別紙様式第４

学　位　論　文　要　旨
Academic degree dissertation abstract

博士課程 (甲)・乙 Doctoral Course 【Type of degree】 1. Full-time 2. Dissertation-only	第　号 No.	氏　名 Name	Maya Shofa

[論文題名]
猫ヘパドナウイルスと B 型肝炎ウイルスは NTCP を共通のレセプターとして用いる

Dissertation title
Conserved use of the sodium/bile acid cotransporter (NTCP) as an entry receptor by hepatitis B virus and domestic cat hepadnavirus

Antiviral Research, 217:1-13, 2023, DOI: 10.1016/j.antiviral.2023.105695

[要　旨]
Abstract

The *Orthohepadnaviridae* genus is a group in the *Hepadnaviridae* family with small, partially double-stranded DNA genomes and includes members that infect mammals, particularly affecting the liver. The most important pathogen of this viral genus is the hepatitis B virus (HBV), which poses a significant global health threat due to its potential to cause life-threatening liver diseases in humans, possibly resulting in severe liver conditions, such as cirrhosis and hepatocellular carcinoma. Eradicating chronic infection of this virus remains a challenge despite effective vaccines.

In 2018, a novel member of the *Orthohepadnavirus* genus, the Domestic cat hepatitis B virus, also known as domestic cat hepadnavirus (DCH), was discovered in Australia from a cat that died of lymphoma. Following its initial detection, subsequent surveys have been conducted to determine its prevalence in other countries. Our research group also reported its first identification in Japan and Taiwan. The whole genome sequencing analysis revealed that DCH was genetically similar to HBV. DCH infection is also linked to chronic hepatitis and has been associated with feline

retrovirus infections in cats, suggesting a clinical similarity with HBV pathogenesis. However, the viral replication mechanism for DCH is largely unknown. Therefore, it is necessary to investigate the replication mechanism of DCH, particularly the mechanism for the entry pathway into the target cells. HBV has been shown to use the sodium/bile acid cotransporter (NTCP) as a major cellular entry receptor. Since the equivalent receptor for DCH remains unknown, we sought to identify the cellular entry receptor for DCH.

HBV encodes three envelope glycoproteins: Large (preS1 + preS2 + S), Middle (preS2 + S), and Small (S only) proteins for infection. Of them, preS1 is responsible for binding with NTCP. The phylogenetic analysis of the preS1 sequence of *Orthohepadnavirus* species suggests that several strains of DCH are genetically closer to HBV than *Orthohepadnavirus* in other host species. The N-terminally myristoylated preS1 domain (myristoylated 2-48 peptide) of the hepatitis B surface has been reported to mediate the specific attachment to hepatocytes. Therefore, we synthesized fluorescein amidite (FAM)-labeled myristoylated preS1 peptides derived from both HBV and DCH. As a negative control, we synthesized preS1 peptide with the Asparagine (N) to Lysine (K) mutation at the ninth residue (N9K) that has been reported to abolish the binding activity to NTCP. We used these peptides to probe the binding of preS1 peptide to NTCP molecule. Huh7 cells, a hepatocyte-derived carcinoma cell line, were transiently transfected with a plasmid encoding NTCP molecules and tested for binding to myristoylated preS1 peptides. To distinguish between transfected and untransfected cells, we cotransfected a plasmid encoding mCherry2 fluorescent protein and measured the percentage of FAM-positive cells in the mCherry2-positive population, which should express each NTCP molecule via fluorescent microscopy and flow cytometry.

By binding assay, we observed that HBV-derived preS1 peptide efficiently bound to Huh7 cells expressing HumanNTCP. Moreover, the HBV-derived preS1 peptide showed significant binding to CatNTCP to a level similar to HumanNTCP. As previously reported, cynomolgus monkey NTCP (CmNTCP) did not support the binding of the HBV-derived preS1 peptide. Furthermore, we found that the DCH-derived preS1 peptide efficiently bound to both CatNTCP and HumanNTCP but not to CmNTCP. Similar to the HBV preS1 peptide, the DCH-derived preS1 peptide harboring the N9K substitution failed to bind to any NTCPs. These findings suggest that both viruses utilize NTCP as a binding receptor and more importantly, the HBV-derived preS1 peptide can bind to CatNTCP, while the DCH-derived preS1 peptide can bind to HumanNTCP. Furthermore, we demonstrated that the DCH-derived preS1 peptide binds to NTCPs derived from a broad range of animal species, suggesting that DCH has a

potentially broad host tropism. Interestingly, although DCH has been identified in dogs, dog NTCP minimally supported the binding of the HBV-derived and the DCH-derived preS1 peptides.

A previous study demonstrated that several amino acids of NTCP were under positive selection during the evolution, suggesting that NTCP has been a frontline for arms races against hepadnaviruses. Prior research conducted by another group has shown that Glycine (G) at the residue 158 of NTCP (G158) is critical for interaction with the HBV-derived preS1 peptide. Intriguingly, it has been reported that CmNTCP possess Arginine (R) at the residue 158 of NTCP (R158), conferring them resistance to HBV infection. Our result demonstrated that NTCP with non-G158 residues in several animal species did not support the binding of HBV or DCH-derived preS1 peptide. We also demonstrated that mutagenesis of Glycine at NTCP position 158 to Arginine (G158R) caused a loss in binding to the DCH-derived preS1 peptide for both HumanNTCP (G158R) or CatNTCP (G158R), suggesting the shared nature of the binding pattern of DCH- and HBV-derived preS1 peptide to the NTCP receptors.

Moreover, to elucidate the functional interaction between DCH envelope L protein and NTCP molecules, we conducted an infection assay using hepatitis D virus (HDV) particles enveloped with L proteins derived from HBV, DCH (Rara) or DCH (Sydney). The HDV particles were generated by contrasfecting the pSVL (D3) plasmid together with a plasmid encoding the L proteins derived HBV, DCH (Rara strain) or DCH (Sydney strain) into Huh7 cells. Consistent with previous studies, HDV particles enveloped with HBV L protein infected Huh7 cells expressing HumanNTCP. Notably, HDV particles enveloped with L proteins derived from DCH (Rara) or DCH (Sydney) established infection on Huh7 cells expressing either HumanNTCP or CatNTCP. In addition, we used primary human hepatocytes (PHHs) to test the infections of HDV particles enveloped with L proteins derived from HBV, DCH (Rara) or DCH (Sydney). HDV particles enveloped with L proteins derived from HBV or DCH (Rara) established infection in PHHs, suggesting that DCH (Rara) can utilize HumanNTCP as a functional receptor. These findings suggest that HumanNTCP and CatNTCP are functional receptors for DCH.

Considerable effort has been made to interrupt the interaction between preS1 and NTCP to develop an entry inhibitor. Herein, we examined the effect of an entry inhibitor, Myrcludex B, which has been approved in Europe. Myrcludex B is a synthetic lipopeptide derived from the preS1 domain of the HBV envelope protein, specifically targeting hepatocytes to block *de novo* HBV infection efficiently. Our study demonstrated that Myrcludex B efficiently inhibited the interaction of the DCH-derived

preS1 peptide with CatNTCP.

In this study, we used synthesized lipopeptides to test the interaction between preS1 and the NTCP molecules and investigated the viral entry pathway. While we showed that HDV particles enveloped with L proteins derived from DCH (Rara) or DCH (Sydney) established infection on Huh7 cells expressing either HumanNTCP or CatNTCP, the interaction between the DCH and NTCPs requires experimental verification using an infectious virus. Consequently, we are currently trying to isolate infectious DCH. Future studies using infectious DCH will support our present findings.

In summary, our investigation revealed that CatNTCP is a functional receptor for HDV particles enveloped with DCH L proteins, suggesting that CatNTCP is a cellular receptor for DCH. Additionally, the specificity of binding for the DCH preS1 peptide is determined by residue 158 of NTCP proteins, highlighting species-specific interactions. Myrcludex B, an HBV entry inhibitor, blocked the binding of the DCH preS1 peptide, suggesting that Myrcludex B can be used to treat cats chronically infected with DCH. Thus, DCH and HBV may share cell entry molecules, suggesting a possibility of inter-species transmission.