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EDITORIAL

HNRNPM Regulates HCC Tumorigenesis and Cancer Stemness: Identification of a Novel Therapeutic Target?

Hepatocellular carcinoma (HCC) is the fourth most common cause of cancer-related deaths worldwide, and there are few therapeutic options for patients.[1] Cancer stem cells (CSCs) promote HCC progression, metastasis, and immune system evasion, potentially lending to a worse prognosis.[2] CSCs are associated with HCC immunosuppression, and targeting stemness-related pathways reduces CSC tumorigenicity.[2,3] The transcription factors octamer-binding transcription factor 4 (OCT4) and sex-determining region Y-box 2 (SOX2) are well-known as regulators of pluripotency in stem cells as well as CSCs.[4,5] Multiple transcription factors and other post-translational gene modifiers, including the splicing factor heterogeneous nuclear ribonucleoprotein M (HNRNPM), have been linked to cell stemness and self-renewal. Specifically, HNRNPM has been identified as a component of a heterogeneous nuclear ribonucleoprotein complex that interacts with SOX2 to mediate stemness in glioblastoma.[6] In addition, HNRNPM controls CD44 (CSC marker) expression in aggressive forms of breast cancer.[7] However, the exact roles of HNRNPM in CSCs and the potential to target these pathways to block HCC progression are not well-known. In the article by Zhu et al[8] published in the current issue of *Cellular and Molecular Gastroenterology and Hepatology*, the authors aimed to identify the molecular mechanisms regulating cancer stemness and immune evasion in HCC.

HNRNPM was found to be highly expressed in mouse and human fetal liver as compared with adult liver. Interestingly, HNRNPM expression was increased in HCC tissues and significantly correlated with overall survival, high tumor grade, microvascular invasion, poor tumor stage, multiple tumors, proliferative capacity, and tumor size. These findings suggest that HNRNPM levels may be a prognostic marker for HCC. Importantly, HNRNPM expression was higher in HCC cell lines that showed greater metastatic potential than HCC cell lines with less metastatic capacity. Overall, the authors conclude that HNRNPM is an oncofetal protein regulating HCC progression.

The authors then evaluated the mechanisms regulating HNRNPM expression in HCC. *In vitro*, OCT4 and SOX2 were found to control HNRNPM expression, and bioinformatic analysis confirmed OCT4 and SOX2 binding sites in the HNRNPM promoter. Additionally, data from The Cancer Genome Atlas database identified that there was a significant correlation between OCT4 and SOX2 expression with HNRNPM, and binding was verified with luciferase assay. On the basis of this information, the stemness-related transcription factors OCT4 and SOX2 promote HNRNPM expression during HCC.

The studies thus far indicate that HNRNPM maintains stemness during HCC. Indeed, HNRNPM overexpression in HCC cells increased sphere formation, cell proliferation, and colony formation *in vitro*, identifying the proliferative role of HNRNPM in HCC. In addition, HNRNPM overexpression promoted cell migration and invasion in HCC cells *in vitro*, further identifying the role of HNRNPM on metastasis. Xenograft and orthotopically injected mouse models receiving HNRNPM overexpressing HCC cells developed larger tumors that contain CSC-like cells. Oppositely, knockdown of HNRNPM in HCC cells decreased sphere formation, cell growth, survival, and cell migration and invasion *in vitro* and decreased tumor formation, metastasis, and CSC-like properties *in vivo*.

HNRNPM is linked with stemness and CSC-like properties in HCC. Interestingly, the authors noted that HNRNPM was highly expressed in embryonic stem cells and HCC samples but down-regulated in endoderm cells, liver progenitor cells, premature hepatocytes, and hepatocytes. This finding was accompanied with increased expression of genes related to pluripotency, stemness, and self-renewal (OCT4, SOX2, and E2F1) in embryonic stem cells and HCC samples. The transcription factor E2F1 was found to be an upstream activator of HNRNPM, and expression of E2F1 significantly and positively correlated with HNRNPM expression in HCC samples. Binding of HNRNPM and E2F1 was confirmed with bioinformatic approaches and chromatin immunoprecipitation.

RNA-sequencing (RNA-seq) was used in control and HNRNPM knockdown HCC cell lines to identify the transcriptomic landscape. Kyoto Encyclopedia of Genes and Genomes analysis found that HNRNPM was associated with Wnt/β-catenin signaling, mitophagy, and transforming growth factor-β signaling. Furthermore, HNRNPM-associated RNA peaks were enriched in introns, promoters, 5'UTRs and 3 ' UTRs, and motif analysis found that GU-enriched patterns were most associated with HNRNPM-dependent RNA peaks. HNRNPM had a high degree of interaction with methyl-CpG binding domain protein 2 (MBD2), and interestingly, overexpression of HNRNPM increased MBD2a expression but decreased MBD2c expression in HCC cells. MBD2 binding to HNRNPM was confirmed, and overall, the results indicate that HNRNPM promotes MBD2 splicing leading to more MBD2a but less MBD2c. Similar to HNRNPM, MBD2a expression was up-regulated in fetal liver tissues and HCC samples, with an opposite trend shown for MBD2c. Additionally, high expression of MBD2a and low expression of MBD2c were associated with poorer prognosis in HCC patients,

identifying that these factors may be important prognostic indicators.

Both *in vitro* and *in vivo* experiments showed that MBD2a overexpression enhanced tumorigenesis and CSC phenotypes in HCC cells, whereas these effects were diminished in MBD2c overexpressing HCC cells. Upstream, OCT4 and SOX2 down-regulated MBD2a and up-regulated MBD2c expression, which is important because these transcription factors can also regulate HNRNPM. RNA-seq identified that HCC cells with MBD2a knockdown or MBD2c overexpression had overlapping pathways involved in tumor proliferation, metastasis, cell cycle, cellular component organization, and biological adhesion. Furthermore, gene enrichment analysis of the RNA-seq data confirmed that MBD2a knockdown or MBD2c overexpression was positively associated with focal adhesion and transforming growth factor-β signaling. When evaluating the top 20 genes from the RNA-seq data, MBD2a was found to enhance and MBD2c was found to reduce expression of β-catenin, snail, OCT4, and SOX2 in HCC cells. These genes were also up-regulated after HNRNPM overexpression in HCC cells. Data demonstrated that MBD2a and MBD2c could competitively bind to the frizzled 3 (FZD3) promoter to exert opposing effects, which is key considering thatFZD3 is an important upstream regulator of Wnt/β-catenin signaling. Overall, MBD2a and MBD2c have opposing functions regarding CSC phenotypes in HCC progression.

The authors next found that FZD3 promoted epithelial to mesenchymal transition and self-renewal in HCC cells. MBD2a knockdown or MBD2c overexpression enhanced methylation of the FZD3 promoter, indicating that MBD2a and MBD2c inversely regulate FZD3 and subsequent β-catenin activity by competitive binding. In turn, β-catenin bound to the promoters of OCT4 and SOX2 in HCC cells, thereby demonstrating a positive feedback loop comprising OCT4, SOX2, HNRNPM, MBD2a, and FZD3 during HCC tumorigenesis.

Lastly, the authors validated the therapeutic potential of targeting HNRNPM *in vitro* and *in vivo* using antisense oligonucleotides (ASOs). HNRNPM ASOs blocked the downstream expression of OCT4, SOX2, MBD2a, and FZD3 and down-regulated Wnt/β-catenin signaling. HNRNPM ASOs further reduced CSC number and significantly decreased tumorigenesis and growth. Interestingly, combined therapy using HNRNPM ASO and PD-1 inhibitor reduced tumor size and promoted antitumor immunity. Overall, inhibition of HNRNPM may enhance PD-1 inhibitor immunotherapy in HCC samples with active Wnt signaling.

Cancer stemness and immune evasion are important concepts in HCC research. This study was key in identifying a signaling mechanism that promotes CSC-like properties and may in turn impact immune surveillance. The authors identify a novel feedback loop whereby enhanced HNRNPM expression regulates OCT4, SOX2, MBD2a, and Wnt signaling, thus modulating HCC progression. Considering that these factors can target many other genes and proteins, it would be of interest to evaluate changes in other downstream targets to see whether there are other cell responses being mediated by this pathway. The findings further indicate that HNRNPM, MBD2a, and MBD2c could potentially be used as prognostic markers in HCC, and the use of HNRNPM ASO demonstrates the important therapeutic potential of targeting this factor. It would be important for the development of a molecular inhibitor that could be used in HCC patients to understand whether this therapeutic can be translated to human use. Also, some HCC patients can be unresponsive to PD-1 inhibitor immunotherapy because of immune evasion; therefore, it would be important to demonstrate whether HNRNPM inhibition could sensitize this subset of patients to immunotherapies. This mechanistic study identified a new pathway regulating HCC tumorigenesis and CSC properties and may help in the development of new therapeutic targets and prognostic markers.

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