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Distinct notch signaling expression patterns between nucleoside and nucleotide analogues treatment for hepatitis B virus infection

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ABSTRACT

Nucleos(t)ide analogues therapies are currently approved for the treatment of chronic hepatitis B virus (HBV) infection, which effectively suppress HBV replication and correlate with the anti-HBV-specific immune response. Notch signaling serves pleiotropic roles in the immune system that also contribute to virus-specific immunity. In this study, we assessed Notch signal-related gene expression after administrating nucleoside or nucleotide analogues to HBV-replicating cells and clinical liver tissues. We found distinct Notch signaling expression patterns under nucleos(t)ide analogues therapies, with high expression for nucleotide analogues (adefovir pivoxil or tenofovir disoproxil fumarate) and low expression for nucleoside analogues (lamivudine or entecavir) in the presence of HBV infection. Furthermore, activation of mammalian target of rapamycin (mTOR)-Akt (Ser473) phosphorylation was also observed after nucleotide analogue treatment. In conclusion, nucleoside and nucleotide analogues displayed different patterns of Notch signaling activity under HBV infection, and the induction of mTORC2-Akt (Ser473) phosphorylation may contribute to nucleotide analogues-mediated Notch signaling activation.

1. Introduction

Hepatitis B virus (HBV) infects more than 350 million people worldwide, increasing their risk of liver cirrhosis and hepatocellular carcinoma (HCC) [1]. High rates of chronic infection are maintained mostly due to vertical transmission from infected mothers to neonates, presumably because neonates have an immature immune system [2]. However, infection in most adults results in acute hepatitis, which always appears as a self-limited, transient liver disease due to the presence of antiviral-specific immunity, except for some cases who develop inactive HBV infection or fulminant hepatitis, resulting in mortality [2]. In general, there are distinct innate and adaptive immune responses during HBV infection that may be related to differential antiviral immunity and pro-inflammatory capacity.

Nucleos(t)ide analogues (NAs) therapies are currently approved for the treatment of chronic HBV infection that effectively suppress HBV replication without serious adverse effects, including nucleoside analogues (lamivudine [LAM] and entecavir [ETV]) and nucleotide analogues (adefovir pivoxil [ADV] and tenofovir disoproxil fumarate [TDF]). NAs directly inhibit the action of reverse transcriptase, leading to a reduction of HBV replication and a decrease of serum HBV-DNA below detection limits [3]. However, it is still difficult to achieve clinical cure of chronical hepatitis B because these NAs do not target HBV covalently closed circular DNA (cccDNA) directly, which serves as a template for the transcription of viral mRNA, enabling viral rebound upon discontinuation of antiviral treatment [4]. Despite acting by the same mechanism, there are differences between nucleoside and nucleotide analogues on HBV infection, such as stronger cccDNA inhibition with ETV and LAM [4–6], shorter time for HBsAg loss with TDF, and better serological effect with ADV [7]. There is a negative correlation between HBV-DNA replication and immune activation during NA treatment, in which ETV induces higher interferon (IFN)-γ production and may be able to break virus-mediated immune tolerance [8]. These differences may be explained by their different potencies in inhibiting reverse transcriptase or an additional effect of nucleotide analogues in inducing IFN-λ3 [7,9].

Notch signaling is an intercellular signaling pathway that

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controls a wide range of crucial effects in liver development involved in liver repair, inflammation, metabolism, and HCC [10,11]. Moreover, recent evidence also showed an essential role for Notch signaling in influencing HBV infection and viral persistence [12,13]. Notch signaling has also been reported to serve pleiotropic roles in the immune system through a canonical pathway on regulatory T cell (Treg) regulation or a non-canonical pathway with the mammalian target of rapamycin (mTOR)-Akt cascade on the virus-specific immune response [14–17].

Knowing the different effects of nucleoside or nucleotide analogues on signal transduction or induction of the immune response can be beneficial in the clinical setting when selecting a treatment option. On the basis of these findings, we hypothesized that Notch signaling may have an important regulatory role during NAs treatment, which would contribute to a better understanding of the possible mechanism underlying the differences between NAs. To test this hypothesis, we examined the changes of Notch signaling-related gene expression after administrating nucleoside or nucleotide analogues, and compared the differences between these two types of NAs as well as the possible mechanism in HBV-replicating cells and clinical liver tissues.

2. Materials and methods

2.1. Cell culture

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. HepG2.2.15.7 cells, subcloned from HepG2.2.15 cells, produce a higher titer of HBV than HepG2.2.15 cells [18]. HepAD38, a HepG2-derived cell line, has a stable integration of the entire genome of HBV under tetracycline control [19]. These cell lines were cultured in DMEM/F12 medium (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 100 U/mL penicillin, 100 μg/mL streptomycin, 400 μg/mL G418, 10 mM HEPES buffer solution, and 5 μg/mL insulin in a 5% CO_2 incubator at 37 °C.

2.2. NAs administration

Adherent cells were seeded at a density of 1.0×10^5 cells/well. Each cell line was incubated with ADV (0.74 μM), TDF (0.13 μM), LAM (0.51 μM), or ETV (0.03 μM) for 120 h. The medium was changed at 24, 48, 72, and 96 h. All drugs were purchased from Sigma. The molar NA concentrations were determined based on their reported EC90 values in HepAD38 cells, which effective reduce the HBV level by 90% [20,21].

2.3. Hirt DNA extraction and real-time detection PCR (RTD-PCR) quantification of HBV-DNA and HBV cccDNA

The Hirt protein-free DNA extraction procedure was used to isolate HBV cccDNA from HBV-infected cells [22]. For cccDNA detection, Hirt-extracted DNA was first pre-treated with Plasmid-Safe DNase (Epicenter, Madison, WI) as previously reported [23]. HBV-DNA and cccDNA expression was quantified with TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, Waltham, MA) using a specific HBV DNA probe (5′-FAM-TATCGCTG-GATGTGTCTGCGGCGT-TAMRA-3′), forward primer (5′-ACTCAC-CAACCTCCTCCTGTCCT-3′), and reverse primer (5′-GACAAACGGGCAACATACCT-3′), and cccDNA probe (5′-FAM-ACCACCGTGAACGCCCA-MGB-3′), forward primer (5′-GCGCACCTCTCTTTACGCG-3′), and reverse primer (5′-GCCCCAAAGCCACCCA AG-3′) (FASMAC, Kanagawa, Japan) as follows: 50 °C for 2 min, 95 °C for 10 min, and 45 cycles of 95 °C for 10 min and 65 °C for 30 s. HBV-DNA and cccDNA copy number was quantified relative to the known copy number of HBV-DNA.

2.4. RNA extraction and RTD-PCR analysis

Total RNA was isolated using a High Pure RNA Isolation Kit (Roche, Basel, Switzerland) after cell collection at the indicated time points. A High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used for first-strand cDNA synthesis with 0.1 μg total RNA from each sample. RTD-PCRs were conducted using TaqMan Gene Expression Assay Identification according to the manufacturer's instructions. The expression data were normalized using the reference gene GAPDH (Thermo Fisher Scientific).

2.5. Western blot analysis

Protein extracts were prepared with RIPA Lysis Buffer (Merck Millipore, Temecula, CA) containing protease inhibitor cocktail and phosphatase inhibitor tablets (Sigma). Protein content was determined using a protein assay kit (Bio-Rad, Hercules, CA). Western blotting was performed as described previously [24]. All primary and secondary antibodies, except anti-Jagged1 (Abcam, Cambridge, UK), were purchased from Cell Signaling Technology (Danvers, MA).

2.6. Immunohistochemical staining of clinical samples

Surgical samples from HBV-related HCC patients were stained immunohistochemically to confirm whether NA administration was related to Notch signaling activity. All study participants provided informed consent, and the study design was approved by the medical ethics committee of Kanazawa University. Formalin-fixed, paraffin-embedded background liver samples were analyzed according to the antiviral therapy of each patient (Table 1). The slides were incubated with primary (Abcam, Cambridge, UK) and secondary (Dako, Santa Clara, CA, USA) antibodies. Western blot analysis was performed to analyze related protein expression in these clinical samples.

2.7. Statistical analysis

All pairwise data are presented as mean ± standard deviation and were analyzed by Student's *t*-test using SPSS 22.0 (SPSS, Inc., Chicago, IL). Statistical significance was preset at $P < 0.05$.

3. Results

3.1. Notch signaling activity depending on NA type

We first investigated HBV-DNA and HBV cccDNA levels after NAs treatment. In accordance with a previous report, NA therapy effectively reduced HBV-DNA in these HBV-replicating cells, but it could not completely eradicate HBV cccDNA (Fig. 1A and B). To identify the involvement of the Notch signaling pathway after NA treatment, we compared Notch1 levels among the HBV-replicating cells after NA treatment. Interestingly, higher Notch1 mRNA and protein levels were found in HBV-replicating cells treated with nucleotide analogues (ADV or TDF); however, lower levels were detected in cells treated with nucleoside analogues (ETV or LAM) compared with the control group (Fig. 1C and F). Notch signaling is initiated by binding with its corresponding ligand (Delta-like1, 3, 4 or Jagged1, 2), resulting in the release of the Notch intracellular domain, which is subsequently translocated into the nucleus to modulate downstream gene expression, including Hes1 [25]. To