LINE(1)s of Evidence in HBV-Driven Liver Cancer

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Hepatitis B virus (HBV) integration in hepatocellular carcinoma (HCC) is a poorly understood event. In a recent *Cancer Cell* paper, Lau et al. (2014) describe a HBV-human fusion transcript (HBx-LINE1) that functions as a lncRNA, influences the epithelial-mesenchymal transition, and correlates with reduced patient survival and tumor formation in mice.

Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer and the fifth-most common cause of cancer-related deaths in humans. HCC can be induced by several etiologies including alcohol or drug abuse, inflammatory liver disease, and a high-fat or high-sugar diet. The most relevant etiology driving liver cancer, however, is chronic infection with hepatitis C or B viruses (HCV; HBV). Worldwide, approximately 240 million people are chronically infected with HBV, and chronic HBV carriers are at high risk for developing cancer (El-Serag and Rudolph, 2007). In most cases, chronic HBV infection induces chronic liver inflammation and liver injury, which drives the development of liver fibrosis and subsequent cirrhosis, ultimately leading to HCC. Several epidemiologic and experimental studies have indicated that expression of HBV-derived proteins can directly promote HCC formation—even in the absence of chronic liver injury (Na et al., 2011; Murakami et al., 2005). In addition, HBV integration into the host genome has been linked to liver cancer formation (Matsubara and Tokino, 1990), since 85%–90% of HBV-associated HCCs contain at least one HBV integration site (Bréchot et al., 2000). Initially, it was proposed that HBV integrations occur randomly without preferred integration sites. However, recent high-throughput next-generation sequencing studies identified recurrent insertion sites with many HBV integrations occurring within or near-repetitive, noncoding sequences, such as LINEs (long interspersed nuclear elements), SINEs (short interspersed nuclear elements), and Alu (named after the restriction enzyme derived from the bacterium *Arthrobacter luteus*, specifically cutting those sequences) (Ding et al., 2012). LINE and SINE are retrotransposons, making up 17% and 11% of the human genome, respectively.

By applying an algorithm (ViralFusion-Seq) enabling efficient and unbiased detection of possible fusions between viral and human sequences (Li et al., 2013), Lau et al. (2014) describe the existence of a viral-human hybrid RNA. Starting with HBV-positive HCC cell lines, the authors identified a LINE(1) sequence that was cotranscribed from an HBV insertion detected in chr.8p11. This LINE(1)-rich region of chromosome 8 is placed within a transcriptionally silent heterochromatin containing no transcription factor binding sites. The transcript identified turned out to be a fusion of the human LINE(1) and the HBV encoded X protein (HBx-LINE1$^{1-674}$ further denoted as HBx-LINE[1]) transcripts, presumably being driven by the HBx promoter (Figure 1). HBx-LINE(1) transcription was detected in 23.3% of the examined HCCs, correlating with a shorter overall survival of HCC patients.

In a series of well-conducted and controlled in vitro experiments, Lau et al. (2014) show that HBx-LINE(1) expression results in a long noncoding (lnc) RNA that drives migration and invasion of tumor cell lines through the induction of epithelial-mesenchymal transition (EMT) (Figure 1). In order to determine whether the virally encoded part, the human sequence, or the full-length HBx-LINE(1) determines the described functionality, the full-length HBx-LINE1$^{1-674}$ and several variants were expressed and investigated for their biological effects. The authors demonstrate that exclusively the full-length HBVx-LINE(1) induces EMT and nuclear translocation of β-catenin. To confirm that the observed phenotype stemmed from the HBx-LINE(1) mRNA and not pro-

tein expression, Lau et al. (2014) introduced a stop codon in the HBx-LINE(1) RNA at the beginning of the HBx encoded sequence, inhibiting protein translation but still enabling transcription of the HBx-LINE(1) fusion mRNA. Unambiguously, the authors show that colony formation, cell migration, and induction of EMT depend on mRNA function and not protein expression of HBx-LINE(1). Additionally, the authors ruled out the contribution of potential microRNAs generated from HBx-LINE(1). In vitro experiments using Drosha and Dicer, which are essential for microRNA production, showed that there was no cleavage of full-length HBx-LINE(1) into smaller 18–22 nt fragments, suggesting a lack of hairpin folding of HBx-LINE(1) to allow microRNA maturation. Thus, the biological activity of full-length HBx-LINE(1) is very likely mediated as a lncRNA and not as a microRNA.

Finally, the authors show that expression of HBx-LINE(1) as a transgene in livers of mice increases the incidence and number of HCCs after treatment with the chemical carcinogen diethylnitrosamine (DEN). They also observed enhanced nuclear β-catenin localization in livers and HCC nodules in HBx-LINE(1) transgenic mice. Although these data suggest a role of HBx-LINE(1) mRNA expression in DEN-induced liver cancer formation, it would be interesting to learn whether HCC in HBx-LINE(1) transgenic mice differ from those of control mice in terms of growth pattern and transcriptional/genomic profile, and whether comparable profiles are found in HCC of patients with HBx-LINE(1) mRNA expression.

As indicated by in situ experiments with livers of HBx-LINE(1) transgenic mice, high HBx-LINE1 mRNA expression is exclusively found in DEN-induced HCC
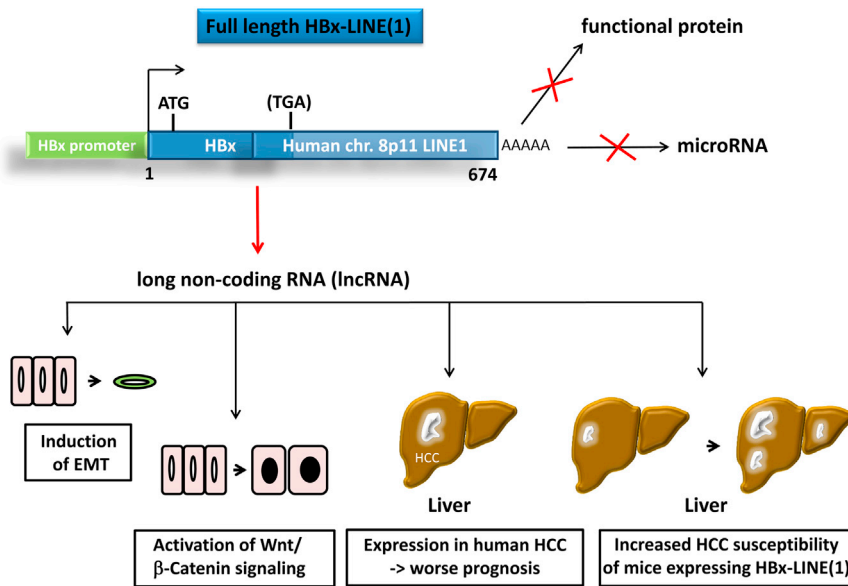
Figure 1. Biological Consequences of HBx-LINE1 Expression
Schematic drawing of the HBx-LINE1 mRNA and its biological effects as a lncRNA on epithelial-mesenchymal transition (EMT) transition, nuclear β-catenin translocation, as well as HCC development in human patients and diethyl-nitrosamine (DEN)-treated HBx-LINE1 transgenic mice.

biological function and presumably also a different outcome upon therapeutic intervention.

In conclusion, the study by Lau et al. (2014) characterizes the biological function of a class of biologically active, chimeric RNAs affecting HBV-induced liver carcinogenesis (Figure 1). At the same time, the exact mode of action of HBx-LINE(1) in HCC development needs to be defined in more detail. Future studies may characterize additional viral-human gene fusions and their biological function—and hopefully pave the way to exploit these as therapeutic targets.

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nodules and not in other regions of the transgenic liver. Thus, it appears that HBx-LINE1 reaches high expression levels and exerts it biological effects exclusively in an oncogenic environment. Since presumably expression of HBx-LINE(1) alone does not result in liver damage or induce liver cancer, what are the cofactors contributing to HBV-associated HCC development in humans? Accordingly, it will be important to investigate the underlying mechanisms of HBx-LINE(1) RNA activity in an environmental context. Along these lines, it will be equally important to see if HBx-LINE(1) transcripts can exert protumorigenic functions in HCC mouse models with chronic inflammation (Wolf et al., 2010)—as found in most chronic HBV carriers.

Finally, it will be important to identify the underlying molecular cues of how HBx-LINE(1) transcripts induce EMT transition and nuclear β-catenin translocation. What are the potential direct and indirect cellular and even viral binding partners (proteins, RNAs) for HBx-LINE(1) transcripts? Does the fusion transcript also bind to nucleosomes and influence the chromatin microenvironment and centromere formation as described for other LINE(1) transcripts (Chueh et al., 2009)?

From a clinical perspective the presented data on frequent expression of HBx-LINE(1) in human HCC and its consequences on patient survival are very intriguing. Although analysis for HBx-LINE(1) mRNA expression and correlation with clinical data need to be confirmed in other patient cohorts, this finding may be the starting point to identify subtypes within HBV-induced HCC with distinct