IDENTIFICATION OF NOVEL VIRAL-HOST INTERACTIONS IN HEPATITIS B VIRUS REPLICATION AND PATHOGENESIS OF HEPATITIS B VIRUS-RELATED HEPATOCELLULAR CARCINOMA

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Chronic infection with HBV (CHB) is the predominant risk factor for hepatocellular carcinoma (HCC). HCC is the most common type of liver cancer, associated with poor clinical outcome and a high mortality rate. Hence, it is important to understand host-virus interactions governing the role of HBV in hepatocarcinogenesis (detailed in section I) and HBV replication (detailed in section II), so as to identify novel biomarkers and develop better interventions to improve prognosis in HBV-related HCC.

(I) The Hepatitis B small surface antigen (HBsAg) is an HBV-encoded secretory protein and a major structural component of the HBV proteolipid envelope. Clinical and *in vivo* studies strongly support the role of HBsAg in hepatocarcinogenesis, however the underlying mechanisms remain largely unknown. Here, we tested the ability of HBsAg to regulate long non-coding RNAs (lncRNAs) in hepatocytes, and further investigated the role of HBsAg-mediated lncRNAs in HCC development. Publicly available microarray data from HepG2 cells overexpressing HBsAg identified LINC00665 as an HBsAg-regulated lncRNA. Furthermore, gene expression data from liver samples of HBV and HCC patients demonstrated that LINC00665 is upregulated in HBV infection and HCC, specifically in HBV-related HCC. This was supported by our *in vitro* data which demonstrated that LINC00665 expression was upregulated in the presence of HBsAg in HepG2 and Huh7 cells, as well as in the presence of the HBV whole-genome in 3 different HBV cell culture models. Next, we evaluated the oncogenic potential of LINC00665 by its overexpression and CRISPRi-based knock-down in various cell-based assays. LINC00665 promoted cell proliferation, migration and colony formation, but inhibited cell apoptosis *in vitro*. Taken together, these results identified LINC00665 as a novel lncRNA through which HBV, specifically HBsAg can drive hepatocarcinogenesis in HBV-related HCC.

We wanted to further understand the mechanism by which HBsAg regulates oncogenic lncRNAs. The Nuclear Factor-κB (NF-κB) family of transcription factors (NF-κB1, NF-κB2, RelA, RelB, and c-Rel) are master regulators of inflammation, regeneration and immunomodulation in the liver. Previous studies suggest that LINC00665 is an NF-κB responsive lncRNA. Furthermore, HBsAg has been shown to activate NF-κB signaling in clinical studies, as well as in mouse models. Based on these findings, we hypothesized that HBsAg acts through the NF-κB pathway to regulate LINC00665 expression. We first confirmed that HBsAg activates NF-κB signaling *in vitro*, by demonstrating that HBsAg promoted the nuclear translocation of NF-κB factors. Next, we observed that supressing NF-

κB activity nullified HBsAg-induced LINC00665 transcription, as well as the HBsAg-mediated activation of the LINC00665 promoter in luciferase assays. This suggests that HBsAg acts through the NF-κB pathway to regulate LINC00665. Furthermore, we identified multiple NF-κB binding sites in the LINC00665 promoter and demonstrated their functional relevance. We used reporter assays to show that disrupting κB sites abrogated the HBsAg-mediated activation of the LINC00665 promoter. Finally, we used ChIP to demonstrate HBsAg promotes enrichment of NF-κB factors at the LINC00665 promoter in the presence of HBsAg. Taken together, this data suggests that HBsAg positively regulates oncogenic LINC00665 through the NF-κB pathway, thereby identifying a novel mechanism by which HBsAg drives hepatocarcinogenesis. This work identifies HBsAg/NF-κB/LINC00665 axis as a novel viral-host interaction driving hepatocarcinogenesis, thereby substantiating further studies on LINC00665 as a biomarker in HBV-related HCC.

(II) In addition to HBV-host interactions in HCC, we wanted to investigate novel-virus host interactions regulating HBV replication. Numerous studies have shown that nuclear receptors are essential modulators of HBV transcription, translation and ultimately HBV replication. However, not all members of this superfamily have been explored for their ability to regulate HBV activity. Clinical studies support the role of Vitamin D and its cognate nuclear receptor, Vitamin D receptor (VDR), in regulating HBV activity in CHB. This antiviral role of vitamin D is widely attributed to VDR/Retinoid X Receptor (RXR)-mediated regulation of host immunomodulatory genes through Vitamin D Response Elements (VDREs) in their promoters. Here, we investigated the ability of calcitriol (metabolically activated Vitamin D) to directly regulate HBV transcription, translation and replication through the VDR signaling pathway. We observed that calcitriol selectively inhibited only the HBV-core promoter without affecting the HBV-PreS1, HBV-PreS2/S, or HBx promoters. We then identified a VDRE-cluster in the HBV-core promoter that is highly conserved across most HBV genotypes. Disruption of this VDRE-cluster abrogated calcitriol-mediated suppression of the HBV-core promoter, demonstrating its functional relevance. Furthermore, we use binding assays to show that VDR interacts directly with the VDRE-cluster in the HBV-core promoter independent of RXR. This demonstrates that calcitriol inhibits HBV-core promoter activity through a non-canonical calcitriol-activated VDR-pathway. Finally, we observed that calcitriol suppressed expression of the canonical HBV-core promoter transcripts, pregenomic RNA (pgRNA) and precore RNA (pcRNA) in multiple HBV cell culture models. Additionally, calcitriol inhibited the secretion of HBeAg and HBsAg (HBV-encoded proteins linked to poor disease prognosis), without affecting virion secretion. These results were corroborated in an HBV drug-resistant mutant, where calcitriol inhabited pgRNA, pcRNA, HBeAg and HBsAg, but not virion secretion. The ability of vitamin D to reduce HBeAg and HBsAg levels suggests a potential role for this micronutrient as a supplement along with anti-viral therapies for HBV, especially in drug-resistant mutants. Furthermore, this work identifies VDR as a novel nuclear receptor regulating HBV-core promoter activity and conceptually advances our understanding of the antiviral role of vitamin D from an immunomodulator to a direct regulator of viral activity.