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Core-APOBEC3C chimerical protein inhibits hepatitis B virus replication

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We tested the capsid targeted viral inactivation method as an anti-HBV strategy. HepG2 cells were cotransfected with HBV expression plasmid and the plasmid encoding fusion protein of either Core-A3C or Core-humanized renilla GFP (hrGFP). Core-A3C had substantial effect on HBV DNA levels. In the HepG2 cells expressing Core-A3C, the number of G-to-A mutations increased dramatically, whereas other nucleotide substitutions were rare. In addition, Core-A3C substantially inhibited HBV production intracellularly and in culture supernatant. These results suggest that Core-A3C may be a candidate as a novel antiviral agent against human HBV infection.

Keywords: APOBEC3C (A3C)/hepatitis B virus (HBV) core protein/recombinant HBV vector/capsid targeted viral inactivation (CTVI).

Abbreviations: HBV, Hepatitis B Virus; HBsAg, Hepatitis B surface antigen; APOBEC, apolipoprotein B mRNA-editing enzyme-catalytic polypeptide; A3C, APOBEC3C; Core-A3C, HBV core APOBEC3C chimerical protein; hrGFP, humanized renilla Green Fluorescent Protein; CTVI, capsid targeted viral inactivation; SN, staphylococcal nuclease.

Hepatitis B virus (HBV) is a major cause of liver disease and has infected approximately 2 billion worldwide (*1*). The limited success of current therapies for HBV infection has prompted the discovery of alternative strategies. An antiviral strategy known as capsid targeted viral inactivation (CTVI) is shown to be efficient in interfering with viral replication *(2)*. The Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide (APOBEC) family (including APOBEC1, APOBEC2 and APOBEC3 subgroups) can deaminate cytidine in RNA and/or DNA, and has diverse physiological functions (*3*). APOBEC3 subgroup, in particular APOBEC3B/C/F/G, exhibits anti-retroviral effect, and inhibits human immunodeficiency virus (HIV) production by reducing the accumulation of viral reverse transcriptase and trigger lethal G-to-A hypermutation (*4*). However, this cellular innate defence can be thwarted by virion infectivity factor (Vif) encoded by HIV, resulting in decreased editing effect of human deaminase APOBEC3 on HIV DNA (*5*). APOBEC3G (A3G) inhibited HBV replication *(6*, *7)*, while APOBEC3C (A3C) has little effect on HBV DNA synthesis (*7*). Compared to HBV core-A3G, HBV core-A3C complex was much more stable in response to ribonuclease treatment (*7*). A3C induced extensive G-to-A mutations in the majority of the newly synthesized HBV DNA genomes (*7*, *8*). These suggest that HBV is susceptible to the editing effect of endogenous A3C, and A3C may elicit an anti-HBV host response.

In this study, we used the CTVI approach as an antiviral strategy to construct expression plasmids encoding Core-A3C, and constructed plasmids encoding Core-hrGFP fusion proteins. Our results suggest that Core-A3C may be a candidate anti-viral agent against human HBV infection.

A3C gene was amplified by polymerase chain reaction (PCR) using A3C expression plasmid (pc-A3C-HA) as a template. On the basis of the wild-type HBV expression plasmid pCH-3093, having a two base pair deletion before the HBV pre-genomic RNA transcription start of pCH-9/3091 (*9*), pCH-Core-A3C and pCH-Core-hrGFP were constructed for replication-defective HBV as well as fusion protein expression. Plasmids of pCMV-A3C, pCMV-hrGFP and pCMV-hrGFP-A3C (fusion of hrGFP and A3C) were constructed on pcDNA3.1 for non-CTVI controls. Plasmids were transfected into HepG2 cells. Ninety-six hours after transfection, cell culture supernatants or cytoplasmic lysates of transfected HepG2 cells were used for the following analysis. Cells transfected with wild-type HBV expression plasmid (pCH-3093) served as control. For experiments, the supernatant (cytoplasmic fraction) was collected and HBV DNA was extracted for Southern blot analysis Nucleocapsid-associated viral DNA from cytoplasmic lysates was amplified with forward primer HBV1501 (5′-TRACAARAATCCTCACAATAC-3′) and reverse primer HBV1848(-) (5′-AAYAGGAGGGATAYA TAGAG-3′). PCR fragments were purified and ligated into the pGEM-T cloning vector. Individual clones were sequenced with T7 promoter sequencing primer.

We first investigated the effect of Core-A3C fusion protein on the accumulation of viral DNA replicative intermediates and viral particles released into the supernatant of transfected cells. Although Core-hrGFP results in only a minor suppression of HBV replication [a 20% decrease in the intracellular HBV DNA level versus cells transfected with pCH-3093 only

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(a mean of two independent experiments)], Core-A3C had substantial effect on the HBV DNA levels [an 84% decrease in the intracellular HBV DNA level versus control cells (a mean of two independent experiments)] (Fig. 1A). Similar results were obtained for the effects of Core-hrGFP and Core-A3C on HBV particle release into the supernatant of transfected cells, as assessed by Southern blot analysis (Fig. 1A; 30 and 91% decreases for Core-hrGFP and Core-A3C, respectively). Next, we investigated the effect of pCH-Core-hrGFP, pCH-CoreA3C, pCMV-A3C, pCMV-hrGFP and pCMV-hrGFP-A3C plasmids on HBV particle release in cytoplasmic and supernatant of transfected cells. The results show that pCMV-A3C, pCMV-hrGFP and pCMV-hrGFP-A3C did not have any effect on HBV particle release (Fig. 1B). In order to rule out the possibility of transfection efficiency on the results, we performed ELISA experiments for hepatitis B surface antigen (HBsAg) to confirm equal transfection efficiency in HepG2 cells. The results showed the transfection efficiency were the same in HepG2 cells (Fig. 1C). In order to investigate the mechanism underlying the inhibitory effect of Core-A3C on HBV production, the ability of Core-A3C on editing synthesized encapsidated HBV DNA was compared. The newly synthesized nucleocapsid-associated HBV

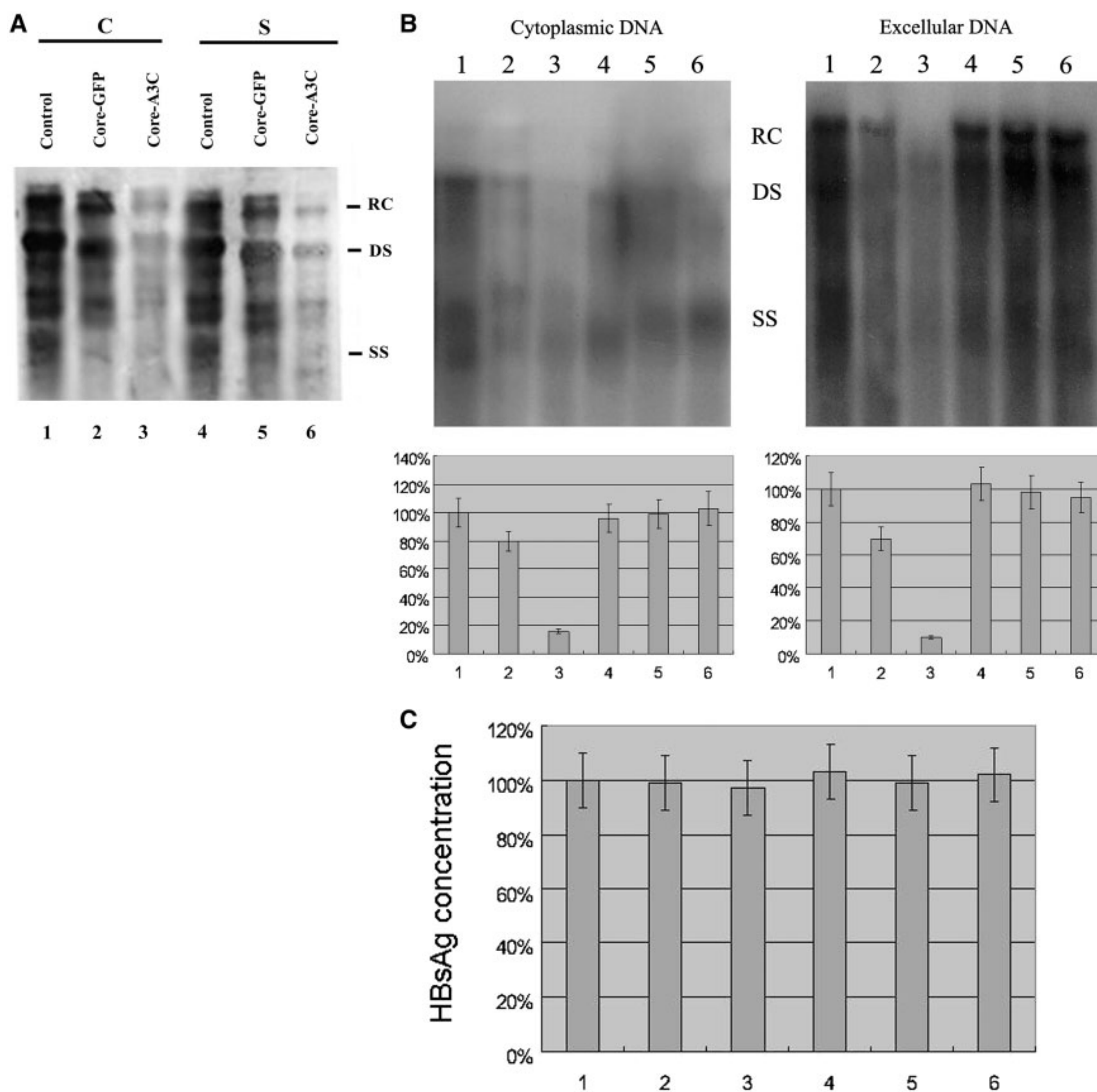

Fig. 1 Southern blot analysis of effects of Core-hrGFP and Core-A3C on the accumulation of cytoplasmic viral DNA replicative intermediates and viral particles released into the supernatant of transfected cells. (A) HepG2 cells were cotransfected with wild-type (wt) HBV expression plasmid and expression constructs encoding Core-hrGFP and Core-A3C, respectively, as indicated in lanes 1–6. HepG2 cells transfected with wild-type HBV expression plasmid served as control. The accumulation of HBV replicative intermediates in cytoplasmic lysates (shown in lanes 1–3) was analysed by Southern blot analysis of nuclease-resistant HBV DNA. The release of viral particles into the culture supernatant of transfected cells (shown in lanes 4–6) was analysed by Southern blot of HBV DNA isolated from HBV particles from the culture supernatant. (B) Effect of plasmids on HBV particle release in cytoplasmic and supernatant of transfected cells. Lane 1, control; Lane 2, pCH-Core-hrGFP; Lane 3, pCH-Core-A3C; Lane 4, pCMV-A3C; Lane 5, pCMV-hrGFP; Lane 6, pCMV-hrGFP-A3C. Band intensities were quantified using Labworks Image Analysis software. (C) ELISA experiments for hepatitis B surface antigen (HBsAg) to confirm equal transfection efficiency in HepG2 cells. Lane 1, control; Lane 2, pCH-Core-hrGFP; Lane 3, pCH-Core-A3C; Lane 4, pCMV-A3C; Lane 5, pCMV-hrGFP; Lane 6, pCMV-hrGFP-A3C. Core-GFP, pCH-Core-hrGFP plasmid; Core-A3C, pCH-Core-A3C plasmid; C, cytoplasmic hepatitis B virus DNA; S, particle-associated HBV DNA purified from cell culture supernatants; RC, relaxed circular HBV DNA; DS, double-stranded HBV DNA; SS, single-stranded HBV DNA.

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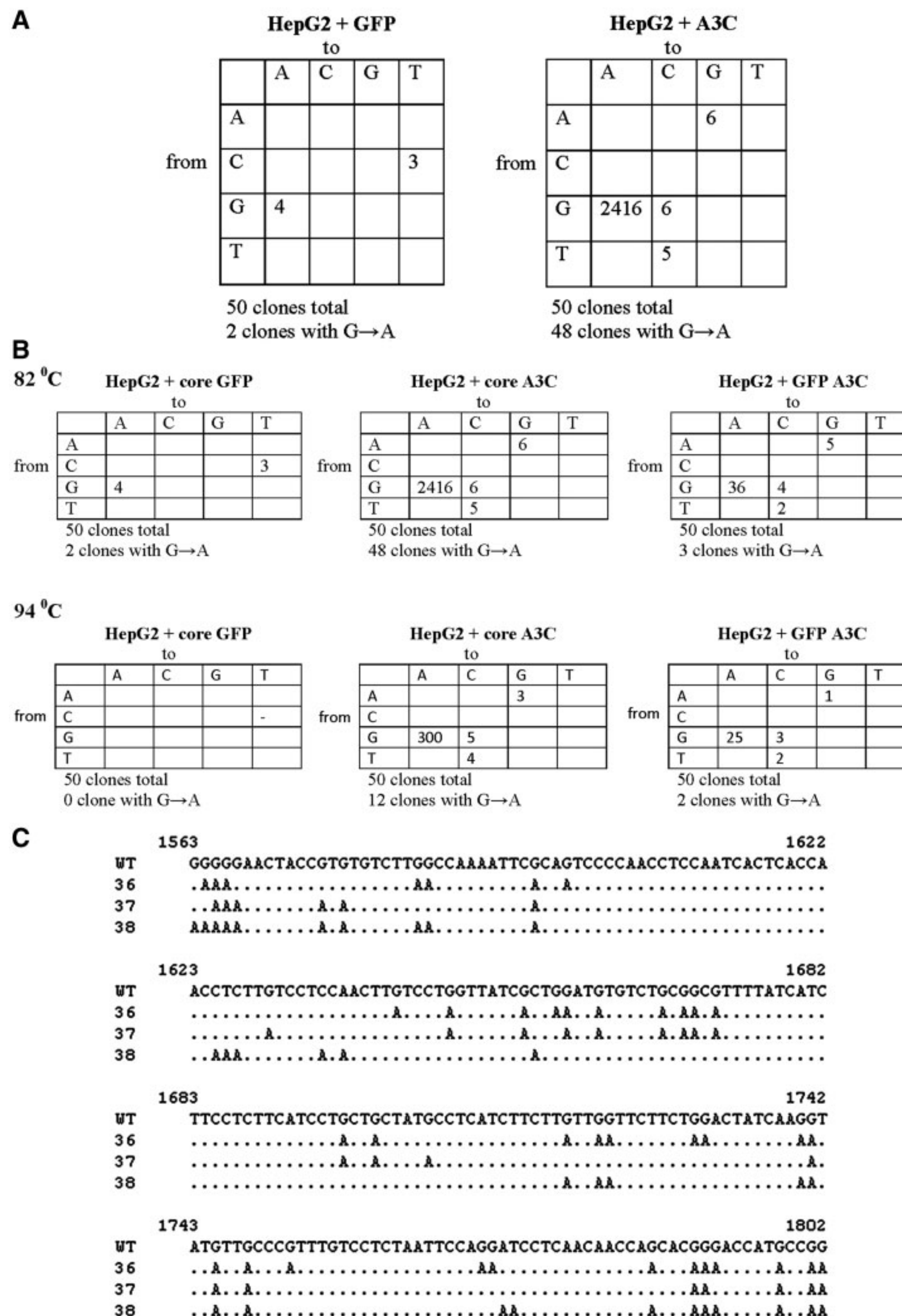

Fig. 2 G-to-A mutations in newly synthesized HBV DNA produced in HepG2 hepatocellular carcinoma cells in the presence of pCH-Core-hrGFP or pCH-Core-A3C. (A) HepG2 cells were cotransfected with wild-type HBV expression plasmid pCH-3093 and pCH-Core-hrGFP or pCH-Core-A3C, respectively. Nucleocapsid-associated HBV DNA was amplified by PCR, cloned and sequenced with T7 promoter sequencing primer (about 345 nucleotides per clone). The footnotes indicate the total number of sequenced clones and the number of clones with G-to-A mutations. The boxes show the total number of respective mutations. (B) HBV DNA obtained non-biased PCR (94°C for denature temperature) and low temperature PCR (82°C for denature temperature). (C) Nucleotide sequence of three individual PCR-amplified HBV clones (#36, 37 and 38) produced in HepG2 hepatocellular carcinoma cells cotransfected with wild-type HBV expression plasmid pCH-3093 and pCH-Core-A3C plasmid. Nucleocapsid-associated HBV DNA was amplified by PCR, cloned and sequenced. Mutations are depicted with respect to the wild-type sequence. Dashed lines represent nucleotide identity. The numbers indicate the nucleotide positions with respect to the start codon of the core protein.

DNA was amplified by PCR from nuclease-digested cell lysates (cytoplasmic fraction) of cotransfected HepG2. Fifty individual clones were randomly selected for DNA sequencing analysis. In the presence of Core-hr-GFP, G-to-A mutations were rare (only 2 out of 50 clones; Fig. 2A, left panel). In contrast, in Core-A3C expressing HepG2 cells, the number of clones bearing G-to-A mutations and the overall number of G-to-A mutations increased dramatically (48 out of 50 clones; Fig. 2A, right panel). We found that the G-A mutations can be seen in GG, GT and GC sequences, with the preferential site being the GG duplet sequence. We also investigated the effect of different temperature on the results (Fig. 2B). The PCR

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condition was the same as 82°C, except for the denature temperature was 94°C. The results show that mutations were much lower in 94°C compared to 82°C (Fig. 2B). In all mutations, G-to-A mutation was the most often observed, whereas other nucleotide substitutions were rare (Fig. 2C).

Core-A3C fusion protein significantly inhibited HBV replication by hypermutating the majority of HBV genomes. CTVI has its own advantages: (i) nuclease is more stable than antisense RNA and siRNA with higher antiviral efficiency. Even if only one nuclease is assembled into a HBV particle, this single nuclease can degrade HBV genomes, resulting in the abolishment of its infectivity of HBV. These inactivated virus-like particles can efficiently induce innate immune response to produce specific antibody to protect the human body. (ii) the envelop protein is essential for viral replication and assembly, and can avoid any sudden changes of the immune-reactivity (*10*).

Beterams and Nassal (*10*) applied CTVI strategy for the first time to an HBV study, by fusing calcium-dependent staphylococcal nuclease (SN) to HBV core protein to yield a chimeric protein, coreSN. The study demonstrated that in HBV co-transfected human hepatoma cells, less than 1 coreSN protein per 10 wild-type core protein subunits significantly reduces titers of enveloped DNA containing virions. This antiviral effect highly requires an enzymatically active SN and on the core domain. CoreSN does not block the assembly of RNA-containing nucleocapsids but interferes with proper synthesis of viral DNA inside the capsid, or leads to rapid DNA degradation (*11*). However, SN requires relatively high concentration of Ca^{2+} to be active. HBV DNA inside of cells remains unaffected in the presence of CoreSN because of extremely low intracellular calcium concentration. CoreSN can degrade HBV DNA only when the viruses are released into the extracellular cell growth medium. Thus, HBV DNA replication inside the cells cannot be inhibited by the above CoreSN-based approach. The activation of A3C does not require Ca^{2+}. In our study, Core-A3C directly inhibits HBV production inside of the human hepatoma cells.

A3C contains 190 amino acids, exhibits a strong inhibitory effect on HBV replication, and does not damage the stability of core–shell complex of HBV, which allows the correct reverse transcription of 'pregenomic' RNA. However, A3C induces G-to-A hypermutation of the newly synthesized HBV DNA, further affecting the replication of offspring HBV. This finally results in dysfunction of various capsid proteins within HBV.

The capsid of HBV is composed of 240 protein subunits (capsomers) arranged with icosahedral symmetry. Assembly of the capsomers around viral nucleic acid was mediated by strong interactions between them. A3C protein has a similar size to SN whose molecular weight is 16.9 kDa (*12*). Fusion of A3C to the C-terminus of HBV core protein should not change the interaction between capsomers. Therefore, it is predicted that A3C can form core particles together with HBV core protein. The chimerical protein fused to C-terminus of HBV core protein can be assembled into core particles, where the chimerical protein functions as deaminase to inhibit the replication of HBV. The detailed mechanism underlying how Core-A3C inhibits the replication of HBV needs further dissection.

In conclusion, a plasmid-encoding Core-A3C chimerical protein was constructed, which substantially inhibited HBV production intracellularly and in cell culture supernatant.

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Conflict of interest

None declared.

References

1. Ganem, D. and Prince, A.M. (2004) Hepatitis B virus infection—natural history and clinical consequences. *N. Engl. J. Med.* **350**, 1118–1129
2. Natsoulis, G. and Boeke, J.D. (1991) New antiviral strategy using capsid-nuclease fusion proteins. *Nature* **352**, 632–635
3. Conticello, S.G., Thomas, C.J., Petersen-Mahrt, S.K., and Neuberger, M.S. (2005) Evolution of the AID/APOBEC family of polynucleotide (deoxy)cytidine deaminases. *Mol. Biol. Evol.* **22**, 367–377
4. Mangeat, B., Turelli, P., Caron, G., Friedli, M., Perrin, L., and Trono, D. (2003) Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* **424**, 99–103
5. Harris, R.S. and Liddament, M.T. (2004) Retroviral restriction by APOBEC proteins. *Nat. Rev. Immunol.* **4**, 868–877
6. Turelli, P., Mangeat, B., Jost, S., Vianin, S., and Trono, D. (2004) Inhibition of hepatitis B virus replication by APOBEC3G. *Science* **303**, 1829
7. Baumert, T.F., Rosler, C., Malim, M.H., and von Weizsacker, F. (2007) Hepatitis B virus DNA is subject to extensive editing by the human deaminase APOBEC3C. *Hepatology* **46**, 682–689
8. Kock, J. and Blum, H.E. (2008) Hypermutation of hepatitis B virus genomes by APOBEC3G, APOBEC3C and APOBEC3H. *J. Gen. Virol.* **89**, 1184–1191
9. Sun, D., Rosler, C., Kidd-Ljunggren, K., and Nassal, M. (2010) Quantitative assessment of the antiviral potencies of 21 shRNA vectors targeting conserved, including structured, hepatitis B virus sites. *J. Hepatol.* **52**, 817–826
10. Ma, L., Jones, C.T., Groesch, T.D., Kuhn, R.J., and Post, C.B. (2004) Solution structure of dengue virus capsid protein reveals another fold. *Proc. Natl Acad. Sci. USA.* **101**, 3414–3419
11. Beterams, G. and Nassal, M. (2001) Significant interference with hepatitis B virus replication by a core-nuclease fusion protein. *J. Biol. Chem.* **276**, 8875–8883
12. Beterams, G., Böttcher, B., and Nassal, M. (2000) Packaging of up to 240 subunits of a 17 kDa nuclease into the interior of recombinant hepatitis B virus capsids. *FEBS Lett.* **481**, 169–76

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