Editorial Commentary

New insights into the impact of impaired epigenetic machinery on liver cancer malignant phenotype

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Hepatocellular carcinoma (HCC) is the most frequent liver malignancy, representing up to 85–90% of all primary hepatic cancers. Its progression and aggressiveness mainly rely on the existence of liver cancer stem cells (LCSCs), which can self-renew and are responsible for the marked inter-individual, inter-tumors in a single patient, and even intra-tumor cellular heterogeneity seen in HCC. This feature contributes to the recurrence often seen in these patients, even after an initial satisfactory response to systemic treatments, which constitutes a clinically serious problem and positions HCC as the third leading cause of cancer-associated death worldwide. The existence of different subpopulations of LCSCs makes the problem more complex. They can be characterized by specific surface markers, such as EpCAM, LGR5, CD133, CD44, and CD24, or using advanced techniques like single-cell RNA sequencing (1,2). Furthermore, molecular studies can also identify characteristic genetic alterations and modifications in specific signaling pathways of cancer cells that contribute to their aggressive behavior.

One primary reason for HCC-associated high mortality (less than 20% survival rate 5 years after diagnosis) is its marked resistance to available chemotherapy, including those based on modern tyrosine kinase inhibitors (TKI), such as sorafenib and lenvatinib. However, even in the best cases, this targeted therapy results in only a moderate improvement in the typically poor outcomes of HCC patients (3). This lack of response is due in part to alterations in signaling pathways, for example, NOTCH1 and Wnt/β-catenin pathways that are crucial for preserving stemness characteristics that are involved in malignant traits of HCC cells, including chemoresistance. Interestingly, the stemness features can also be regulated by epigenetic mechanisms, some of which have been previously related to the development of liver tumors (4). The recently published study by Zhang *et al.* (5) further investigated this clinically relevant issue.

The orchestrated intervention of the epigenetic machinery, involving writer, reader, and eraser elements broadens the phenotypic traits beyond those merely based on information contained in the genome. Epigenetic processes, leading to DNA/RNA methylation, nucleosome remodeling, and histone modification, among others, are involved in the control of many different functions

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in health and disease. More precisely, they play a crucial role in cancer, in general, and HCC in particular (6,7). Epigenetic mechanisms are central to the complex interplay between genetic and environmental drivers of HCC, as they facilitate the integration and amplification of the effects mediated by these factors. HCC progression is often driven by a combination of environmental events, which include recurring inflammatory infections due to hepatitis viruses (mainly B and C), alcohol consumption, and diabetes. Additionally, genetic mutations, such as those affecting tumor suppressor genes and oncogenes also play an important role in HCC development and progression (8). These environmental factors can account for epigenetic changes being induced, such as altered DNA methylation patterns or histone modifications, which in turn affect gene expression and eventually tumor behavior. For instance, the exposure to aflatoxins can affect the DNA methylation patterns affecting the expression/function of the tumor suppressor gene *TP53* and the growth-regulator H19 (9). Furthermore, chronic hepatitis B or C infections can result in aberrant methylation of genes, such as N^6-methyladenosine (m^6A) modification of *PTEN* mRNA (10). Moreover, the advancement in our understanding of epigenetic mechanisms and their essential players has settled the basis for developing novel strategies for sensitizing HCC cells to pharmacological treatments (11). Thus, a novel field of interest in the search for druggable targets is the investigation of proteins involved in the epigenetic machinery. In this direction, Zhang *et al.* (5) have explored the usefulness of manipulating the epigenetic reader encoded by the gene *YTHDF1*, also known as *C20orf21* or *DF1*.

The protein encoded by this gene belongs to the family of the YTH (Yeast Two-Hybrid) domain-containing proteins that participate in numerous RNA processes, such as pre-mRNA splicing, nuclear export, translation, and decay in posttranscriptional regulation. Consequently, they play an essential role in modulating the expression of genes involved in healthy cell cycle progression, inflammation, immunity, and autophagy, as well as in disease-associated processes, such as cancer cell proliferation, migration, and invasion. More precisely, YTH N^6-methyladenosine RNA binding protein 1 (YTHDF1) specifically recognizes and binds m^6A, which is the most prevalent internal (non-cap) modification present in the mRNA (and some noncoding RNAs) of all higher eukaryotes. YTHDF1 acts as a regulator of mRNA stability by promoting the degradation of m^6A-containing mRNAs via interaction with the CCR4-NOT complex YTHDF1 promotes the formation of phase-separated membrane-less compartments, such as cytoplasmic P-bodies and stress granules, by undergoing liquid-liquid phase separation upon binding to mRNAs containing multiple m^6A-modified residues. Moreover, this protein enables ribosome binding activity and positive regulation of translational initiation.

YTHDF1 is a relatively ubiquitous protein with low tissue specificity, although its expression is particularly high in the thyroid. This is consistent with the fact that its high expression has been considered an unfavorable prognostic marker in thyroid cancer, but also in breast cancer and HCC. In this respect, the study by Zhang *et al.* (5) has suggested the role of YTHDF1 in preserving the stemness characteristics of LCSCs and reducing their response to chemotherapy. Moreover, the results of that study revealed that the YTHDF1-m^6A-NOTCH1 epitranscriptomic axis is involved in HCC stemness and drug resistance, contributing to tumorigenesis and recurrence.

Another aspect of consideration in Zhang *et al.*'s study (5) is the investigation of YTHDF1 as a target for chemosensitization using gene therapy tools. The authors proposed a treatment strategy for HCC based on using lipid nanoparticles (LNPs) as carriers for *YTHDF1*-targeted silencing vectors (LNP-siYTHDF1). Due to the suppression of stemness, this manoeuvre potentiated lenvatinib and sorafenib cytostatic effect *in vitro* and their antitumor activity in a mouse orthotopic xenograft model of HCC. These results led the authors to suggest that silencing YTHDF1 is a promising strategy for attenuating malignant characteristics of HCC and enhancing the response of this tumor to targeted therapies.

The role of m^6A modification in the stemness and chemoresistance of gastrointestinal cancer has already been reported. Thus, in gastric adenocarcinoma, these characteristics have been associated with the enhanced activity of the m^6A writer METTL3, leading to LGR5 (leucine rich repeat containing g protein-coupled receptor 5) up-regulation (12). This protein is involved in the canonical Wnt pathway and plays a role in forming and maintaining adult intestinal stem cells during post-embryonic development. Moreover, in HCC, down-regulation of the hepatocyte nuclear factor 3γ (HNF3γ, gene symbol *FOXA3*), which plays an essential role in liver physiology and inhibits stem cell differentiation, has been inversely correlated with tumor malignancy and patient survival. Interestingly, reduced HNF3γ expression in HCC is mediated by METTL14-dependent m^6A methylation of

FOXA3 mRNA (13).

Another valuable contribution of Zhang *et al.*'s study (5) is the demonstration of some relationship between YTHDF1 and the NOTCH1-mediated pathway, which has been directly related to the stemness state of HCC cells (5). Nevertheless, the actual link between both proteins and the role of m^{6}A in this connection has been only partly elucidated.

Similarly to YTHDF1, NOTCH1 is ubiquitously expressed, being fat, the spleen, and the skin, the territories with the highest expression. Regarding the role of both proteins in HCC, it is worth noting that the levels of YTHDF1 and NOTCH1 are not particularly high in healthy livers.

The four members of the NOTCH family (NOTCH1–4) are type I transmembrane proteins sharing structural characteristics, such as an extracellular domain consisting of multiple epidermal growth factor-like (EGF) repeats and an intracellular domain consisting of several different domain types. The function of these proteins is an evolutionarily conserved intercellular signaling pathway that regulates the interaction between physically adjacent cells through binding of these receptors to their membrane-bound ligands jagged-1 (JAG1), jagged-2 (JAG2), delta-1 (DLL1), and delta-4 (DLL4). Following the translation of these proteins, the immature amino acid sequence is proteolytically processed in the trans-Golgi network by a furin-like convertase to generate two different polypeptides that must heterodimerize to form the mature cell-surface receptor. Upon ligand binding, NOTCH1 is cleaved by ADAM family protease to release its intracellular domain (NICD1), which, after nuclear translocation, forms a transcriptional complex with other recruited proteins, such as the DNA-adapter protein RBPJ and chromatin modifiers. This complex acts as a transcription factor that induces the selective expression of target genes involved in cell differentiation, proliferation, and apoptosis activation. Mutations in the *NOTCH1* gene affecting its function have been associated with the deregulation of this intercellular signaling pathway, which favors the malignant transformation of hematologic and solid cancers. Thus, gain-of-function mutations in *NOTCH1* gene are frequent in T-cell acute lymphoblastic leukemia (14) and chronic lymphocytic leukemia. In contrast, mutations in its paralogs *NOTCH2*, *NOTCH3*, and *NOTCH4* are rare events in these malignancies (15).

Although Zhang *et al.* proposed a direct effect of YTHDF1 on the processing of *NOTCH1* pre-mRNA (5), other indirect mechanisms cannot be ruled out. Indeed, different connections between both elements with the maturation of the pre-mRNA of other genes have been reported. Thus, in chronic lymphocytic leukemia, mutations in the gene encoding the spliceosome component SF3B1 have been associated with the appearance of alternative splicing affecting *DVL2* pre-mRNA (16). In turn, through binding to RBPJ, DVL2 acts as a negative regulator for transcription of NOTCH1 target genes. In contrast, the generation of the *DVL2* splice variant results in up-regulation of the NOTCH1 target gene *HES1* (16,17). Whether YTHDF1-dependent pre-mRNA processing is involved in the deregulation of elements participating in the NOTCH1 pathway remains unknown. Similarly, considering the relevance of these epigenetic mechanisms, an essential question to be addressed before envisaging any clinical translation is to fully elucidate the impact on healthy tissues of manipulating the YTHDF1-m^{6}A-NOTCH1 epitranscriptomic axis with non-highly selective tools.

Targeting the molecular pathways and epigenetic modifications that account for malignant traits of LCSC subpopulations is required to modify their impact on HCC treatment response. This is particularly relevant in the case of this cancer, for which m^{6}A methylation is a critical regulator of gene expression (18). The function of m^{6}A methylation regulators, like YTHDF1, which is involved in NOTCH1 up-regulation, and therefore increases stemness and therapeutic resistance, highlights their potential as prognostic indicators and therapeutic targets in HCC. The analysis of the expression levels in tumor tissues of important m^{6}A writers, erasers, and readers enables us to find relationships between this machinery and clinical outcomes like overall survival and recurrence rates (19). Additionally, advances in bioinformatics tools which permit the quick analysis of massive datasets downloaded from sources such as The Cancer Genome Atlas (TCGA) can uncover the relationship between patient prognosis and the expression of these proteins (20). Apart from their prognostic value, m^{6}A regulators exhibit potential as biomarkers for distinguishing HCC subtypes that may exhibit distinct responses to treatment. Thanks to the analysis of the relationship between the expression/activity of these regulators and clinical features/patient stratification a more effective therapy customization can become possible. For instance, patients whose tumor present elevated levels of specific m^{6}A readers or writers may be eligible for tailored treatments aimed at inhibiting these regulators and disrupting the malignant signaling

pathways controlled by them. This can be achieved by using targeted therapies aimed at specifically blocking certain signaling pathways or by using gene editing technologies. Combining these approaches with traditional therapies may result in overcoming chemoresistance and lowering the risk of recurrence (21). Furthermore, studies on the reversibility of epigenetic modifications raise the prospect of creating treatments that allow HCC cells to express their genes normally again. Histone deacetylation or DNA methylation inhibitors are examples of epigenetic therapies that are already demonstrating promising results in treating other types of cancer. Similarly, these strategies could be improved upon and tailored for HCC.

Assessing differentially methylated sites associated with HCC is essential to comprehending the epigenetic alterations that drive the disease. Already existing high-throughput technologies such as array-based methods like the Infinium MethylationEPIC BeadChip (22) or whole-genome bisulfite sequencing (WGBS) (23) could be used to achieve this goal. These techniques enable a comprehensive analysis of DNA methylation patterns throughout the genome, providing detailed maps of methylation changes in tumor versus normal tissue. Following data acquisition, computational tools and algorithms are used to find differentially methylated regions (DMRs), which may be associated with changes in gene expression, tumor suppressor silencing, or oncogene activation. After that, these DMRs undergo additional analysis to determine their functional significance, frequently by combining transcriptomic or proteomic profiles with methylation data to create a connection between methylation alterations and modified cellular pathways (24). Moreover, by linking these differentially methylated sites to patient outcomes, the clinical significance of these sites can be evaluated. For instance, a poor prognosis, therapy resistance, or a higher risk of metastasis may be linked to specific methylation patterns. The generation of methylation-based biomarkers for HCC early detection, prognosis, and individualized treatments may result from the advances in this field (25). In this context, by determining how m^6A methylation affects important pathways like NOTCH1 signaling, the investigation by Zhang *et al.* (5) offers insights into the molecular mechanisms underlying HCC, which can be translated into clinical applications.

In terms of early diagnosis, the results highlight the potential of m^6A methylation markers as biomarkers for early detection of HCC, especially those involving YTHDF1. Increased levels of YTHDF1 or other related m^6A regulators in blood or other accessible biological fluids may become non-invasive diagnostic markers, which could facilitate early intervention before the disease progresses to a more advanced state. This is crucial, as treatment outcomes and survival rates for HCC are greatly improved by early diagnosis.

For disease evaluation, the study by Zhang *et al.* (5) offers a framework for understanding how tumor biology and patient response to treatment are impacted by m^6A-mediated regulation. Hopefully, clinicians will be able to better assess the aggressiveness of the disease and adjust treatment strategies by understanding the role that YTHDF1 and related m^6A modifications play in stemness and therapeutic resistance. With this knowledge, targeted therapies that precisely interfere with these epigenetic mechanisms, such as the LNPs-siYTHDF1 described in that work, could be further developed and improved, increasing the efficacy of current treatments and decreasing relapse rates. Taken together, these can lead to novel theragnostic approaches that simultaneously improve the diagnosis and selection of the best therapeutic option for HCC patients.

In conclusion, although more work is needed, that promising results obtained by Zhang *et al.* (5) have paved the way for developing new sensitizing strategies to enhance the effectiveness of pharmacological treatments for HCC patients.

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