Non-coding RNAs in human liver malignancies: Critical regulators for cancer stemness?

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Cancer stem cells (CSCs) have become one of the essential topics in cancer research over the past few years (1). Present chemotherapy modalities eliminate the bulk of the tumor cells, but cannot eliminate a core of these CSCs that have a high capacity for renewal. Identification of these cells is the first step in the development of therapeutic modalities (2). CSCs have been defined as a unique subpopulation in tumors that have the capacity to initiate tumor growth and sustain tumor self-renewal. Although evidence has been provided to support the existence of CSCs in various solid tumors, the identity and functions of liver CSCs remains unclear. The CSC hypothesis first appeared almost one hundred years ago when a number of pathologists in Europe observed that tumors were composed of a heterogeneous mixture of partially differentiated cell types, similar in many respects to a normal organ. The existence of CSCs was first demonstrated more than a decade ago when John E. Dick *et al.* proved the hypothesis to be largely true for human acute myeloid leukemia (3). The leukemic stem cell, which was recognized by specific markers of $CD34^+CD38^-$, serially reproduced the malignancy in immunodeficient mice, showing properties of longevity and self-renewal (4). The important discovery was subsequently verified in breast and brain tumors (5,6). Despite some limitations, the growth of a side population of tumor cells with specific markers in immunodeficient mice has become the popular standard for identifying a CSC in other solid tumors including GI cancers such as colon, liver and pancreatic cancer (7). In some of these studies as few as 100 cells of the CSC subpopulation induced tumor growth in immunodeficient mice. In addition, it should be noted that there exist some discrepancies for CSC markers among different groups, and few studies have examined specific markers in both human and murine models of disease. The most recent definition of CSCs by the American Association for Cancer Research (AACR) is: cells within a tumor that possess the capacity for self-renewal and that can cause the heterogeneous lineages of cancer cells that constitute the tumor. These mutated cells, which live indefinitely and can seed new tumors, are now suspected of causing many, if not all, cancers. What is worse, these persistent cells are not killed by chemotherapy or other current treatments. Their survival might explain why tumors frequently recur or spread after treatment.

In 1961, Jacob and Monod hypothesized that "Structural genes encode proteins and regulatory genes produce non-coding RNAs". It has been recognized that the normal function of a cell depends on the accurate expression of a large number of protein-coding RNAs (mRNAs) and non-protein coding RNAs (ncRNAs), the RNA molecules that are not translated into proteins. More than 95% of the genome is made up of non-coding DNA and large and growing families of transcribed ncRNAs have now been identified in mammalian cells (8). For a long time ncRNAs have been judged to be evolutionary junk, and they have been erroneously considered to be the result of transcriptional noise. This may be partly related to the lack of understanding their complex and subtle roles in gene regulation and the inability to determine their functional roles using conventional protein based

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approaches. Therefore, a major challenge for the future is to identify ncRNA that are functional, and to clarify their functions (9,10). Thousands of ncRNAs have been identified and an appreciation of their regulatory roles is now emerging (8,11,12). ncRNA can be arbitrarily separated based on size of 200 base pairs into small and large ncRNA. Small ncRNAs are now demonstrated as essential regulators of gene expression through the recent discoveries of RNA interference and of miRNAs (13). Both of these involve the recognition of mRNAs by small ncRNAs and their subsequent degradation or inhibition of translation. The mechanisms by which large ncRNAs modulate gene expression are unknown, and their functions remain indefinable. The long-held dogma of human malignancy as a genetic disease involving protein encoding tumor suppressor and oncogenic genes (14-16) has been challenged by information about the contribution of ncRNAs in cancer, and of stemness mechanisms. The involvement of ncRNA in tumor biology has been most actively investigated for the small RNAs including miRNAs and Transcribed Ultra Conserved Regions (T-UCRs). Despite of a number of large ncRNAs in cancer cells have been identified, their functional role in cancer development and progression is still not clearly clarified.

Liver cancer/hepatocellular carcinoma (HCC) is a complex genetic disease caused by the accumulation of mutations that lead to the deregulation of gene expression and to uncontrolled cell proliferation. The classical models of HCC tumorigenesis postulate alterations in protein-coding oncogenes and tumor suppressor genes. miRNAs can also contribute to hepatic oncogenesis, functioning as tumor suppressors [e.g., *miR-122a* and *miR-125a*], or as oncogenes [e.g., *miR-21*, the *miR-221*, and *miR-143*]. Relatively minor variations in the levels of expression of miRNAs or mutations that moderately influence the conformation of miRNA-mRNA pairing could have important consequences for the cell because of the large number of targets of each miRNA. Additional studies have demonstrated that miRNAs play a critical role in HCC tumor initiation and progression and those miRNA alterations are ubiquitous in human liver cancers. Consequently, events activating or inactivating miRNAs are now viewed as cooperating with abnormal protein-coding genes in human hepatic tumorigenesis (17,18). However, much less was known about the upstream regulation of miRNA in cancer cells until a recent series of publications demonstrated that the tumor suppressor gene, *TP53*, regulates the transcription of the *miR-34* family (18) and that the *miR-34* family subsequently mediates the induction of apoptosis, cell cycle arrest, and senescence. Using quantitative real-time polymerase chain reaction, it was demonstrated that *miR-34a* was highly up-regulated in a human cancer cell line, which was treated with a DNA-damaging agent, doxorubicin (19). Additionally, the basic helix-loop-helix transcription factor Twist, an organizer of the epithelial mesenchymal transition (EMT) and newly discovered marker for human HCC, has been found to be the regulator of the expression of specific miRNA cluster (20,21). Furthermore, it was shown that widespread miRNA repression by the *c-Myc* oncogenic transcription factor contributes to tumorigenesis in general (22) and to repression of the *miR-17-92* cluster in particular. However, the extent of miRNA regulation by various transcriptions factors in hepatocellular cancer cells, especially in HCC stem cells is not yet known.

Although miRNAs represent the most widely studied category of the non-coding RNAs, other non-coding RNAs that might be involved in tumorigenesis are Transcribed Ultra Conserved Regions (T-UCRs), which are a subset of transcripts of genomic sequences that are located in both intra- and intergenic regions and that are absolutely (100%) conserved between orthologous regions of the human, rat, and mouse genomes (23,24). These T-UCRs represent a subset of ncRNA that can be transcribed, and have been implicated in human cancers (17). We have observed a consistent alteration in T-UCR in a high percentage of analyzed human malignancies. Meanwhile, a unique expression of T-UCR in HCC cancer stem cells compared to HepG2 HCC cells as well as normal human hepatocytes has been uncovered. Some T-UCRs have been involved in HCC cancer cell survival and invasion. These data suggest that alterations in T-UCR expression may contribute to hepatic malignant processes. As a matter of fact, researchers have proposed a model in which both coding and non-coding genes contribute to human cancer development and progression. Therefore, our observations provide the justification for focused studies of ncRNA such as T-UCR and their functional role in mediating gene expression in HCC cancer stem cells. In spite of their highly conserved nature, there may be some functional redundancy since targeted deletion of some T-UCR in mice results in viable animals. These paradoxical observations may also reflect new roles of mechanisms in the regulation of gene expression. Although it is expected that T-UCR may act as regulatory ncRNA, their precise functions are still undefined. It is hypothesized that putative functions of T-UCR may include either an anti-sense inhibitory role for other ncRNAs or protein coding genes, or a role as non-specific microRNAs in modulating gene expression, or as an enhancer of gene expression (25,26). Some T-UCRs are found within exons of protein coding genes with potential cancer relevance. Moreover, correlations between the expression of T-UCRs and miRNA in response to TGF-β/Twist1 raise the intriguing possibility of complex functional regulatory pathways in which these two types of ncRNAs, or others, may interact and influence cell-signaling pathways, cellular effects or phenotype

associated with TGF-β/Twist in HCC cancer stem cells.

In summary, the molecular mechanisms by which ncRNAs can modulate cancer stem cells mediate tumor growth, chemoresistance or metastases are still under investigation. It has been shown that several ncRNAs are aberrantly expressed in malignant hepatocytes as well as cholangiocytes, and in human HCC compared with matching non-tumoral tissue (27). Moreover, it has been demonstrated that specific miRNAs expressed in HCC stem cells, like let-7 and miR-181 promotes cell invasion, migration, and growth via repression of target genes and modulation downstream signaling pathways, as well as matrix metalloproteinases (MMPs), the key enzymes in tumor invasion (28). The identification of ncRNAs as an important regulator of cancer stem cell proliferation, migration, and invasion *in vitro*, as well as their upstream modulators and interaction with T-UCRs will emphasize an essential role of the specific ncRNAs in mediating hepatic oncogenesis and tumor behavior, and provides insight into the involvement of altered ncRNA expression in contributing to the tumor phenotype.

Future perspectives: Hepatocellular cancer (HCC) is the fifth most common malignancy in the world, accounting for approximately one million deaths with an increasing trend of new incidences annually in United States. HCC is associated with altered expression of inflammation-associated cytokines and of RNA genes that are involved in cancer stem cell survival and tumor spread. Cancer stem cells are the major contributor to the pathogenesis of liver cancer. We believe that abnormalities in cytokine dependent expression of RNA genes are central to cancer stem cell survival, tumor growth and recurrence. Characterizing non-coding RNAs in HCC stem cells may lead to larger studies on patients, examining diagnostic testing or possibly searching for genetic causes of specific hepatocellular carcinoma. Conceivably, these studies could lead to the identification of potential therapies for patients with resistance to chemotherapy. A long term benefit of the research will be the application of what is learned in these projects to aid in the development of anti-non-coding RNA (pre-non-coding RNA) target therapy for other indications.

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