Commentary

The Wnt/β-catenin pathway is activated by miR-1246 in liver cancer stem cells

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The Wnt/β-catenin signaling pathway drives stem cell proliferation and self-renewal (1). As the key transducer of the Wnt signal, the β-catenin transcriptional coactivator is tightly regulated (2). In the absence of Wnt, cytoplasmic β-catenin is found in a complex containing axis inhibition proteins 1 and 2 (AXIN1/2), adenomatous polyposis coli (APC), glycogen synthase kinase 3 beta (GSK3β), and casein kinase 1 epsilon (CK1ε). Here, it is phosphorylated on its amino terminal residues, ubiquitinated by beta-transducin repeat containing protein, and targeted for proteasomal degradation. When Wnt ligand is present, β-catenin levels are stabilized and it translocates into the nucleus to activate target gene expression.

Mutations in Wnt/β-catenin signaling components render the pathway aberrantly active in many cancers, including hepatocellular carcinoma (HCC) (3). HCC is associated with a high mortality rate because 80% of diagnoses occur after tumors have progressed to a late stage (4). In addition, HCC patients suffer from a 70% relapse rate within 5 years (5). This high HCC relapse rate can be attributed in part to a population of $CD133^+$ liver cancer stem cells (CSCs) that have aberrant activation of stem-cell self-renewal pathways, including Wnt/β-catenin signaling (6). Around 20% of HCCs have mutations in the region of the *CTNNB1* gene encoding its amino terminal phosphodegron (7), but these mutations are thought to arise late in carcinogenesis and also correlate with less aggressive disease (7,8). Despite this, β-catenin accumulation in the nucleus is found in up to 70% of HCCs, suggesting an alternative mechanism by which Wnt signaling is activated in liver CSCs (9).

A recent study by Chai *et al*. sought to identify how Wnt/β-catenin signaling is deregulated in liver CSCs (10). By sequencing small RNAs isolated from $CD133^+$ and CD133- subsets of the PLC8024 HCC cell line, expression of the microRNA, miR-1246, was found to be elevated in the $CD133^+$ subset. Because miR-1246 was predicted to target *AXIN2* and *GSK3β*, the authors reasoned that downregulation of these genes by miR-1246 could drive HCC. After demonstrating that *AXIN2* and *GSK3β* were directly targeted by miR-1246, they found that shRNA-mediated depletion of miR-1246 in HCC cells increased AXIN2 and GSK3β protein levels in the cytoplasm, reduced β-catenin levels in the nucleus, and decreased Wnt target gene expression.

Chai *et al*. then addressed the role of miR-1246 in controlling liver CSC-associated properties *in vitro*. First, they found that loss of miR-1246 inhibited HCC cell self-renewal, as assessed by hepatosphere formation after serial passage. Second, reducing miR-1246 levels sensitized HCC cells to apoptosis induced by both a general chemotherapeutic, cisplatin, and the primary targeted therapy in HCC, sorafenib. Finally, miR-1246 was shown to be required for the invasive, migratory, and angiogenic properties of HCC cells, reflecting its importance to the aggressive spread of HCC. Importantly, over-expressing miR-1246 in HCC cells exacerbated these phenotypes. Thus, miR-1246 is crucial for HCC cell tumorigenesis, drug resistance, and invasiveness *in vitro*.

The role of miR-1246 in driving tumorigenesis *in vivo* was assessed using a xenograft tumor mouse model. Strikingly, miR-1246 knockdown in Hep3B and Huh7

HCC cell lines abrogated tumor formation. The tumor-initiating potential of a more aggressive HCC cell line, BEL7402, was calculated in primary and secondary implants. As expected, depleting miR-1246 in BEL7402 cells dramatically reduced the rate of tumor formation. Additionally, the authors used orthotopic liver injections of luciferase-labeled control and knockdown HCC cells to demonstrate that reduction of miR-1246 levels resulted in significantly fewer lung metastases. This finding agrees with reports that aberrant activation of Wnt/β-catenin signaling correlates with metastasis in HCC patients (11). Chai *et al*. confirmed that miR-1246 is activating Wnt/β-catenin signaling in the orthotopic liver tumors by demonstrating increased levels of AXIN2 and GSK3β and decreased levels of β-catenin.

After elucidating the role for miR-1246 in promoting HCC in both cell culture and mouse xenograft models, the authors also demonstrated its importance in patients, which added substantially to the clinical relevance of this study. Compared to adjacent normal liver tissue, levels of miR-1246 in HCC tumors are dramatically increased. Increased levels of miR-1246 negatively correlated with both overall survival and disease-free survival. Notably, the authors detected miR-1246 in the serum of HCC patients and found that it acts as a highly sensitive biomarker, clearly distinguishing control individuals from HCC patients. This is a significant finding given the existing troubles detecting HCC at a stage where therapeutic intervention may be more effective. Additionally, the authors show that miR-1246 overexpression and β-catenin mutation in HCC are mutually exclusive events, suggesting that miR-1246 may be driving oncogenic Wnt/β-catenin signaling in tumors that do not harbor stabilizing β-catenin mutations. This mutual exclusivity is supported by findings that altering miR-1246 levels in HCC cell lines containing mutant β-catenin abrogates the tumorigenic, drug-resistant, and invasive phenotypes described previously.

This cumulative data raises the question of how miR-1246 levels are elevated in HCC. By analyzing transcription factor binding motifs in the miR-1246 promoter region, Chai *et al*. identified four putative octamer-binding transcription factor 4 (OCT4) binding sites. Subsequently, OCT4 binding to three of these sites was confirmed and a positive correlation between *OCT4* and miR-1246 expression in HCC tumors was reported. Additionally, OCT4 is expressed more abundantly in $CD133^+$ CSCs cells relative to non-CSCs, and its depletion reduced both miR-1246 and β-catenin expression. While OCT4 is a stem cell transcription factor that is associated with self-renewal transcriptional programs and has been identified as an oncogene in HCC (12,13), it is unknown how it's expression is elevated in HCC liver CSCs. Interestingly, OCT4 has previously been shown to directly activate expression of Wnt target gene, *CCND1* (13). Perhaps OCT4 activates a subset of Wnt target genes both directly and indirectly through upregulation of miR-1246.

Cumulatively, the results presented in Chai *et al*. reveal a novel mechanism for β-catenin accumulation in $CD133^+$ liver CSCs. Overexpression of the transcription factor OCT4 in HCC cells leads to elevated levels of miR-1246, which targets *AXIN2* and *GSK3β* mRNA. The subsequent reduction in levels of these regulatory proteins allows accumulation of nuclear β-catenin and aberrant activation of genes controlling self-renewal, drug-resistance, tumorigenicity, and metastasis (*Figure 1*). Importantly, miR-1246 may also serve as a highly sensitive serum biomarker for early detection of HCC. While early detection is ideal, this study may also contribute to the development of novel therapeutics. The current treatment for late-stage HCC, sorafenib, only prolongs survival three months (14). Given that Chai *et al*. demonstrate increased sensitivity to sorafenib in miR-1246 depleted HCC cells, combining sorafenib and a miR-1246 targeted therapy could improve outcomes. Interestingly, a combination therapy clinical trial of sorafenib and a Wnt pathway inhibitor is currently underway (NCT02069145). The Wnt inhibitor in this trial, OMP-54F28, is a frizzled family receptor 8 decoy receptor that acts as a Wnt ligand sponge, preventing pathway activation by exogenous Wnt ligand.

In summary, Chai *et al*. have elucidated an important mechanism driving HCC. This study raises many new questions that should be addressed in future research. First, whether miR-1246 is therapeutically targetable is unknown. Several miRNA-targeted therapies have had success, yet difficulties remain in preventing off-target effects and assuring effective delivery (15). Targeting upstream of miR-1246, for example OCT4, may prove to be effective although the results presented in the current study do not preclude the involvement of another transcriptional regulator of miR-1246 expression. Indeed, p53 has previously been shown to induce miR-1246 expression in HCC cell lines (16). Second, it will be interesting to determine what other targets of miR-1246 are implicated in HCC development. Cell adhesion molecule 1 (CADM1) and nuclear factor 1 B (NF1B) have also been confirmed as miR-1246 targets in HCC (16,17). Finally, Wnt/β-catenin

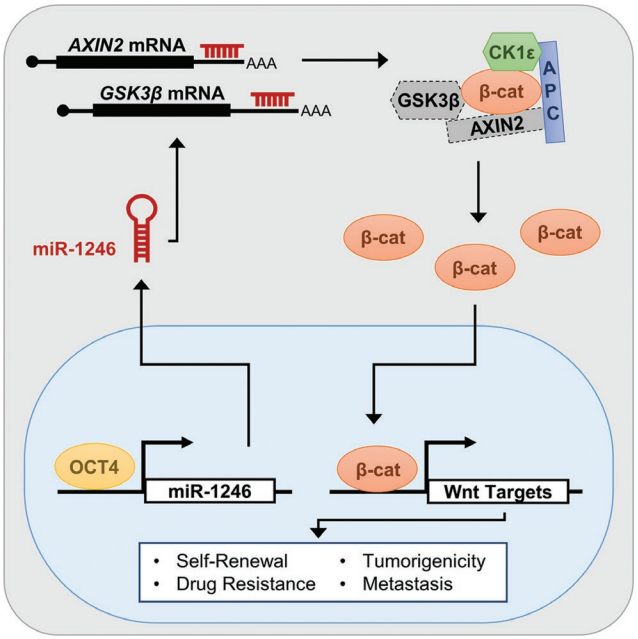

Figure 1 Wnt/β-catenin activation by miR-1246 in CD133$^+$ liver CSCs. OCT4 increases expression of miR-1246, which targets *AXIN2* and *GSK3β* mRNAs, and decreases AXIN2 and GSK3β protein levels in the cytoplasm (indicated by dashed outlines). As a consequence, β-catenin accumulates in the cytoplasm and subsequently translocates into the nucleus where it activates downstream target gene expression. These direct Wnt/β-catenin target genes potentiate self-renewal, drug resistance, tumorigenic, and metastatic properties of liver CSCs. CSC, cancer stem cell; OCT4, octamer-binding transcription factor 4.

signaling is deregulated in numerous cancer subtypes as well as a myriad of diseases (18). Therefore, while important for HCC diagnosis and treatment, the novel mechanism of Wnt/β-catenin pathway activation by miR-1246 uncovered in this study may have a more global impact on human health and disease.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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