorescence staining assays showed that knockdown of miR-1246 resulted in a substantial increase in cytoplasmic AXIN2 and GSK3β expression, concomitant with a marked decrease in nuclear accumulation of β-catenin in Hep3B and Huh7 cells (Fig. 2B). Hep3B and Huh7 cells with miR-1246 stably repressed also resulted in enhanced β-catenin ubiquitylation and degradation as determined by ubiquitination assay (Fig. 2C), decreased transactivating activity of β-catenin as determined by TOP/FOP reporter assay (Fig. 2D), and reduced transcription of Wnt target genes, including CYCLIN D1 (CCND1), CMYC, and matrix metalloproteinase 7 (MMP7; Fig. 2E). On overexpressing miR-1246, a reversed phenomenon was observed (Fig. 2B,D). RNA-immunoprecipitation analysis using anti-Ago2 antibody following the overexpression or silencing of miR-1246 further demonstrated that the messenger RNAs (mRNAs) of these target genes could be specifically recruited to the miRNP complex (Supporting Fig. S1). Consistently, immunofluorescence analysis revealed that the expression levels of both AXIN2 and GSK3β were elevated in CD133− and miR-1246 low/absent HCC cells Huh7 and PLC8024, as compared to their corresponding CD133+ and miR-1246-expressing counterparts (Fig. 2F).

miR-1246 ENHANCES CANCER AND STEM-CELL-LIKE PROPERTIES IN HCC IN VITRO

To understand the biological effect of miR-1246 deregulation in promoting cancer and stem-cell-like properties in CD133+ HCC, in vitro loss-of-function analyses were performed using a knockdown strategy through the lentivirally stable suppression of miR-1246 in human HCC cells Hep3B and Huh7 (Supporting Fig. S2A,B), which express high levels of both CD133 and miR-1246. Knockdown of miR-1246 led to a marked decrease in the cells’ ability to initiate hepatosphere formation and serially passage in secondary transplantations, which suggest a diminished ability of the cells to self-renew (Fig. 3A). Furthermore, knockdown of miR-1246 also resulted in the sensitization of HCC cells to commonly used chemo (5-fluorouracil and cisplatin) and molecular targeted therapeutic drugs (sorafenib). Flow cytometry analysis for percentage of dead cells demonstrated decreased survival ability and lower resistance to these drugs following miR-1246 knockdown (Fig. 3B). In addition, Matrigel-coated (for invasion) or -uncoated (for migration) Transwell assays showed that miR-1246 knockdown drastically decreased the invasiveness and migration of Hep3B and Huh7 cells (Fig. 3C,D). Conditioned medium collected from Hep3B and Huh7 cells with miR-1246 stably repressed was also less efficient in inducing tube formation in HUVEC cells as compared to conditioned medium collected from control cells (Fig. 3E). Conversely, on overexpressing miR-1246 in Huh7 cells with miR-1246 stably repressed (Supporting Fig. S3), reciprocal changes with significantly augmented self-renewal, migration, invasion, and angiogenic capabilities were observed (Fig. 3A-E). Note that because miR-1246 in our tested HCC cell line panel was already detected at a relatively abundant expression level, we had difficulty in further overexpressing miR-1246 in HCC cells. Collectively, our data suggest that miR-1246 greatly contributes to the development of HCC and stemness.

miR-1246 AUGMENTS TUMOR-INITIATING AND METASTATIC POTENTIAL IN VIVO

Given that the in vitro experiments revealed that miR-1246 overexpression is associated with pro-tumor-initiating and -metastatic traits, we next asked whether miR-1246 could promote tumorigenicity and metastasis in vivo. The in vivo tumorigenic role of miR-1246 was assessed by subcutaneous xenograft models, where the knockdown group failed to initiate tumor formation in Hep3B and Huh7 cells. On the contrary, Hep3B and Huh7 control (ZIP) cell xenotransplants generated 100% tumor formation (n = 5; Fig. 4A). To further validate whether the absence of miR-1246 is responsible for this observed attenuated tumorigenicity, we overexpressed miR-1246 or empty
vector control plasmids in Huh7 cells with miR-1246 stably repressed and repeated the experiment. Reexpression of miR-1246 in Huh7 cells with miR-1246 originally repressed partially rescued the ability of cells to initiate tumor formation (n = 5/10) as compared to cells transduced with empty vector (EV) controls (n = 1/10; Fig. 4A). Given that miR-1246 knockdown in Hep3B and Huh7 cells failed to give rise to tumors for serial transplantation experiments, we then performed the same experiment in the more-aggressive BEL7402 HCC cells. Limiting dilution assays using decreasing inoculation doses of BEL7402 with miR-1246 depleted led to increasing latency and reducing incidence of tumor initiation and tumor volume (Fig. 4B, C). Estimated tumor-initiating frequency was reduced from 1 in 1 to 1 in 54,568 and 1 in 6,189 to 1 in 21,080 in primary and secondary implantations, respectively (Fig. 4C). Histological analyses of the xenograft tumors found that decreased miR-1246 in BEL7402 knockdown cells displayed enhanced expressions of AXIN2 and GSK3β, concomitant with decreased β-catenin expression. Reciprocal changes were observed in xenograft tumors formed by BEL7402 control cells (Fig. 4D).

In vivo metastasis was then assessed by orthotopic implantation of luciferase-labeled BEL7402 HCC cells directly into the liver. miR-1246 repression led to a slight decrease in the ability of cells to initiate tumor formation in the liver (n = 4/6) and a prominent reduction to metastasize to the lung (n = 0/6; Fig.
FIG. 4
4E). In contrast, BEL7402 control cells led to 100\% tumor formation in the liver (n = 6/6), of which 3 of these mice went on to develop lung metastasis (Fig. 4E). Hematoxylin-eosin (H&E) staining of xenograft tumors confirmed the bioluminescence signals observed to indeed represent tumor cells, and that there is altered ability of the cells to metastasize to the lung (Fig. 4E). Immunohistochemistry (IHC) analyses also found decreased miR-1246 in BEL7402 knockdown cells to display a relatively elevated AXIN2 and GSK3\beta expression, as compared to control cells, paralleled with a marked reduction in \( \beta \)-catenin expression (Fig. 4F). Together, these data indicate that miR-1246 plays a pivotal role in promoting tumor initiation and metastasis in vivo.

**miR-1246 REGULATES CANCER STEMNESS AND THE Wnt/\( \beta \)-CATENIN PATHWAY IN HCC BY TARGETING AXIN2 AND GSK3\beta OF THE \( \beta \)-CATENIN DEGRADATION COMPLEX**

To further validate the role of \( \beta \)-catenin activation in miR-1246-induced tumorigenicity, metastasis, and stemness, we analyzed the impact of introducing a constitutively active \( \beta \)-catenin (\( \Delta 45\beta \)-cat) into Huh7 cells with miR-1246 stably repressed on these altered phenotypes. Constitutively active \( \beta \)-catenin contains a mutation at serine 45 and, as a result, cannot be phosphorylated nor undergo ubiquitination and degradation.\(^{13}\) Wild-type (WT) \( \beta \)-catenin was also overexpressed as a control. Immunofluorescence staining assay showed successful transduction of both WT and constitutively active \( \beta \)-catenin expression 2 days posttransfection in Huh7 cells with miR-1246 stably repressed. But at 14 days posttransfection, intensity of \( \beta \)-catenin expression significantly subsided in cells transfected with WT \( \beta \)-catenin, whereas \( \beta \)-catenin expression was still abundantly expressed in cells transfected with constitutively active \( \beta \)-catenin (Fig. 5A). This is expected given that transfection was performed in Huh7 cells with miR-1246 stably repressed, where AXIN2 and GSK3\beta were demonstrated to be up-regulated, thus resulting in the assembly of \( \beta \)-catenin degradation complexes for ubiquitination and degradation of WT \( \beta \)-catenin. Consistent with this observation, transactivating activity of \( \beta \)-catenin was significantly elevated when Huh7 cells with miR-1246 repressed were transfected with \( \Delta 45\beta \)-cat, as compared to WT \( \beta \)-catenin, as demonstrated by TOP/FOP reporter assay (Fig. 5B). Functionally, when \( \Delta 45\beta \)-cat was ectopically overexpressed in miR-1246 repressed Huh7 cells, miR-1246-attenuated hepatosphere formation, cell motility, invasion, angiogenesis, and tumor-initiating activity in vivo were, at least partly, rescued. Overexpression of \( \Delta 45\beta \)-cat in Huh7 cells with miR-1246 stably repressed resulted in successful tumor formation in 7 of 10 mice, as compared to 1 of 10 mice when the same cells were transfected with WT \( \beta \)-catenin. H&E staining of harvested xenografted subcutaneous tumors confirmed a primary HCC phenotype (Fig. 5C-F). These results demonstrated that AXIN2, GSK3\beta, and \( \beta \)-catenin were functionally important for miR-1246-attenuated tumorigenicity, metastasis, and stemness in HCC cell lines.

**ENDOGENOUS AND SECRETORY miR-1246 OVEREXPRESSION IS TIGHTLY ASSOCIATED WITH ADVERSE HCC SURVIVAL**

To further understand the clinical relevance of the above findings in human HCC, expression of miR-1246 was examined in 114 human HCC tissue specimens as well as their matched adjacent nontumor counterparts by miRNA in situ hybridization (ISH). Grading of miR-1246 expression was divided into

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**FIG. 4.** miR-1246 augments tumor-initiating and metastatic potential in vivo. (A) Representative xenograft tumors derived from Hep3B and Huh7 cells after stable miR-1246 knockdown or in Huh7 cells with stable miR-1246 knockdown and then overexpressed, compared to controls, 5 weeks after subcutaneous inoculation in nude mice (n = 5). (B) IHC analysis of BEL7402 cells subcutaneously inoculated in NOD-SCID mice, and tumor volume, incidence, and latency were monitored. Representative xenograft tumors derived from BEL7402 cells after stable miR-1246 knockdown (1246 KD), compared to control (ZIP; n = 5). (D) H&E images of xenograft tumors harvested. ISH of miR-1246 and IHC analyses of AXIN2, GSK3\beta, and \( \beta \)-catenin expression in serial sections of subcutaneous xenograft tumors. (E) Ex vivo imaging of liver (top) and lung (bottom) harvested from nude mice that received orthotopic liver injections of luciferase-labeled BEL7402 cells stably transduced with miR-1246 knockdown (1246 KD) or control (ZIP; n = 6). Luciferase signals shown as dot plots. H&E images of liver and lung tissues harvested. Tumor nodules denoted as “T”. (F) ISH of miR-1246 and IHC analyses of AXIN2, GSK3\beta, and \( \beta \)-catenin expression in serial sections of orthotopic liver xenograft tumors.
low, medium, and high (Fig. 6A). Expression of miR-1246 in paired nontumor portions of human HCC tissues was comparatively low. Overall, 53.52% (61 of 114) showed low and 46.5% (53 of 114) showed medium expression, whereas none of the samples displayed high miR-1246 expression. In comparison, only 13.2% (15 of 114) showed low miR-1246 expression in the HCC counterparts, whereas 66.7% (76 of 114) and 20.2% (23 of 114) showed medium and high expression, respectively (Fig. 6B; $P < 0.0001$). Expression of miR-1246 was found to correlate with serum alpha fetoprotein (AFP) level (Fig. 6B, right; Supporting Table S6; $P = 0.001$). Patients with high miR-1246 expression was also associated with worse overall survival, with an estimated mean of 13.1 months, as compared to 47.3 months ($P = 0.001$) and 33.4 months ($P = 0.002$) in HCC patients that displayed low or medium miR-1246 expression, respectively (Fig. 6C, left; Supporting Table S6). Moreover, high miR-1246 expression also correlated with worse disease-free survival, with an estimated mean of 14.8 months, as compared to 43.5 ($P = 0.024$) and 29.3 months ($P = 0.055$) in patients displaying low and medium miR-1246 expression, respectively (Fig. 6C, right; Supporting Table S6).

miR-1246 was also found to be an independent prognostic marker in both overall and disease-free survival by Cox regression analysis (Supporting Table S7). However, no significant correlation was identified between high endogenous miR-1246 expression and
miR-1246 has previously been reported to be detectable in a circulating form in the blood of patients diagnosed with myeloma or oesophageal squamous cell carcinoma.\(^{14,15}\) Thus, we also set forth to examine the clinical relevance of circulating miR-1246 in HCC. A cohort containing 62 plasma samples collected from HCC patients and 24 plasma samples collected from healthy individuals were used in this study. Expression of miR-1246 was significantly elevated in plasma collected from HCC as compared to healthy individuals (Fig. 6D, left; \(P < 0.001\)). By receiver-operating characteristic (ROC) curve analysis, miR-1246 was found to be a sensitive and specific marker for HCC diagnosis.
to be an extremely sensitive and specific biomarker for HCC diagnosis, with an area under curve (AUC) of 0.982 (95% confidence interval [CI], 0.96-1.00; Fig. 6D, right).

Last, we examined whether miR-1246 overexpression and its mediation of Wnt/β-catenin pathway activation were clinically relevant in HCC, and whether miR-1246 overexpression and β-catenin mutation were mutually inclusive or exclusive events in HCC. In a cohort of eight HCC clinical samples that all expressed high levels of nuclear β-catenin (based on subcellular fractionation then western blotting), a higher proportion of tumor specimens expressing higher levels of miR-1246 showed absent β-catenin mutation (based on sequencing) compared with those with lower miR-1246 ($P = 0.0003$; Fig. 6E). An inverse correlation between expression levels of miR-1246 and its targets, AXIN2, GSK3β, and β-catenin, was also observed in our cohort (Fig. 6F).
Oct4 ACTIVATES miR-1246 EXPRESSION THROUGH miR-1246 PROMOTER BINDING AND COOPERATIVELY DRIVE β-CATENIN ACTIVATION IN HCC

To determine the mechanism of miR-1246 up-regulation in HCC, the miR-1246 upstream region (−1 to −30,000) was analyzed using JASPAR (http://jaspar.genereg.net), and four binding sites of Oct4 were predicted on the upstream region of miR-1246 (sites 1, 3, 4, and 7), using a cut-off score of 10 and relative profile score threshold of 80% (Fig. 7A). Notably, Oct4 is also a self-renewal gene that is preferentially overexpressed in the CD133+/ liver CSC subset (Fig. 7B). Chromatin immunoprecipitation (ChIP) assays showed high physical binding affinity of endogenous Oct4 to miR-1246 in Huh7 cells in two of these sites (sites 1 and 3; Fig. 7C, right). To delineate whether Oct4 plays a specific role, Oct4 was transiently silenced in Huh7 cells, which led to decreased binding in site 3 of miR-1246 (Fig. 7C, left). A positive correlation between miR-1246 and Oct4 expression was also substantiated in 14 HCC clinical samples (r = 0.6451; P = 0.0127; Fig. 7D). Transient knockdown of Oct4 in Huh7 and Hep3B cells not only resulted in a marked reduction of miR-1246 expression, but also led to a concomitant decrease in transcription of CCND1, a Wnt target gene (Fig. 7E). Overall, our results demonstrate that the Oct4/miR-1246 signaling axis drives Wnt/β-catenin activation in CD133+ HCC by targeting the β-catenin degradation complex (AXIN2 and GSK3β), thereby enhancing cancer and stem-cell-like traits and ultimately leading to therapy resistance and poor prognosis in HCC patients (Fig. 7F).

Discussion

Constitutive activation of Wnt signaling is closely associated with tumor progression of HCC. (16) Percentage of human HCC with β-catenin protein accumulation is high, with IHC study showing β-catenin overexpression in 50%-83% of HCC. (17-19) However, somatic mutations of β-catenin have been shown to occur in only ~10%-20% of HCC. (20-22) AXIN1 and AXIN2 gene mutations are unlikely to contribute significantly, given that they have only been reported in around 5%-10% of HCCs. (23) Inactivating mutations of the APC gene are rarely identified in HCC. (12,24) Thus, other undefined mechanisms are believed to be involved in β-catenin stabilization and accumulation. Here, we demonstrate that the Oct4/miR-1246 signaling axis activates Wnt signaling in CD133+ liver CSCs and HCC by simultaneously suppressing multiple Wnt inhibitors that play key roles in the β-catenin degradation complex, providing a new layer of mechanism by which the Wnt-mediated stem-cell-like properties of HCC cells are developed. Our findings also identify a novel mechanism by which two self-renewal pathways work cooperatively to promote HCC stemness using miR-1246 as a link.

GSK3β and AXIN2 are negative regulators of canonical Wnt signaling and tumor suppressors in HCC. (16) A key role of GSK3β and AXIN2 is to control the β-catenin pool by establishing a β-catenin degradation complex with APC. We found this complex to be disrupted in the presence of miR-1246 and the assembly of the degradation complex to be impeded as a result of miR-1246 targeting AXIN2 and GSK3β. Because of the tight correlation of high miR-1246 with low AXIN2 and GSK3β expression in HCC specimens without β-catenin mutations in exon 3, aberrant expression of miR-1246 may serve as a novel mechanism underlying activation of Wnt/β-catenin signaling that is equally as important as β-catenin mutation in this cancer type. Yet, further analysis involving a larger cohort of clinical samples is needed.

In our study, we provide compelling biological and clinical evidence that both endogenous and secretory miR-1246 expression is markedly up-regulated in HCC and that miR-1246 acts to maintain stem-cell-like traits and to promote tumorigenesis, metastasis, self-renewal, angiogenesis, and treatment resistance in HCC. In fact, there have been increasing reports of miR-1246 overexpression and its oncogenic function in various cancer types in recent years. Both endogenous and secretory miR-1246 have been found to be frequently overexpressed in multiple myeloma as well as cancers of the colon, pancreas, cervix, oral squamous, and esophagus. (14,15,25-29) Pancreatic cancer cells that express elevated miR-1246 are capable of promoting self-renewal and chemoresistance through targeting cyclin G2. (30) miR-1246 promotes proliferation, migration and invasion in human cervical carcinoma cells though targeting thrombospondin 2. (31) Up-regulation of miR-1246 in human neuroblastoma cells is associated with disc large homolog 3. (32) miR-1246 have also been found to promote angiogenesis by activating small mothers of decapentaplegic 1/5/8 signaling elicited by promyelocytic leukemia down-regulation in endothelial cells. (33) In HCC, miR-1246 has been
shown to enhance metastasis by targeting cell adhesion molecule 1 (CADM1), a tumor-suppressor gene involved in cell-cell interactions. Circulating miR-1246 has also been recently shown to predict tumor recurrence and survival in HCC patients after liver transplantation. In contrast, miR-1246 expression was shown to be decreased in cervical cancer tissues and to inhibit HCC cell growth by targeting nuclear factor I/B and in a p53-induced manner. Taking these findings and ours together, miR-1246 appears to play a dual role as both a tumor-promoting and tumor-suppressing miRNA. In such a context, both genetic/epigenetic and microenvironmental cues are believed to contribute to determining whether and how a miRNA molecule functions to promote or suppress cancer development and progression. Further investigations of the molecular mechanisms mediating the differential biological effects and targets of miR-1246 in HCC and other cancer types remain important. Upstream mechanisms other than Oct4, as reported in our current study, by which miR-1246 is differentially regulated, also remains to be elucidated. Whether miR-1246 is secreted as exosomes in HCC and whether secreted miR-1246 contributes to cell-cell communication would also be important and interesting to follow. Whether and how miR-1246 and known biomarkers of HCC (i.e., AFP, glypican 3) are associated with patient outcome, and whether they can be used separately or together in the clinic needs to be further investigated with large-scale, prospective, clinical trials. It should also be noted that miR-1246-mediated β-catenin activation is likely not the only mechanism critical for regulating cancer stemness in HCC. This is not surprising, given that miRNAs are known to target multiple targets. For instance, and as mentioned above, miR-1246 has also been reported to enhance aggressive cancer phenotype by targeting the tumor suppressor, CADM1. We speculate that there may be other miRNAs/pathways that miR-1246 targets to regulate cancer stemness in HCC. This current study focuses on the importance and contribution of miR-1246-mediated Wnt/β-catenin in this aspect. We found miR-1246 to be preferentially overexpressed in the CD133 liver CSC subset, which there is now ample evidence to show that it represents the root of tumor recurrence and therapy resistance in HCC. Targeting CSCs and the Wnt pathway that is preferentially activated in these cells represents a promising therapeutic strategy to address tumor recurrence following resistance to therapy. In fact, gene therapies delivering AXIN2, which was demonstrated to be a target of miR-1246 in this study, are being clinically tested in colon cancer (NCT01882660). There is also a phase I clinical trial on the combination of therapy using gemcitabine- and GSK3β-inhibiting drugs for gemcitabine-resistant advanced pancreatic cancer. In addition, analogs of the β-catenin antagonist, ICG-001, which is able to target and eliminate drug-resistant CSCs in several types of tumor models, including leukemia, glioblastoma, and colon cancer, are currently being tested in a phase I clinical trial. However, effective small-molecule drugs targeting Wnt signaling usually act against only a single molecule. Extensive research has demonstrated that antagonists might represent a class of potentially efficient, specific, and long-lasting silencers of endogenous miRNAs in vivo. Our current study revealed that forced expression of miR-1246 in HCC cells might subject cells to confer cancer and stemness features by causing simultaneous inhibition of AXIN2 and GSK3β and constitutive activation of the Wnt signaling pathway, thus promoting tumor-initiating and therapy resistance in HCC. Meanwhile, antagonizing miR-1246 caused multilevel inactivation of Wnt signaling and had an obvious inhibitory effect on CSCs and tumorigenesis. These results suggest that miR-1246 may be a novel prototype therapeutic agent that can target Wnt signaling in liver CSCs to suppress tumorigenesis and relapse. Taken together, our data provide a clinical and biological basis for the potential use of miR-1246 as a novel diagnostic biomarker and an anticancer stemness target in HCC.

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