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Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3137184 A1 2020/10/22

(21) 3 137 184

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2020/04/20
(87) Date publication PCT/PCT Publication Date: 2020/10/22
(85) Entrée phase nationale/National Entry: 2021/10/15
(86) N° demande PCT/PCT Application No.: KR 2020/005203
(87) N° publication PCT/PCT Publication No.: 2020/214003
(30) Priorité/Priority: 2019/04/18 (US62/835,628)

(51) Cl.Int./Int.Cl. *C12N 15/10* (2006.01), *A61K 31/7088* (2006.01), *A61K 48/00* (2006.01), *A61P 31/20* (2006.01), *C12N 15/113* (2010.01), *C12N 15/63* (2006.01), *C12N 15/86* (2006.01), *C12N 9/22* (2006.01)

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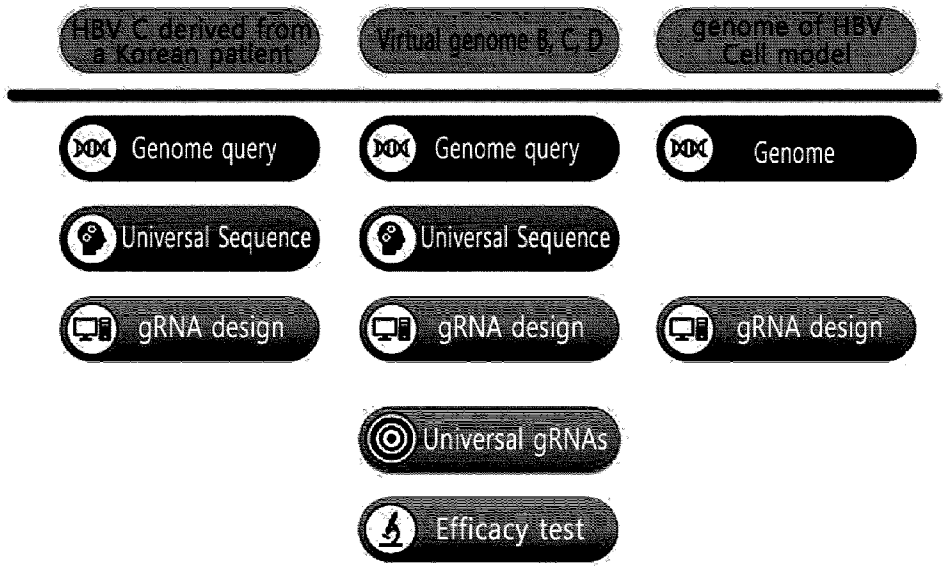
(54) Titre : COMPOSITION ET PROCEDE D'INHIBITION DE LA PROLIFERATION DU VIRUS DE L'HEPATITE B
(54) Title: COMPOSITION AND METHOD FOR INHIBITING PROLIFERATION OF HEPATITIS B VIRUS

[Figure 1]



(57) Abrégé/Abstract:
The present application relates to a composition and method for inhibiting proliferation of hepatitis B virus.

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(13) A1

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[ABSTRACT]

The present application relates to a composition and method for inhibiting Hepatitis B virus (HBV) proliferation.

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[DESCRIPTION]

[Invention Title]

COMPOSITION AND METHOD FOR INHIBITING PROLIFERATION OF HEPATITIS B VIRUS

[Technical Field]

The present application relates to a method for inhibiting the proliferation of various hepatitis B virus genotypes.

The present application relates to a composition for inhibiting the proliferation of various hepatitis B virus genotypes.

The present application relates to various uses of a composition for inhibiting the proliferation of various hepatitis B virus genotypes.

[Background Art]

Hepatitis B is an inflammation generated by the hepatitis B virus (herein referred to as HBV).

HBV infection not only has a high incidence worldwide, but can also occur in anyone regardless of age or race, and there are various routes of infection.

Hepatitis B therapeutics developed so far only improve symptoms for some of the various HBV genotypes, and thus hepatitis B often recurs.

In addition, various documents (Patent No. EP2014830911; World J Gastroenterol. 2015 Aug 28; 21(32): 9554-9565.) which obtained through research on the inhibition of HBV proliferation using a CRISPR/Cas system to reduce HBV activity also show that an HBV inhibitory effect depends on a target region, and varies for each of the HBV genotypes.

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As such, in addition to efforts to reduce the recurrence of HBV, there is still a need for inhibition of the proliferation of various HBV genotypes.

[Disclosure]

[Technical Problem]

One aspect of the present application is to providing a method for inhibiting HBV proliferation by targeting conserved regions of various HBV genotypes.

Another aspect of the present application is to providing a composition for targeting conserved regions of various HBV genotypes.

Still another aspect of the present application is to providing conserved sequences in conserved regions of various HBV genotypes.

[Technical Solution]

To solve the above-described problems, the present application may provide a method for inhibiting HBV proliferation. Here, the HBV includes at least two HBV genotypes selected from HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F and HBV genotype G,

The method may be provided that it comprises:

introducing into a subject a composition comprising:

a) a guide nucleic acid, or a nucleic acid sequence encoding the same; and

b) a Cas protein, or a nucleic acid sequence encoding the same;

wherein the guide nucleic acid selected from SEQ ID NO.51, 52, 56, 57, 69, 70, 73, 74, 75, 76, 80, 85, 87 and 89. In particular, the method may be provided that wherein thereby inducing an indel in the nucleic acid sequence in the gene of the

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hepatitis B infection.

To solve the above-described problems, the present application may provide a method for inactivating a conserved sequence in an HBV gene. Here, the HBV gene is one or more selected from the HBV genotypes consisting of HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F and HBV genotype G, and the conserved sequence is a sequence conserved in the HBV genotype sequences. Here, the conserved sequence is one or more selected from SEQ ID NOs. 1 to 45, and inactivation is cleavage of the conserved sequence.

The method may be provided that it comprises:

introducing into a subject a composition comprising:

a) a guide nucleic acid, or a nucleic acid sequence encoding the same; and

b) a Cas protein, or a nucleic acid sequence encoding the same;

the guide nucleic acid is a sequence having a complementarity or homology with the conserved sequence in part or in whole,

wherein the guide nucleic acid selected from SEQ ID NO.51, 52, 56, 57, 69, 70, 73, 74, 75, 76, 80, 85, 87 and 89.

[Advantageous Effects]

According to the present application, the following effects are shown.

First, according to the present application, by targeting conserved regions of various HBV genotypes, a method for inhibiting HBV proliferation without being significantly limited by genotype can be provided.

Second, according to the present application, a composition for targeting conserved regions of various HBV genotypes can be provided. Here, therapeutic effects on the various HBV genotypes can be obtained using the same composition.

Third, according to the present application, conserved regions of various HBV genotypes can be provided.

[Description of Drawings]

FIG. 1 is a schematic diagram for the design of CRISPR gRNAs which are capable of targeting HBV genotypes B, C and D.

FIG. 2 shows the virtual universal HBV genome of HBV genotype B manufactured based on an open database.

FIG. 3 shows the virtual universal HBV genome of HBV genotype C manufactured based on an open database.

FIG. 4 shows the virtual universal HBV genome of HBV genotype D manufactured based on an open database.

FIG. 5 shows the gRNA distribution of SpCas9(Streptococcus pyogenes Cas9) targeting virtual universal HBV genotypes B, C or D.

FIG. 6 shows the distribution of CjCas9(Campylobacter jejuni Cas9) targeting virtual universal HBV genotypes B, C or D.

FIG. 7 shows the gRNA distribution of SpCas9 targeting a commonly conserved region in sequences targeting the obtained open database-based conserved regions, sequences of Korean patient-derived HBV genotype C, and available HBV cell models.

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FIG. 8 shows the gRNA distribution of CjCas9 targeting a commonly conserved region in sequences targeting the obtained open database-based conserved regions, sequences of Korean patient-derived HBV genotype C, and available HBV cell models.

FIG. 9 shows target regions of HBV gRNA of the present application.

FIGS. 10 to 13 and 18 show results of measuring HBsAg and HBeAg of the HBV D genotype in gRNA candidates (Cj#06, Cj#45, Cj#47, Cj#57) for CjCas9.

FIGS. 14 to 17 and 19 show results of measuring HBsAg and HBeAg of the HBV D genotype in gRNA candidates (Sp#17, Sp#20, Sp#89, Sp#90, Sp#154, Sp#159, Sp#193, Sp#194, Sp#196, Sp#197) for SpCas9.

FIGS. 20 and 21 show results of measuring HBsAg and HBeAg of Huh7 (HBV D genotype) and HepG2 (HBV D genotype) cell lines in gRNA candidates (Sp#17, Sp#90, Sp#193, Sp#197, Sp#17+90+193, Sp#17+90+197, Sp#17+193+197, Sp#90+193+197) for SpCas9.

FIG. 22 is a table that summarizes the results of FIGS. 20 and 21.

FIGS. 23 to 26 show results of measuring HBsAg and HBeAg of each of HBV genotype A, HBV genotype B, HBV genotype C and HBV genotype D in gRNA candidates (Sp#193, Sp#197, Sp#17+90+197, Sp#17+193+197) for SpCas9.

FIG. 27 is a vector map which is designed to express one SpgRNA, Cas9 and a fluorescent protein in one plasmid.

FIG. 28 is a vector map which is designed to express one CjgRNA, Cas9 and a fluorescent protein in one plasmid.

FIG. 29 is a vector map which is designed to express three SpgRNAs, Cas9 and a fluorescent protein in one plasmid.

[Modes of the Invention]

Unless defined otherwise, all technical and scientific terms used in the specification have the same meanings as commonly understood by one of ordinary skill in the art to which the present invention belongs. Although methods and materials similar or equivalent to those described in the specification can be used in the practice or experiments of the present invention, suitable methods and materials are described below. All publications, patent applications, patents and other references mentioned in the specification are incorporated by reference in their entirety. In addition, the materials, methods and examples are merely illustrative and not intended to be limiting.

Hepatitis B is not only one of the most prevalent infectious diseases worldwide, but also a disease which is highly likely to develop into chronic hepatic diseases such as cirrhosis and liver cancer.

A virus causing hepatitis B, HBV, is a DNA virus belonging to the Hepadnaviridae family, and an incomplete double-stranded virus consisting of approximately 3,200 base pairs (3.2 kb).

HBV has plus-strand DNA with an incomplete complementary structure located inside minus-strand DNA with a complete circular structure.

In the HBV genome, there are four overlapping open reading frames (ORFs), and the frames have genes designating pre-S/S, C, P and X, respectively. The ORFs of the HBV genome have more than 50% overlapping parts. Particularly, HBV polymerase overlaps the entire pre-S/S, and overlaps other ORFs. Thus, mutations

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that occur in HBV replication may affect one or more ORFs, causing mutations to easily and particularly occur during HBV replication.

To date, it is known that there are a total of 7 types of various HBV genotypes A to G. Each subtype has an 8% or more difference in the entire base sequence of HBV.

The HBV genotype has a distinct distribution pattern depending on a region. Genotypes A and D are the major types in Western Europe and North America, and genotypes B and C are known as major types in Asia. GenoType E is only limited to Africa, and type F is common in Central America. Particularly, it has been reported that the virus proliferation of HBV genotypes B and C is maintained longer than that of other genotypes of HBVs (J Infect Dis 1997; 176:851-858).

Since there are various HBV genotypes, an effective method for inhibiting HBV proliferation without being limited by such genotypes is needed.

- Difficulty in inhibiting proliferation of various HBV genotypes

As described above, HBV is not only the cause of diseases such as hepatitis and liver cancer, but also known to have a total of 7 genotypes.

When HBV enters the blood, it is generally located in liver cells, causing an immune response in our body to eliminate the HBV. Therefore, interferon α, which is the first developed hepatitis B therapeutic, was developed to promote the expression of a major histocompatibility complex I (MHC I) antigen in the HBV-infected liver cells and promote the disruption of the HBV-infected liver cells.

However, interferon α is effective only on some genotypes, not on all of the various HBV genotypes, and does not inactivate activated HBV DNA by integration into liver cells. Thus, there is a possibility of recurrence.

Recently, research to inhibit HBV proliferation by inactivating the integrated HBV DNA using a CRISPR/Cas system is being conducted (EP2014830911, World J Gastroenterol 2015 August 28; 21(32): 9554-9565).

Specifically, in EP2014830911, hundreds of guide nucleic acid candidates, which target a conserved region of HBV are disclosed. Among these, approximately 24 guide nucleic acid candidates selected for screening to confirm whether HBV proliferation is inhibited are disclosed. In addition, as a result of screening, among the approximately 24 guide nucleic acid candidates, only about three guide nucleic acids are reported to substantially inhibit HBV proliferation.

In World J Gastroenterol 2015 August 28; 21(32): 9554-9565, approximately 15 guide nucleic acid candidates targeting a conserved region of HBV are also disclosed. In this document, approximately 15 guide nucleic acids are also used alone or in combination for screening to confirm HBV inhibition, and as in EP2014830911, it is reported that the guide nucleic acid candidates have different effects. In addition, among the combination of specific guide nucleic acids, it has been reported that only some of them have various HBV genotypes inhibitory effects.

As such, according to several documents, among even the compositions targeting a conserved region of HBV, only some exhibit HBV proliferation inhibitory effects, and only a few guide nucleic acids can be applied to various HBV genotypes.

In other words, even in the case of targeting a conserved region of HBV, the HBV proliferation inhibitory effect appears only in specific sequences. Therefore,

according to these known facts, it can be seen that only with effects of inhibiting the proliferation of some of various HBV genotypes, the effects of inhibiting the proliferation of other HBV genotypes cannot be easily expected.

Accordingly, in research to inhibit HBV proliferation by inactivating HBV DNA in the art, it is required to find a specific target sequence having a substantial proliferation inhibitory effect on various HBV genotypes.

In response to this need, the present application provides a method and composition for inhibiting the proliferation of various HBV genotypes. Particularly, the present application uses another specific target sequence having a substantial proliferation inhibitory effect on various HBV genotypes by extracting a conserved region of HBV from more various subjects than before and designing it to target these subjects.

● Technical features of the present application

The present application relates to a composition for targeting a specific conserved region of HBV and a method using the same, and particularly, to a composition and method for simultaneously inhibiting various HBV genotypes.

In the present application, a specific region on the targeted HBV genome is obtained by a method different from the related art, and such a specific region determined in the present application is clearly different from the region disclosed in the related art.

Specifically, a subject for extracting a specific conserved region of HBV is obtained by combining information of a conventional database, information derived from HBV-infected patients and information derived from HBV-injected cell lines.

By targeting a specific region on the HBV genome determined by the above-described method, a proliferation inhibitory effect on all of the various HBV genotypes may be exhibited.

Accordingly, in the present application, a treatment method effective on all genotypes using the same sequence (region), rather than a treatment method considering the sequence of each of various HBV genotypes, for example, HBV type A, HBV type B, HBV type C, HBV type D, HBV type E, HBV type F and HBV type G, may be applied. That is, HBV proliferation may be inhibited with one type of composition without specific limitation to various HBV genotypes.

Therefore, the present application has the following technical features.

First, the method for inhibiting HBV proliferation of the present application inactivates a specific sequence conserved in an HBV gene. Here, the specific sequence conserved is a sequence conserved in a region in which mutations do not easily occur between sequences of various HBV genotypes. Thus, the method for the present application may be applied without limitation to various HBV genotypes. In the present application, at least two HBV genotypes selected from HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F and HBV genotype G may be simultaneously inhibited.

Second, according to the composition and method for inhibiting HBV proliferation by targeting a specific region of the present application, a therapeutic effect may be exhibited using one type of specific region of various HBV genotypes.

That is, using the same composition and method, hepatitis B caused by HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F or HBV genotype G may be treated.

Hereinafter, a method for inhibiting the proliferation of various HBV genotypes having the technical features of the present application will be described in further detail.

Proliferation inhibitory method using common target for hepatitis B caused by various HBV genotypes

One aspect of the present application relates to a method for inhibiting the proliferation of various HBV genotypes using a common target region.

Specifically, the method for inhibiting HBV proliferation of the present application is to inhibit HBV proliferation using a technique of targeting a specific region commonly conserved in various HBV genotypes.

The HBV genotype may be selected from HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F and HBV genotype G, but the present invention is not limited thereto.

The method for inhibiting HBV proliferation of the present application may simultaneously inhibit the proliferation of one or more HBV genotypes selected from HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F and HBV genotype G.

The term “conserved region” used herein should be interpreted as follows. The conserved region refers to a part in which mutations do not easily occur between

sequences of at least two genotypes selected from HBV genotypes such as HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F and HBV genotype G. Particularly, the conserved region refers to a conserved region between base sequences of an HBV genotype selected from targets for obtaining base sequences of HBV genotypes by considering all of HBV-infected patient-derived cells and HBV-injected cell models as well as a database. In the specification, the nucleic acid sequence in the conserved region is referred to as a "conserved sequence."

The conserved sequence of the present application may be obtained using the following method.

According to one embodiment, a method for finding a conserved sequence of the various HBV genotypes may include:

aligning base sequences of HBV genotype B, HBV genotype C and HBV genotype D obtained from a database; and

finding conserved sequences by comparing the aligned HBV genotype B, HBV genotype C and HBV genotype D sequences.

The conserved sequence obtained by the above-described method may be one or more selected from SEQ ID NOs. 1 to 45 of Table 1 below.

[Table 1]

Name	**sequence (5'→ 3')**	**PAM**
Sp20-viHBV-B-#10	GTAACACGAGCAGGGGTCCT **(SEQ ID NO. 1)**	AGG
Sp20-viHBV-B-#11	CCCCGCCTGTAACACGAGCA **(SEQ ID NO. 2)**	GGG

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Sp20-viHBV-B-#12	ACCCCGCCTGTAACACGAGC **(SEQ ID NO. 3)**	AGG
Sp20-viHBV-B-#13	AGGACCCCTGCTCGTGTTAC **(SEQ ID NO. 4)**	AGG
Sp20-viHBV-B-#14	ACCCCTGCTCGTGTTACAGG **(SEQ ID NO. 5)**	CGG
Sp20-viHBV-B-#17	CACCACGAGTCTAGACTCTG **(SEQ ID NO. 6)**	TGG
Sp20-viHBV-B-#20	GGACTTCTCTCAATTTTCTA **(SEQ ID NO. 7)**	GGG
Sp20-viHBV-B-#52	CCTACGAACCACTGAACAAA **(SEQ ID NO. 8)**	TGG
Sp20-viHBV-B-#53	CCATTTGTTCAGTGGTTCGT **(SEQ ID NO. 9)**	AGG
Sp20-viHBV-B-#54	CATTTGTTCAGTGGTTCGTA **(SEQ ID NO. 10)**	GGG
Sp20-viHBV-B-#89	GGGTTGCGTCAGCAAACACT **(SEQ ID NO. 11)**	TGG
Sp20-viHBV-B-#90	TTTGCTGACGCAACCCCCAC **(SEQ ID NO. 12)**	TGG
Sp20-viHBV-B-#101	TCCGCAGTATGGATCGGCAG **(SEQ ID NO. 13)**	AGG
Sp20-viHBV-B-#102	AGGAGTTCCGCAGTATGGAT **(SEQ ID NO. 14)**	CGG
Sp20-viHBV-B-#103	TCCTCTGCCGATCCATACTG **(SEQ ID NO. 15)**	CGG
Sp20-viHBV-B-#113	CGTCCCGCGCAGGATCCAGT **(SEQ ID NO. 16)**	TGG
Sp20-viHBV-B-#117	CCGCGGGATTCAGCGCCGAC **(SEQ ID NO. 17)**	GGG
Sp20-viHBV-B-#118	TCCGCGGGATTCAGCGCCGA **(SEQ ID NO. 18)**	CGG
Sp20-viHBV-B-#119	CCCGTCGGCGCTGAATCCCG **(SEQ ID NO. 19)**	CGG
Sp20-viHBV-B-#138	GTAAAGAGAGGTGCGCCCCG **(SEQ ID NO. 20)**	TGG
Sp20-viHBV-B-#140	GGGGCGCACCTCTCTTTACG **(SEQ ID NO. 21)**	CGG
Sp20-viHBV-B-#142	GAAGCGAAGTGCACACGGTC **(SEQ ID NO. 22)**	CGG
Sp20-viHBV-B-#143	GGTCTCCATGCGACGTGCAG **(SEQ ID NO. 23)**	AGG
Sp20-viHBV-B-#154	AATGTCAACGACCGACCTTG **(SEQ ID NO. 24)**	AGG
Sp20-viHBV-B-#159	AGGAGGCTGTAGGCATAAAT **(SEQ ID NO. 25)**	TGG
Sp20-viHBV-B-#186	CGGAAGTGTTGATAAGATAG **(SEQ ID NO. 26)**	GGG
Sp20-viHBV-B-#187	CCGGAAGTGTTGATAAGATA **(SEQ ID NO. 27)**	GGG
Sp20-viHBV-B-#193	GCGAGGGAGTTCTTCTTCTA **(SEQ ID NO. 28)**	GGG
Sp20-viHBV-B-#194	GACCTTCGTCTGCGAGGCGA **(SEQ ID NO. 29)**	GGG
Sp20-viHBV-B-#196	GATTGAGACCTTCGTCTGCG **(SEQ ID NO. 30)**	AGG

Sp20-viHBV-B-#197	CTCCCTCGCCTCGCAGACGA **(SEQ ID NO. 31)**	AGG
Sp20-viHBV-B-#198	GATTGAGATCTTCTGCGACG **(SEQ ID NO. 32)**	CGG
Sp20-viHBV-B-#199	GTCGCAGAAGATCTCAATCT **(SEQ ID NO. 33)**	CGG
Sp20-viHBV-B-#200	TCGCAGAAGATCTCAATCTC **(SEQ ID NO. 34)**	GGG
Cj22-viHBV-B-#06	TGTCAACAAGAAAAACCCCGCC **(SEQ ID NO. 35)**	TGTAACAC
Cj22-viHBV-B-#20	AAGCCCTACGAACCACTGAACA **(SEQ ID NO. 36)**	AATGGCAC
Cj22-viHBV-B-#23	TTACCAATTTTCTTTTGTCTTT **(SEQ ID NO. 37)**	GGGTATAC
Cj22-viHBV-B-#40	ACGTCCCGCGCAGGATCCAGTT **(SEQ ID NO. 38)**	GGCAGCAC
Cj22-viHBV-B-#44	GTGCACACGGTCCGGCAGATGA **(SEQ ID NO. 39)**	GAAGGCAC
Cj22-viHBV-B-#45	GTGCCTTCTCATCTGCCGGACC **(SEQ ID NO. 40)**	GTGTGCAC
Cj22-viHBV-B-#46	CGACGTGCAGAGGTGAAGCGAA **(SEQ ID NO. 41)**	GTGCACAC
Cj22-viHBV-B-#47	TGCGACGTGCAGAGGTGAAGCG **(SEQ ID NO. 42)**	AAGTGCAC
Cj22-viHBV-B-#48	GACCGTGTGCACTTCGCTTCAC **(SEQ ID NO. 43)**	CTCTGCAC
Cj22-viHBV-B-#57	ATGTCCATGCCCCAAAGCCACC **(SEQ ID NO. 44)**	CAAGGCAC
Cj22-viHBV-B-#67	GACCACCAAATGCCCCTATCTT **(SEQ ID NO. 45)**	ATCAACAC

As another example, the method for finding the conserved sequence of the various HBV genotypes may include:

obtaining base sequences of the various HBV genotypes from the following base sequences, such as

i) base sequences derived from databases of HBV genotype B, HBV genotype C and HBV genotype D, respectively,

ii) a base sequence of an HBV genotype D cell model, and

iii) a base sequence derived from an HBV type C-infected patient;

aligning the base sequences of the i), ii), iii); and

finding conserved sequences in the aligned sequences.

The conserved sequence of the present application obtained by the above-described method may be any one or more selected from SEQ ID NOs. 6, 7, 11, 12, 24, 25, 28, 29, 30, 31, 35, 40, 42 and 44 in Table 1.

The most preferable method for finding a conserved sequence of various HBV genotypes according to the present application is, as described above, to obtain a conserved sequence by expanding subjects acquiring base sequences of various HBV genotypes.

According to this method, the common conserved region of the present application, which may exhibit an inhibitory effect on various HBV genotypes at the same time, may be determined. The determined conserved sequence may be sequences selected from SEQ ID NOs. 1 to 45, and more preferably, sequences selected from SEQ ID NOs. 6, 7, 11, 12, 24, 25, 28, 29, 30, 31, 35, 40, 42 and 44; and a sequence having 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, or 100% homology with respect to the sequences.

Likewise, the conserved sequence of HBV targeted by the method described above, preferably, base sequence data for various HBV genotypes may be obtained from a combination of various subjects derived from databases, patients, and cell models.

The specific conserved sequence determined as described above may be a target capable of inhibiting HBV proliferation without limitation to an HBV genotype unlike the conventional case.

A composition of the present application which will be described in further detail below is designed to target the conserved region of the various HBV genotypes.

One embodiment of the present application relates to a composition for inhibiting the proliferation of various HBV genotypes.

The composition targets conserved regions of various HBV genotypes.

The term "targets" refers to a mechanism showing an HBV proliferation inhibitory effect or killing effect by inducing the modification or inactivation of function of a sequence of the conserved region of HBV.

The composition of the present application for inhibiting the proliferation of various HBV genotypes is not limited as long as it has a function of targeting the common specific region determined above, that is, the conserved region (sequence). For example, the composition of the present application may include both of a composition capable of inducing a mutation of a conserved region on HBV genomic DNA, and a composition capable of inhibiting the translation of a conserved region on HBV mRNA.

In one embodiment, the composition of the present application includes a CRISPR system.

Specifically, a guide nucleic acid which target the conserved region of the present application and a Cas protein may be included.

The CRISPR system is a system that is capable of introducing an artificial mutation by targeting a target sequence around a proto-spacer-adjacent motif (PAM) sequence on genomic DNA. Specifically, the guide nucleic acid and the Cas protein may bind to (or interact with) each other, thereby forming a guide nucleic acid-Cas protein complex, and a mutant indel may be induced on the genomic DNA by cleaving a desired DNA sequence. The composition of the present application may inhibit the proliferation or function of HBV by introducing an indel into the above-described conserved region.

For more detailed description on the guide nucleic acid, the Cas protein and the guide nucleic acid-Cas protein complex, Korean Patent No. 10-2017-0126636 may be referred to.

In the present application, the Cas protein may include one or more proteins selected from a *Streptococcus pyogenes* (Sp)-derived Cas9 protein, and a *Campylobacter jejuni* (Cj)-derived Cas9 protein, or one or more selected from nucleic acid sequences encoding the same. The Cas9 protein serves to cleave a DNA sequence at a PAM sequence-adjacent position by recognizing a PAM(proto-spacer-adjacent Motif) sequence on the genomic DNA sequence of a subject and interacting with a guide nucleic acid.

In the present application, the guide nucleic acid may complementarily bind to the conserved sequence of the present application, as a target sequence, of the HBV genomic DNA sequence.

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The guide nucleic acid includes a site complementarily binding to the target sequence (referred to as a guide region) and a site involved in forming a complex with a Cas protein (referred to as a complex-forming region).

Here, the guide region may be a sequence having homology or complementarity with a sequence selected from SEQ ID NOs. 1 to 45, more preferably, SEQ ID NOs. 6, 7, 11, 12, 24, 25, 28, 29, 30, 31, 35, 40, 42 and 44. Although the guide region includes 0 to 5 mismatches while binding to the target sequence, it is not a big issue if the complex function is not affected.

The sequence targeting the conserved sequence of HBV may be any one selected from SEQ ID NOs.46 to 90 in Table 2.

In one example, the guide nucleic acid may be any one interacting with a SpCas9 protein and selected from SEQ ID NOs.46 to 79.

In another example, the guide nucleic acid may include any one interacting with a CjCas9 protein and selected from SEQ ID NOs.80 to 90.

Here, the complex-forming region may be determined by the type of Cas9 protein-derived microorganism. For example, in the case of the guide nucleic acid interacting with the SpCas9 protein, the complex-forming region may include 5'-GUUUUAGUCCCUGAAAAGGGACUAAAAUAAAGAGUUUGCGGGACUCU GCGGGGUUACAAUCCCCUAAAACCGCUUUU-3', and in the case of the guide nucleic acid interacting with the CjCas9 protein, include 5'-GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUC AACUUGAAAAAGUGGCACCGAGUCGGUGC-3'.

[Table 2]

NO.	Name	sequence (5'→ 3')
SEQ ID NO. 46	Sp20-viHBV-B-#10G	GUAACACGAGCAGGGGUCCU
SEQ ID NO. 47	Sp20-viHBV-B-#11G	CCCCGCCUGUAACACGAGCA
SEQ ID NO. 48	Sp20-viHBV-B-#12G	ACCCCGCCUGUAACACGAGC
SEQ ID NO. 49	Sp20-viHBV-B-#13G	AGGACCCCUGCUCGUGUUAC
SEQ ID NO. 50	Sp20-viHBV-B-#14G	ACCCCUGCUCGUGUUACAGG
SEQ ID NO. 51	Sp20-viHBV-B-#17G	CACCACGAGUCUAGACUCUG
SEQ ID NO. 52	Sp20-viHBV-B-#20G	GGACUUCUCUCAAUUUUCUA
SEQ ID NO. 53	Sp20-viHBV-B-#52G	CCUACGAACCACUGAACAAA
SEQ ID NO. 54	Sp20-viHBV-B-#53G	CCAUUUGUUCAGUGGUUCGU
SEQ ID NO. 55	Sp20-viHBV-B-#54G	CAUUUGUUCAGUGGUUCGUA
SEQ ID NO. 56	Sp20-viHBV-B-#89G	GGGUUGCGUCAGCAAACACU
SEQ ID NO. 57	Sp20-viHBV-B-#90G	UUUGCUGACGCAACCCCCAC
SEQ ID NO. 58	Sp20-viHBV-B-#101G	UCCGCAGUAUGGAUCGGCAG
SEQ ID NO. 59	Sp20-viHBV-B-#102G	AGGAGUUCCGCAGUAUGGAU
SEQ ID NO. 60	Sp20-viHBV-B-#103G	UCCUCUGCCGAUCCAUACUG
SEQ ID NO. 61	Sp20-viHBV-B-#113G	CGUCCCGCGCAGGAUCCAGU
SEQ ID NO. 62	Sp20-viHBV-B-#117G	CCGCGGGAUUCAGCGCCGAC
SEQ ID NO. 63	Sp20-viHBV-B-#118G	UCCGCGGGAUUCAGCGCCGA
SEQ ID NO. 64	Sp20-viHBV-B-#119G	CCCGUCGGCGCUGAAUCCCG
SEQ ID NO. 65	Sp20-viHBV-B-#138G	GUAAAGAGAGGUGCGCCCCG
SEQ ID NO. 66	Sp20-viHBV-B-#140G	GGGGCGCACCUCUCUUUACG
SEQ ID NO. 67	Sp20-viHBV-B-#142G	GAAGCGAAGUGCACACGGUC
SEQ ID NO. 68	Sp20-viHBV-B-#143G	GGUCUCCAUGCGACGUGCAG
SEQ ID NO. 69	Sp20-viHBV-B-#154G	AAUGUCAACGACCGACCUUG
SEQ ID NO. 70	Sp20-viHBV-B-#159G	AGGAGGCUGUAGGCAUAAAU

SEQ ID NO. 71	Sp20-viHBV-B-#186G	CGGAAGUGUUGAUAAGAUAG
SEQ ID NO. 72	Sp20-viHBV-B-#187G	CCGGAAGUGUUGAUAAGAUA
SEQ ID NO. 73	Sp20-viHBV-B-#193G	GCGAGGGAGUUCUUCUUCUA
SEQ ID NO. 74	Sp20-viHBV-B-#194G	GACCUUCGUCUGCGAGGCGA
SEQ ID NO. 75	Sp20-viHBV-B-#196G	GAUUGAGACCUUCGUCUGCG
SEQ ID NO. 76	Sp20-viHBV-B-#197G	CUCCCUCGCCUCGCAGACGA
SEQ ID NO. 77	Sp20-viHBV-B-#198G	GAUUGAGAUCUUCUGCGACG
SEQ ID NO. 78	Sp20-viHBV-B-#199G	GUCGCAGAAGAUCUCAAUCU
SEQ ID NO. 79	Sp20-viHBV-B-#200G	UCGCAGAAGAUCUCAAUCUC
SEQ ID NO. 80	Cj22-viHBV-B-#06G	UGUCAACAAGAAAAACCCCGCC
SEQ ID NO. 81	Cj22-viHBV-B-#20G	AAGCCCUACGAACCACUGAACA
SEQ ID NO. 82	Cj22-viHBV-B-#23G	UUACCAAUUUUCUUUUGUCUUU
SEQ ID NO. 83	Cj22-viHBV-B-#40G	ACGUCCCGCGCAGGAUCCAGUU
SEQ ID NO. 84	Cj22-viHBV-B-#44G	GUGCACACGGUCCGGCAGAUGA
SEQ ID NO. 85	Cj22-viHBV-B-#45G	GUGCCUUCUCAUCUGCCGGACC
SEQ ID NO. 86	Cj22-viHBV-B-#46G	CGACGUGCAGAGGUGAAGCGAA
SEQ ID NO. 87	Cj22-viHBV-B-#47G	UGCGACGUGCAGAGGUGAAGCG
SEQ ID NO. 88	Cj22-viHBV-B-#48G	GACCGUGUGCACUUCGCUUCAC
SEQ ID NO. 89	Cj22-viHBV-B-#57G	AUGUCCAUGCCCCAAAGCCACC
SEQ ID NO. 90	Cj22-viHBV-B-#67G	GACCACCAAAUGCCCCUAUCUU

A guide region sequence (SEQ ID NOs. 46 to 90) of the guide nucleic acid of the present application is any one of the conserved sequences listed in Table 1 above, and designed by considering the PAM sequence located adjacent to the conserved sequence.

As the PAM sequence, when the spCas9 protein is used, NGG (N is A, T, C or G) is considered, and when the cjCas9 protein is used, NNNNRYAC (N is each independently A, T, C or G, R is A or G, and Y is C or T) is considered.

The composition may include one or more guide nucleic acids.

In one example, the guide nucleic acid of the present application may be used by selecting one or more from SEQ ID NOs. 46 to 90 of Table 2. Preferably, the guide nucleic acid of the present application is used by selecting a combination of two or more of the above sequences.

In one embodiment, the guide nucleic acid of the present application may be used by selecting one or a combination of two or more of SEQ ID NOs. 51, 52, 56, 57, 69, 70, 73, 74, 75, 76, 80, 85, 87 and 89. In one embodiment, the guide nucleic acid of the present application may use a combination of SEQ ID NOs. 51, 57 and 73 or a combination of SEQ ID NOs. 51, 73 and 76.

The composition of the present application may include, for example,

a guide nucleic acid including one or more selected from SEQ ID NOs. 51, 52, 56, 57, 69, 70, 73, 74, 75 and 76 or a nucleic acid sequence encoding the same; and a SpCas protein or a nucleic acid sequence encoding the same.

Alternatively, the composition of the present application may include a guide nucleic acid including one or more selected from SEQ ID NOs. 80, 85, 87 and 89 or a nucleic acid sequence encoding the same; and a CjCas protein or a nucleic acid sequence encoding the same.

The composition of the present application may target

all of two or more conserved regions of HBV genotypes selected from HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F and HBV genotype G.

In one example,

all of the conserved regions of the HBV genotype B, HBV genotype C and HBV genotype D may be targeted.

A form of the composition of the present application may be a vector or ribonucleoprotein (RNP) form. Therefore, the composition includes

a) a guide nucleic acid or a nucleic acid sequence encoding the guide nucleic acid; and

b) a Cas protein or a nucleic acid sequence encoding theCas protein.

Here, a composition in the form of a vector may be a vector simultaneously or separately including a guide nucleic acid and/or a nucleic acid sequence encoding a Cas protein.

For example, the vector may include both of a guide nucleic acid and a nucleic acid sequence encoding a Cas protein, and as another example, the vector may individually include a guide nucleic acid and a nucleic acid sequence encoding a Cas protein.

Here, one or more guide nucleic acids and/or Cas proteins may be used. In one embodiment, two or three guide nucleic acids may be combined.

The vector may be a viral vector or a plasmid.

The viral vector may be one or more selected from the group consisting of a retrovirus, a lentivirus, an adenovirus, an adeno-associated virus (AAV), a vaccinia virus, a pox virus and herpes simplex virus.

In one embodiment, in the composition,

the guide nucleic acid in a) may be any one selected from SEQ ID NOs. 51, 52, 56, 57, 69, 70, 73, 74, 75 and 76, and

the Cas protein in b) may include a *Streptococcus pyogenes*-derived Cas9 protein. Here, in one embodiment, the guide nucleic acid may use a combination of SEQ ID NOs. 51, 57 and 73 or a combination of SEQ ID NOs. 51, 73 and 76.

In another embodiment, the composition may include

the guide nucleic acid in a) may be any one selected from SEQ ID NOs. 80, 85, 87 and 89, and

the Cas protein in b) may include a *Campylobacter jejuni*-derived Cas9 protein.

Meanwhile, the composition may be prepared in the form of an RNP.

This means a complex form in which the guide nucleic acid RNA sequence of the present application binds to (or interacts with) a Cas protein.

The composition of the present application may inhibit the proliferation of the virus regardless of the genotype of a virus by targeting specific conserved regions of various HBV genotypes.

The complex of the guide nucleic acid and the Cas protein of the composition targets a conserved region in HBV genomic DNA to cleave the double strands in the conserved region. As a result, due to an indel in the conserved region, that is, an artificial mutation, the virus is inactivated, thereby inhibiting HBV proliferation.

As a result of the inactivation, both of knockdown and knockout are included.

The "inactivation" refers to the prevention of normal expression of a target gene or a nucleic acid encoding the same in cells, or disabling or inhibition of the function thereof. The inactivation may be performed by inducing a state in which a target gene or nucleic acid is not transcribed and/or translated.

The "knockout" includes particularly a method for deleting genetic information, for example, cleaving a fragment of genetic DNA. Through the knockout, it is possible to inhibit the transcription and/or translation of a disease-causing gene or a gene with an abnormal function.

The "knockdown" particularly refers to blocking of the translation of a gene into a protein, and may include blocking of the generation of a protein from mRNA using a specific material such as iRNA or miRNA.

The composition of the present application, particularly, may generate an indel by NHEJ on an HBV genome by introducing DNA cleavage in a target sequence. Accordingly, an effect of inhibiting the proliferation or function of HBV may be exhibited.

In this application, when the conserved region of HBV is targeted, HBV proliferation may be inhibited, and the generation or expression of an HBV antigen may be inhibited.

The composition of the present application may inhibit simultaneous proliferation of two or more HBV genotypes selected from HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F

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and HBV genotype G. In addition, the generation or expression of one or more HBV antigens selected from HBsAg, HBeAg and HBcAg may be inhibited.

Another aspect of the present application relates to a method for inhibiting HBV proliferation using the composition.

The method for inhibiting HBV proliferation of the present invention may be performed by introducing or administering the composition of the present application to a subject, and here, the composition may include:

a) a guide nucleic acid or a nucleic acid sequence encoding theguide nucleic acid; and

b) a Cas protein or a nucleic acid sequence encoding theCas protein.

Here, the guide nucleic acid may include any one or more selected from SEQ ID NOs. 51, 52, 56, 57, 69, 70, 73, 74, 75, 76, 80, 85, 87 and 89. Here, an indel is formed in a nucleic acid sequence in the HBV gene by the composition. According to this method, the proliferation of two or more HBV genotypes selected from HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F and HBV genotype G may be inhibited at the same time.

The method for the present application may inhibit proliferation of several types of HBVs of the various HBV genotypes with the same composition of the present application.

Meanwhile, the present application relates to a method for inactivating a conserved sequence in an HBV gene.

The method is characterized by targeting a specific conserved sequence regardless of an HBV genotype. The conserved sequence is a sequence conserved between sequences of the HBV genotype to inactivate, for example, one or more selected from the conserved sequences of SEQ ID NOs. 1 to 45, and preferably, SEQ ID NOs. 6, 7, 11, 12, 24, 25, 28, 29, 30, 31, 35, 40, 42 and 44. Here, the inactivation includes cleavage of the conserved sequence.

This method may inactivate one or more types of viruses selected from HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F and HBV genotype G regardless of a genotype.

In one embodiment of the present application, to perform this method,

a) a guide nucleic acid or a nucleic acid sequence encoding the guide nucleic acid; and

b) a Cas protein or a nucleic acid sequence encoding the cas protein may be introduced or administered to a subject. In one embodiment, the guide nucleic acid may include one or more selected from SEQ ID NOs. 51, 52, 56, 57, 69, 70, 73, 74, 75, 76, 80, 85, 87 and 89. In one embodiment, the guide nucleic acid may use a combination of SEQ ID NOs. 51, 57 and 73 or a combination of SEQ ID NOs. 51, 73 and 76.

The method of the present application inactivates a conserved sequence in the HBV gene. Here, since this method uses a conserved sequence in which mutations between sequences of various HBV genotypes do not occur easily, HBV proliferation may be inhibited by applying the method of the present application without limitation to various HBV genotypes.

The method of the present invention has an effect of treating hepatitis B.

The method of the present invention may be performed by introducing or administering the composition of the present application to a subject in need of administration thereof.

The subject may be an HBV-infected mammal, for example, a human, a monkey, a mouse or a rat, but the present invention is not limited thereto.

Here, the method of the present application may be performed when the subject is infected by any of HBV genotype A, HBV genotype B, HBV genotype C, HBV genotype D, HBV genotype E, HBV genotype F and HBV genotype G. Although unusual, the subject may be co-infected by two or more thereof.

That is, since the present application has an effect of simultaneously inhibiting various HBV genotypes, when the composition and method of the present application are used, HBV proliferation may be effectively inhibited and hepatitis B may be treated regardless of a specific type of the infected HBV.

The administration may be performed by any convenient method such as injection, transfection, implantation or transplantation. The administration route may be selected from subretinal, subcutaneous, intradermal, intraocular, intravitreal, intratumoral, intranodal, intramedullary, intramuscular, intravenous, intralymphatic, and intraperitoneal administrations.

During administration, a dose (a pharmaceutically effective amount to obtain a desired effect) of the composition may be selected from all integers within the numerical ranges including approximately 10^4 to 10^9 cells, e.g., 10^5 to 10^6 cells/kg (body weight of a subject), but the present invention is not limited thereto. The dose

of the present application may be suitably prescribed by considering the age, health and body weight of a subject, the type of concurrent treatment, if any, treatment frequency, or the characteristics of a desired effect.

[Examples]

Hereinafter, the present invention will be described in further detail with reference to examples.

The examples are merely provided to more specifically explain the present invention, and it will be obvious to those of ordinary skill in the art that the scope of the present invention is not limited to the examples according to the gist of the present invention.

The diagram for designing a composition for targeting various HBV genotypes according to the present application is shown in FIG. 1. FIG. 1 is a schematic diagram for designing CRISPR gRNAs which are able to target HBV genotypes B, C and D.

As shown in FIG. 1, a genetic scissors which is able to multi-target all of genomes of Korean patient-derived HBV genotype C, open database-derived HBV genotypes B, C and D, and an HBV cell model.

The schematic diagram of FIG. 1 will be described in detail below.

Example 1. Genome query & alignment for HBV genotypes B, C and D

For the genome query for HBV types B, C and D, 1638, 2136 and 923 full sequences of HBV types B, C and D, respectively, were collected from an HBV

genome data base (HBVdb, https://hbvdb.lyon.inserm.fr/HBVdb/HBVdbIndex). A virtual HBV genome (hereinafter, vHBV) sequence was made through sequence alignment of the collected sequences.

The virtual HBV genome sequence type B, C or D was manufactured based on a region with 80% sequence homology among aligned sequences of each of the full sequences of HBV type B, C or D (FIGS. 2 to 4).

SEQ ID NO. 91 corresponds to the sequence of FIG. 2, SEQ ID NO. 92 corresponds to the sequence of FIG. 3, and SEQ ID NO. 93 corresponds to the sequence of FIG. 4. In the sequences of SEQ ID NOs. 91 to 93, the region indicated by capital letters has 80% or more homology with a sequence analyzed in the present application, and the region indicated by small letters has 80% or less homology.

[Table 3]

virtual HBV B genotype sequence (FIG. 2) SEQ ID NO. 91
CTCCACCACTTTCCACCAAACTCTTCAAGATCCCAGAGTCAGGGCcCTGTACT
TTCCTGCTGGTGGCTCCAGTTCAGGAACAGTGAGCCCTGCTCAGAATACTGtCTCTGCC
ATATCGTCAATCTTATCGAAGACTGGGGACCCTGTACCGAACATGGAGAACATCGCAT
CAGGACTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAAAATC
CTCACAATACCACAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGAA
CACCCGTGTGTCTTGGCCAAAATTCGCAGTCCCAAATCTCCAGTCACTCACCAACcTGT
TGTCCTCCAATTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTC
TGCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTG
CCCGTTTGTCCTCTAATTCCAGGATCATCAACaACCAGCACCGGACCATGCAAAACCTG
CACaACTCCTGCTCAAGGAACCTCTATGTTTCCCTCATGTTGCTGTACAAAACCTACGG
ACGGAAACTGCACCTGTATTCCCATCCCATCATCTTGGGCTTTCGCAAAATACCTATGG
GAGTGGGCCTCAGTCCGTTTCTCTTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTT
CGTAGGGCTTTCCCCCACTGTCTGGCTTTCAGTTATATGGATGATGTGGTtTTGGGGGCC

AAGTCTGTACAACATCTTGAGTCCCTTTATGCCGCTGTTACCAATTTTCTTTTGTCTTTG

GGTATACATTTAAACCCTCAcAAAACAAAAAGATGGGGATATTCCCTTAACTTCATGG

GATATGTAATTGGGAGTTGGGGCACaTTGCCaCAGGAACATATTGTACAAAAAATCAA

AaTgTGTTTTAGGAAACTTCCTGTAAACAGGCCTATTGATTGGAAAGTATGTCAACGAA

TTGTGGGTCTTTTGGGGTTTGCcGCCCCTTTcACgCAATGTGGATATCCTGCTTTAATGC

CTTTATATGCATGTATACAAGCaAAACAGGCTTTTACTTTCTCGCCAACTTACAAGGCC

TTTCTAAGTAAACAGTATCTGAACCTTTACCCCGTTGCTCGGCAACGGCCTGGTCTGTG

CCAAGTGTTTGCTGACGCAACCCCCACTGGTTGGGGCTTGGCCATAGGCCATCAGCGC

ATGCGTGGAACCTTTGTGTCTCCTCTGCCGATCCATACTGCGGAACTCCTAGCCGCTTG

TTTTGCTCGCAGCaGGTCTGGgGCaAAACTcATCGGGACTGACAATTCTGTCGTgCTcTCC

CGCAAGTATACATCaTTTCCATGGCTGCTAGGCTGTGCTGCCAACTGGATCCTGCGCGG

GACGTCCTTTGTTTACGTCCCGTCGGCGCTGAATCCCGCGGACGACCCCTCCCGGGGCC

GCTTGGGGCTCTACCGCCCGCTTCTCCGcCTGttGTACCGACCGACCACGGGGCGCACCT

CTCTTTACGCGGACTCCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTT

CACCTCTGCACGTCGCATGGAGACCACCGTGAACGCCCACcGGAACCTGCCCAAGGTC

TTGCATAAGAGGACTCTTGGACTTTCAGCAATGTCAACGACCGACCTTGAGGCATACT

TCAAAGACTGTGTGTTTAcTGAGTGGGAGGAGTTGGGGGAGGAGaTTAGGTTAAAGGT

CTTTGTACTAGGAGGCTGTAGGCATAAATTGGTgTGTTCACCAGCACCATGCAACTTTT

TCACCTCTGCCTAATCATCTCaTGTTCATGTCCTACTGTTCAAGCCTCCAAGCTGTGCCT

TGGGTGGCTTTgGGGCATGGACATTGACCCGTATAAAGAATTTGGAGCTTCTGTGGAGT

TACTCTCTTTTTTGCCTTCTGACTTCTTTCCTTCTATTCGAGATCTcCTCGACACCGCCTC

TGCTCTGTATCGGGAGGCCTTAGAGTCTCCGGAACATTGTTCACCTCACCATACGGCAC

TCAGGCAAGCTATTcTGTGTTGGGGTGAGTTgATGAATCTAGCCACCTGGGTGGGAAGT

AATTTGGAAGATCCAGCATCCAGGGAATTAGTAGTCAGCTATGTCAAcGTTAATATGG

GCCTAAAAATCAGACAACTATTGTGGTTTCACATTTCCTGTCTTACTTTTGGgAGAGAA

ACTGTTCTTGAATATTTGGTGTCTTTTGGAGTGTGGATTCGCACTCCTCCTGCATATAG

ACCACCAAATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGACGAaGAG

GCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAGGTCTCAATCGCCGCG

TCGCAGAAGATCTCAATCTCGGGAATCTCAATGTTAGTATTCCTTGGACaCATAAGGTG

GGAAACTTTACGGGGCTTTATTCTTCTACGGTACCTtGCTTTAATCCTaAaTGGCAAACT

CCTTCTTTTCCTGACATTCATTTGCAGGAGGACATTGTTGATAGaTGTAAGCAATTTGTG

GGgCCCCTtACAGTAAATGAAAACAGGAGACTAAAATTAATTATGCCTGCTAGGTTTTA

TCCCAATGTTACTAAATATTTGCCCTTAGATAAAGGgATCAAACCgTATTATCCAGAGc

ATGTAGTTAATCATTACTTCCAGACGaGACATTATTTACAcACTCTTTGGAAGGCGGGtA

TCTTATATAAAAGAGAGtCcACACGTAGCGCCTCATTTTGCGGGTCACCATATTCTTGG

GAACAAGAtCTACAGCATGGGAGGTTGGTCTTCCAAACCTCGAAAAGGCATGGGGACA

AATCTTTCTGTCCCCAATCCcCTGGGATTCTTCCCCGATCATCAGTTGGACCCTGCATTC

AAAGCCAACTCAGAAAATCCAGATTGGGACCTCAACCCgCACAAGGACAACTGGCCG

GACGCCAACAAGGTGGGAGTGGGAGCATTCGGGCCAGGGTTCACcCCTCCCCATGGGG

GACTGTTGGGGTGGAGCCCTCAGGCTCAGGGCaTACTCACAACTGTGCCAGCAGCTCC

TCCTCCTGCCTCCACCAATCGGCAGTCAGGAAGGCAGCCTACTCCCtTATCTCCACCTC

TAAGGGACACTCATCCTCAGGCCATGCAGTGGAA

virtual HBV C genotype sequence (FIG. 3) SEQ ID NO. 92

CTCCAcaACATTCCACCAAGCTCTGCTAGAtCCCAGAGTGAGGGGCCTATAcTTTCCTGC

TGGTGGCTCCAGTTCCGGAACAGTAAACCCTGTTCCGACTACTGCCTCaCCCATATCGT

CAATCTTCTCGAGGACTGGGGACCCTGCACCGAACATGGAGAaCACaACATCAGGATT

CCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAGAATCCTCACAA

TACCACAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGAGCACCCAC

GTGTCCTGGCCAAAATTcGCAGTCCCCAACCTCCAATCACTCACCAACCTCTTGTCCTC

CAATTTGTCCTGGcTATCGCTGGATGTGTCTGCGGCGTTTTATCATaTTCCTCTTCATCCT

GCTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTACCAAGGTATGTTGCCCGTTTG

TCCTCTACTTCCAGGAACATCAACTACCAGCACGGGACCATGCAAGACCTGCACGAtTC

CTGCTCAAGGAACCTCTATGTTTCCCTCTTGTTGCTGTACAAAACCTTCGGACGGAAAc

TGCACTTGTATTCCCATCCCATCATCcTGGGCTTTCGCAAGATTCCTATGGGAGTGGGC

CTCAGTCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGCT

TTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGTCTGT

ACAACATCTTGAgTCCCTTTTTACCTCTATTACCAATTTTCTTTTGTCTTTGGGTATACAT

TTgAACCCTAATAAAACCAAACGTTGGGGCTACTCCCTTAACTTCATGGGATATGTAAT

TGGAAGTTGGGGTACtTTaCCaCAgGAACATATTGTACtAAAAaTCAAgCAATGTTTTCGa

AAACTgCCTgTAAATAGACCTATTGATTGGAAAGTATGTCAaAGAATTGTGGGTCTTtTG

GGCTTTGCTGCCCCTTTTACACAATGTGGcTAtCCtGCcTTaATGCCTTTaTATGCATGTAT

ACAAtCTAAGCAGGCTTTCACTTTCTCGCCAACTTACAAGGCCTTTCTGTGTAAACAAT

ATCTGAACCTTTACCCCGTTGCcCGGCAACGGTCAGGTCTcTGCCAAGTGTTTGCTGAC

GCAACCCCCACTGGaTGGGGCTTGGCcATaGGCCATCggCGCATGCGTGGAACCTTTGTG

GCTCCTCTGCCGATCCATACTGCGGAACTCCTAGCAGCTTGTTTTGCTCGCAGCCGGTC

TGGAGCgAAACTTATCGGaACcGACAACTCTGTTGTCCTCTCTCGGAAATACACCTCCTT

TCCATGGCTGCTaGGgTGTGCTGCCAACTGGATCCTGCGCGGGACGTCCTTTGTCTACG

TCCCGTCGGCGCTGAATCCCGCGGACGACCCGTCTCGGGGCCGTTTGGGaCTCTACCGT

CCCCTTCTTCaTCTGCCGTTCCGGCCGACCACGGGGCGCACCTCTCTTTACGCGGTCTCC

CCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTGCACGTCGC

ATGGAgACCACCGTGAACGCCCaCCAGGTCTTGCCCAAGGTCTTACATAAGAGGACTCT

TGGACTCTCAGCAATGTCAACGACCGACCTTGAGGCATACTTCAAAGACTGTtTgTTTA

AaGACTGGGAGGAGTTGGGGGAGGAGATTAGGTTAAaGaTCTTTGTACTaGGAGGCTGT

AGGCATAAATTGGTCTGTTCACCAGCACCATGCAACTTTTTCACCTCTGCCTAATCATC

TCATGTTCATGTCCTACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTgGGGCAT

GGACATTGACCCGTATAAAGAATTTGGAGCTTCTGTGGAGTTACTCTCTTTTTTGCCTT

CTGACTTCTTTCCTTCTATTCGAGATCTCCTCGACACCGCCTCtGCTCTGTATCGGGAGG

CCTTAGAGTCTCCGGAACATTGTTCACCTCACCATACAGCACTcAGGCAAGCTATTCTG

TGTTGGGGTGAGTTGATGAATCTGGCCACCTGGGTGGGAAGTAATTTGGAAGACCCAG

CATCCAGGGAATTAGTAGTcAGCTATGTCAATGTTAATATGGGCCTAAAAATCAGACA

ACTAtTGTGGTTTCACATTTCCTGTCTTACTTTTGGAAGAGAAACTGTTCTTGAGTATTT

GGTGTCTTTTGGAGTGTGGATTCGCACTCCTcCcGCTTACAGACCACCAAATGCCCCTAT

CTTATCAACACTTCCGGAAACTACTGTTGTTAGACGACGAGGCAGGTCCCCTAGAAGA

AGAACTCCCTCGCCTCGCAGACGAAGGTCTCAATCGCCGCGTCGCAGAAGATCTCAAT

CTCGGGAATCTCAATGTTAGTATCCCTTGGACTCATAAGGTGGGAAACTTTACTGGGCT

TTATTCTTCTACTGTACCTGTCTTTAATCCTGAgTGGCAAACTCCCTCcTTTCCTcACATT

CATTTacAgGAGGAcATTATtAATAGATGTCAACAATATGTGGGCCCTCTTACAGTTAAT

GAAAAAAGGAGATTAAAATTAATTATGCCTGCTAGGTTCTATCCTAACCTTACCAAAT

ATTTGCCCTTgGAcAAAGGCATTAAACCtTATTATCCTGAACATgCAGTTAATCATTACT

TCaAAACTAGGCATTATTTACATACTCTGTGGAAGGCtGGcATTcTATATAAGAGAGAAA

CTACACGCAGCGCCTCATTTTGTGGGTCACCATATTCTTGGGAACAAGAGCTACAGCA

TGGGAGGTTGGTCTTCCAAACCTCGACAAGGCATGGGGACGAATCTTTCTGTTCCCAA

TCCTCTGGGATTCTTTCCCGATCACCAGTTGGACCCtGCGTTCGGAGCCAAcTCAAACA

ATCCAGATTGGGACTTCAACCCCAACAAGGATCAcTGGCCAGaGGCAAAtCAGGTAGGA

GcGGGAGCATTCGGGCCAGGGTTCACcCCACCACACGGCgGTCTTTTGGGGTGGAGCCC

TCAGGCTCAGGGCATATTGACAACAGTGCCAGcAGCaCCTCCTCCTGCCTCCACCAATC

GGCAGTCAGGAAGACAGCCTACTCCCATCTCTCCACCTCTAAGAGACAGTCATCCTCA

GGCCATGCAGTGGAA

virtual HBV D genotype sequence (FIG. 4) SEQ ID NO. 93

CTCCACAACCTTCCACCAAACTCTGCAAGATCCCAGaGTGAGAGGCCTGTATTTCCCTG

CTGGTGGCTCCAGTTCAGGAACAGTAAACCCTGTTCCGACTACTGtCTCTCcCATATCGT

CAATCTTCTCGAGGATTGGGGACCCTGCGCTGAACATGGAGAACATCACATCAGGATT

CCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAGAATCCTCACAA

TACCGCAGAGTCTAGACTCGTGGTGGACTTCTCTCAATTTTCTAGGGGGAACtACCGTG

TGTCTTGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCCTGTCCTCC

AACTTGTCCTGGTTATCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCT

GCTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTATCAAGGTATGTTGCCCGTTTG

TCCTCTAATTCCAGGATCtTCAACCACCAGCaCGGGACCATGCAGAACCTGCACGACTc

CTGCTCAAGGAACCTCTATGTATCCCTCCTGTTGCTGTACCAAACCTTCGGACGGAAAT

TGCACCTGTATTCCCATCCCATCATCCTGGGCTTTCGGAAAATTCCTATGGGAGTGGGC

CTCAGCCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTTCGTAGGGC

TTTCCCCCACTGTTTGGCTTTCAGTTATATGGATGATGTGGTATTGGGGGCCAAGTCTG

TACAGCATCTTGAGTCCCTTTTTACCGCTGTTACCAATTTTCTTTTGTCTTTGGGTATAC

ATTTAAACCCTAACAAAACaAAAAGATGGGGTTACTCTtTAcATTTcATGGGcTATGTCA

TTGGATGTTATGGGTCaTTGCCACAAGATCACATCATACAgAAAATCAAAGAATGTTTT

AGAAAACTTCCTGTTAACAGGCCTATTGATTGGAAAGTcTGTCAACGtATTGTGGGTCT

TTTGGGTTTTGCTGCCCCTTTTACACAATGTGGTTATCCTGCTTTAATGCCcTTGTATGCa

TGTATTCAATCTAAGCAGGCTTTCACTTTCTCGCCAACTTACAAGGCCTTTCTGTGTAA

ACAATACCTGAACCTTTACCCCGTTGCCCGGCAACGGCCAGGTCTGTGCCAAGTGTTT

GCTGACGCAACCCCCACTGGCTGGGGCTTGGTCATGGGCCATCAGCGCATGCGTGGAA

CCTTTCtGGCTCCTCTGCCGATCCATACTGCGGAACTCCTAGCCGCTTGTTTTGCTCGCA

GCAGGTCTGGAGCAAACATTcTCGGGACgGATAACTCTGTTGTTCTCTCCCGCAAATAT

ACATCGTTTCCATGGCTGCTAGGCTGTGCTGCCAACTGGATCCTGCGCGGGACGTCCTT

TGTTTACGTCCCGTCGGCGCTGAATCCCGCGGACGACCCTTCTCGGGGcCGCTTGGGAC

TCTCTCGTCCCCTTCTCCGTCTGCCGTTtCGaCCGACCACGGGGCGCACCTCTCTTTACG

CGGACTCCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCTCTG

CACGTCGCATGGAGACCACCGTGAACGCCCACCAatTCTTGCCCAAGGTCTTACATAAG

AGGACTCTTGGACTCTCTGtAATGTCAACGACCGACCTTGAGGCATACTTCAAAGACTG

TTTGTTTAAaGACTGGGAGGAGTTGGGGGAGGAGATTAGaTTAAaGgTCTTTGTAcTAGG

AGGCTGTAGGCATAAATTGGTCTGCGCACCAGCACCATGCAACTTTTTCACCTCTGCCT

AATCATCTCTTGTTCATGTCCTACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTg

GGgCATGGACATTGAcCCTTATAAAGAATTTGGAGCTaCTGTGGAGTTACTCTCGTTTTT

GCCTTCTGACTTCTTTCCTTCAGTACGAGATCTTCTAGATACCGCCTCAGCTCTgTATCG

GGAAGCCTTAGAGTCTCCTGAGCATTGTTCACCTCACCATACTGCACTCAGGCAAGCA

ATTCTtTGCTGGGGGGAaCTAATGACTCTAGCtACCTGGGTGGGTGgTAATTTGGAAGAT

CCAgcATCcAGGGACCTAGTAGTCAGTTATGTcAACACTAATATGGGCCTAAAGTTCAG

GCAACTaTTGTGGTTTCACATTTCTTGTCTCACTTTTGGAAGAGAAACgGTcaTAGAGTA

TTTGGTGTCTTTCGGAGTGTGGATTCGCACTCCTCCAGCTTATAGACCACCaAATGCCC

CTATCTTATCAACACTTCCGGAGACTACTGTTGTTAGACGACGAGGCAGGTCCCCTAG

AAGAAGAACTCCCTCGCCTCGCAGACGAAGGTCTCAATCGCCGCGTCGCAGAAGATCT

CAATCTCGGGAATCTCAATGTTAGTATTCCTTGGACTCATAAGGTGGGaAACTTTACGG

GGCTTTATTCTTCTACTGTaCCTGTCTTTAACCCTCATTGGAAAACACCcTCTTTTCCTAA

TATACATTTACACCAAGACATTATCAAAAAATGTGAACAaTTTGTAGGCCCACTCACAG

TcAATGAGAAAaGAAGAcTGCAATTgATTATGCCTGCTAGGTTTTATCCAAAtGTTACCA

AATATTTGCCATTgGATAAGGGTATTAAACCTTATTATCCAGAACATCTAGTTAATCAT

TACTTCCAAACcAGACAtTATTTACACACTCTATGGAAGGCGGGTATATTATATAAGAG

AGAAACAACACaTAGCGCCTCATTTTGTGGGTCACCATATTCTTGGGAACAAGAGCTA

CAGCATGGGGCAGAATCTTTCCACCAGCAATCCTCTGGGATTCTTTCCCGACCACCAGT

TGGATCCAGCCTTCAGAGCAAACACCGCAAATCCAGATTGGGACTTCAATCCCAACAA

GGACACCTGGCCAGACGCCAACAAGGTAGGAGCTGGAGCATTCGGGCTGGGaTTCACC

CCACCGCACGGAGGCCTTTTGGGGTGGAGCCCTCAGGCTCAGGGCATAcTACAAACcTT

GCCAGCAAATCCGCCTCCTGCcTCtACCAATCGCCAGTCAGGAAGGCAGCCTACCCCgC

TGTCTCCACCTTTGAGAAACACTCATCCTCAGGCCATGCAGTGGAA

Example 2. Guide RNA (gRNA) design

CRISPR target gRNAs were designed based on virtual universal HBV genotypes B, C and D obtained in Example 1, respectively.

gRNAs for SpCas9 (from *Streptococcus pyogenes* (5'-NGG-3' for target, 5'-NRG-3' for off-target)) and CjCas9 (from *Campylobacter jejuni*: 5'-NNNNRYAC-3') were designed with Cas-Designer(http://www.rgenome.net/cas-designer/), and gRNAs having off-target sequences with 1 and 2 mismatches were excluded. The gRNAs conserved in vHBV genotypes B, C and D are obtained as shown in FIGS. 5 and 6.

As a result of designing, targets (150 gRNAs for SpCas9 and 80 gRNAs for CjCas9) for the virtual universal HBV genotype B, targets (168 gRNAs for SpCas9

and 66 gRNAs for CjCas9) for HBV genotype C and targets (158 gRNAs for SpCas9, 71 gRNAs for CjCas9) for HBV genotype D were obtained. In addition, 34 gRNAs for SpCas9, which were able to target all of the virtual universal HBV genotypes B, C and D, were obtained, and 11 gRNAs for CjCas9, which were able to target all of the virtual universal HBV genotypes B, C and D, were obtained (FIGS. 5 and 6).

The gRNAs of SEQ ID NOs. 46 to 90 in Table 2 are gRNAs capable of targeting at least one of the HBV genotypes B, C and D, and among these, SEQ ID NOs. 46 to 79 are gRNAs for SpCas9, and SEQ ID NOs. 80 to 90 are gRNAs for CjCas9.

Example 3. Selection of gRNAs targeting all of HBV genotypes B, C and D

As gRNAs targeting a common conserved region in a sequence targeting open database-based conserved, Korean patient-derived HBV genotype C and a sequence of an available HBV cell model, 10 gRNAs for SpCas9 and 4 gRNAs for CjCas9 were designed (FIGS. 7 and 8).

gRNAs of SpCas9 capable of targeting all of four viruses, which are patient-derived HBV type C, and two cell lines (type D) generally used in an HBV experiment are SEQ ID NOs. 51, 52, 56, 57, 69, 70, 73, 74, 75 and 76 in Table 2, and gRNAs of CjCas9 capable of targeting all of four viruses, which are patient-derived HBV type C, and two cell lines (type D) generally used in an HBV experiment are SEQ ID NOs. 80, 85, 87 and 89 in Table 2.

Example 4. Screening of HBV target region

As a result of screening an HBV target region targeting gRNAs targeting the common conserved region in the open database-based conserved sequence, Korean patient-derived HBV genotype C and an HBV cell model, which were obtained in Example 3, it was confirmed that one or more of HBV P, X, PreS1, PreS2, C, S and PreC of FIG. 9 are targeted.

The HBV target region targeted by each of the above gRNAs is shown in Table 4.

[Table 4]

NO.	Name	Note.	cccDNA target region
SEQ ID NO. 51	Sp20-viHBV-B-#17G	SpCas9 target	P, PreS1, PreS2, S
SEQ ID NO. 52	Sp20-viHBV-B-#20G		P, PreS1, PreS2, S
SEQ ID NO. 56	Sp20-viHBV-B-#89G		P
SEQ ID NO. 57	Sp20-viHBV-B-#90G		P
SEQ ID NO. 69	Sp20-viHBV-B-#154G		X
SEQ ID NO. 70	Sp20-viHBV-B-#159G		X
SEQ ID NO. 73	Sp20-viHBV-B-#193G		P, SP, PreC, C
SEQ ID NO. 74	Sp20-viHBV-B-#194G		P, SP, PreC, C
SEQ ID NO. 75	Sp20-viHBV-B-#196G		P, SP, PreC, C
SEQ ID NO. 76	Sp20-viHBV-B-#197G		P, SP, PreC, C
SEQ ID NO. 80	Cj22-viHBV-B-#06G	CjCas9 target	P, PreS1, PreS2, S
SEQ ID NO. 85	Cj22-viHBV-B-#45G		P, X
SEQ ID NO. 87	Cj22-viHBV-B-#47G		P, X
SEQ ID NO. 89	Cj22-viHBV-B-#57G		PreC

Example 5. Cell culture and transfection

i) Vectors

Vector construction for introducing the gRNAs obtained in Example 3 into cells was prepared (FIGS. 27 to 29).

200 pmol each of sense and antisense oligonucleotides of gRNA was mixed with a T4 ligase buffer, and subjected to oligo annealing under conditions including heating at 95 °C for 2 minutes, 5 cycles of cooling from 95 to 85 °C at -2 °C/s, and 60 cycles of cooling from 85 to 25 °C at -1 °C/s, followed by insertion behind the U6 promoter of a plasmid using a restriction enzyme.

In FIG. 27, one SpgRNA, Cas9 and a fluorescent protein were designed to be expressed in one plasmid.

In FIG. 28, one CjgRNA, Cas9 and a fluorescent protein were designed to be expressed in one plasmid.

In FIG. 29, three SpgRNAs, Cas9 and a fluorescent protein were designed to be expressed in one plasmid.

ii) Materials

The HBV 1.2 plasmid carrying 1.2-copies of the HBV genome (genotype D, Addgene #51294); pAAV HBV genotypes A, B and C (kindly provided by Pei-Jer Chen, Taipei City, Taiwan); Cj CRISPR Cas9 gRNA plasmids (Cj#06, Cj#45, Cj#47, Cj#57) (Toolgen); and Sp CRISPR Cas9 gRNA plasmids (Sp#17, Sp#20, Sp#89, Sp#90, Sp#154, Sp#159, Sp#193, Sp#194, Sp#196, Sp#197, N#17, N#90, N#193, N#197, N#17-90-193, N#17-90-197, N#17-193-197, N#90-193-197) (Toolgen).

Human liver cancer cell lines Huh7 and HepG2 were obtained from the Korean Cell Line Bank (Seoul, Korea). For cell culture and maintenance, 10% fetal bovine

serum (Capricorn, Ebsdorfergrund, Germany) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA) were added to Dulbecco's modified Eagle's medium (Welgene, Gyeongsan-si, Korea). The cells were maintained in an incubator at 37 °C with 5% CO_2.

4×10^5 of the Huh7 or HepG2 cells were seeded in each well of a 6-well plate, and 1 μg of a replicon and 1 μg of a plasmid expressing CRISPR Cas9 (SpCas9 or CjCas9) and gRNA were transfected using Lipofectamine 2000. After 24 hours, the cell medium was replaced with a fresh medium. After three days, a cell supernatant was obtained for ELISA.

Example 6. Measurement of HBsAg and HBeAg

To measure HBsAg and HBeAg levels in the cell supernatant obtained in Example 5, as commercial ELISA kits, HBeAg ELISA Kit (WB-2496, Wantai Pharm Inc., Beijing, China) and HBsAg ELISA Kit (WB-2296, Wantai Pharm Inc., Beijing, China) were used, and the measurement was performed according to the protocol of the kit. The cell supernatant was used by diluting HBeAg to 1/10 and HBsAg to 1/25 with PBS. The absorbance of HBeAg and HBsAg was measured in a wavelength range of 450 nm using the SPECTRA max PLUS 384 Microplate Reader and the SoftMax Pro 5.2 program.

Result 1) Screening of SpCas9 gRNA candidate and CjCas9 target gRNA candidate

Each of the SpCas9 gRNA candidates and CjCas9 gRNA candidates obtained in Example 3 was used to measure HBeAg and HBsAg levels using HBV D genotype HBV 1.2.

As a result of measuring HBeAg and HBsAg levels of each of the CjCas9 gRNA candidates such as Cj#06, Cj#45, Cj#47 and Cj#57, it can be seen that Cj#47 had the lowest levels of HBeAg and HBsAg (FIGS. 10 to 13, and FIG. 18). That is, it was expected that Cj#47 is CjCas9 target gRNA having the greatest effect.

As a result of measuring HBeAg and HBsAg levels of each of the SpCas9 gRNA candidates such as Sp#17, Sp#20, Sp#89, Sp#90, Sp#154, Sp#159, Sp#193, Sp#194, Sp#196 and Sp#197, most cases had low HBeAg levels, but relatively high HBsAg levels.

When the HBeAg and HBsAg levels of the various gRNA candidates were measured, it was found that the degrees of HBeAg and HBsAg inhibition for each candidate were different (FIGS. 14 to 17, and FIG. 19).

That is, it can be confirmed that even gRNAs targeting the conserved sequence of hepatitis B virus had different HBeAg and HBsAg inhibitory capacities.

Result 2) Confirmation of HBeAg and HBsAg inhibitory capacity in HBV cell model (HBV type D) of SpCas9 gRNA candidates

In each of human liver cancer cell lines Huh7 (pAAV HBV D genotype-introduced cells) and HepG2 (pAAV HBV D genotype-introduced cells), HBeAg and HBsAg levels for Sp#17 (named N#17), Sp#90 (named N#90), Sp#193 (named N#193), Sp#197 (named N#197), Sp#17+Sp#90+Sp#193 combination (named N#17-90-193), Sp#17+Sp#90+Sp#197 combination (named N#17-90-197),

Sp#17+Sp#193+Sp#197 combination (named N#17-193-197) among the SpCas9 gRNA candidates were measured (FIGS. 20 to 22).

In all of the Huh7 and HepG2 cell lines, when a guide nucleic acid of the Sp#17+Sp#90+Sp#197 combination or the Sp#17+Sp#193+Sp#197 combination was used, rather than guide nucleic acids of Sp#17, Sp#90, Sp#193 and Sp#197 alone, it can be seen that the HBeAg and HBsAg inhibitory capacity was excellent.

Result 3) Confirmation of HBeAg and HBsAg inhibitory capacity of HBV genotype A, HBV genotype B, HBV genotype C and HBV genotype D of SpCas9 gRNA candidates

In HBV genotype A, HBV genotype B, HBV genotype C and HBV genotype D cell lines prepared by introducing pAAV HBV genotype A, B, C and D into the human liver cancer cell line Huh7, respectively (respectively referred to as genotype A, genotype B, genotype C and genotype D), HBeAg and HBsAg levels of SpCas9 gRNA candidates Sp#19 3(named N#193), Sp#197 (named N#197), Sp#17+90+197 (named N#17+90+197) and Sp#17+193+197 (named N#17+193+197) were measured.

It can be seen that each of the SpCas9 gRNA candidates had different HBeAg and HBsAg levels in each of the HBV genotype A, HBV genotype B, HBV genotype C and HBV genotype D cell lines.

As comparing all of the results of the genotypes A, B, C and D, it was confirmed that N#193 and N#197 (single gRNA) tended to decrease HBeAg, but did not decrease HBsAg, but N#17-90-193 and N#17-193-197 effectively decreased HBeAg and HBsAg.

That is, it can be seen that N#17-90-193 and N#17-193-197 effectively inhibited HBV genotypes A, B, C and D.

[Industrial Applicability]

The present application relates to a method for inhibiting the proliferation of various HBV genotypes.

[CLAIMS]

【Claim 1】

A method for inhibiting a Hepatitis B virus (HBV) proliferation, comprising

introducing into a subject a composition comprising:

a) a guide nucleic acid, or a nucleic acid sequence encoding the same; and

b) a Cas protein, or a nucleic acid sequence encoding the same;

wherein the hepatitis B virus is at least two or more HBV genotypes selected from HBV type A, HBV type B, HBV type C, HBV type D, HBV type E, HBV type F and HBV type G,

wherein the guide nucleic acid selected from SEQ ID NO.51, 52, 56, 57, 69, 70, 73, 74, 75, 76, 80, 85, 87 and 89,

thereby inducing an indel in the nucleic acid sequence in the gene of the hepatitis B infection.

【Claim 2】

A method for inactivating a conserved sequence in a Hepatitis B virus (HBV) gene, comprising

introducing into a subject a composition comprising:

a) a guide nucleic acid, or a nucleic acid sequence encoding the same; and

b) a Cas protein, or a nucleic acid sequence encoding the same;

the guide nucleic acid is a sequence having a complementarity or homology with the conserved sequence in part or in whole,

wherein the guide nucleic acid selected from SEQ ID NO.51, 52, 56, 57, 69, 70, 73, 74, 75, 76, 80, 85, 87 and 89,

wherein the hepatitis B virus is at least two or more HBV genotypes selected from HBV type A, HBV type B, HBV type C, HBV type D, HBV type E, HBV type F and HBV type G,

wherein the conserved sequence is one or more selected from SEQ ID NO.1 to 45 which is conserved in the HBV genotypes,

the inactivating is being cleaved the conserved sequence.

【Claim 3】

The method of claim 1 or claim 2,

wherein the Cas protein is one or more selected from Cas9 protein derived from Streptococcus pyogenes, Cas9 protein derived from Campylobacter jejuni.

【Claim 4】

The method of claim 1 or claim 2,

wherein the guide nucleic acid of the a) is one selected from SEQ ID NO.51, 52, 56, 57, 69, 70, 73, 74, 75 and 76,

the Cas9 of the b) is Cas9 protein derived from Streptococcus pyogenes.

【Claim 5】

The method of claim 1 or claim 2,

wherein the guide nucleic acid of the a) is one selected from SEQ ID NO.80, 85, 87 and 89,

the Cas9 of the b) is Cas9 protein derived from Campylobacter jejuni.

【Claim 6】

The method of claim 1 or claim 2,

wherein the guide nucleic acid and the Cas protein are introduced in the form of vector.

【Claim 7】

The method of claim 6,

wherein the vector is one or more vectors selected from the group consisting of retrovirus, lentivirus, adenovirus, adeno-associated virus (AAV), vaccinia virus, poxvirus and herpes simplex virus.

【Claim 8】

The method of claim 4,

wherein the guide nucleic acid is introduced with SEQ ID NO.51, 57 and 73.

【Claim 9】

The method of claim 4,

wherein the guide nucleic acid is introduced with SEQ ID NO.51, 73 and 76.

【Claim 10】

The method of claim 1 or claim 2,

wherein the method inhibits one or more selected from HBeAg and HBsAg which are antigens of the hepatitis B virus.

【Claim 11】

A composition for inhibiting a proliferation of a Hepatitis B virus targeting a conserved sequence in the hepatitis B virus (HBV) genome,

the composition comprises

a) a guide nucleic acid, or a nucleic acid sequence encoding the same; and

b) a Cas protein, or a nucleic acid sequence encoding the same;

wherein the guide nucleic acid selected from SEQ ID NO.51, 52, 56, 57, 69, 70, 73, 74, 75, 76, 80, 85, 87 and 89,

wherein the conserved sequence is one or more selected from SEQ ID NO.1 to 45.

【Claim 12】

The composition of claim 11,

wherein the Cas protein is one or more selected from Cas9 protein derived from Streptococcus pyogenes, Cas9 protein derived from Campylobacter jejuni.

【Claim 13】

The composition of claim 11,

wherein the composition is in the form of vector.

【Claim 14】

The composition of claim 11,

wherein the guide nucleic acid of the a) is one selected from SEQ ID NO.51, 52, 56, 57, 69, 70, 73, 74, 75 and 76,

the Cas9 of the b) is Cas9 protein derived from Streptococcus pyogenes.

【Claim 15】

The composition of claim 11,

wherein the guide nucleic acid of the a) is one selected from SEQ ID NO. 80, 85, 87 and 89,

the Cas9 of the b) is Cas9 protein derived from Campylobacter jejuni.

【Claim 16】

The composition of claim 11,

wherein the composition comprises a guide nucleic acid comprising SEQ ID NO.51, 57 and 73.

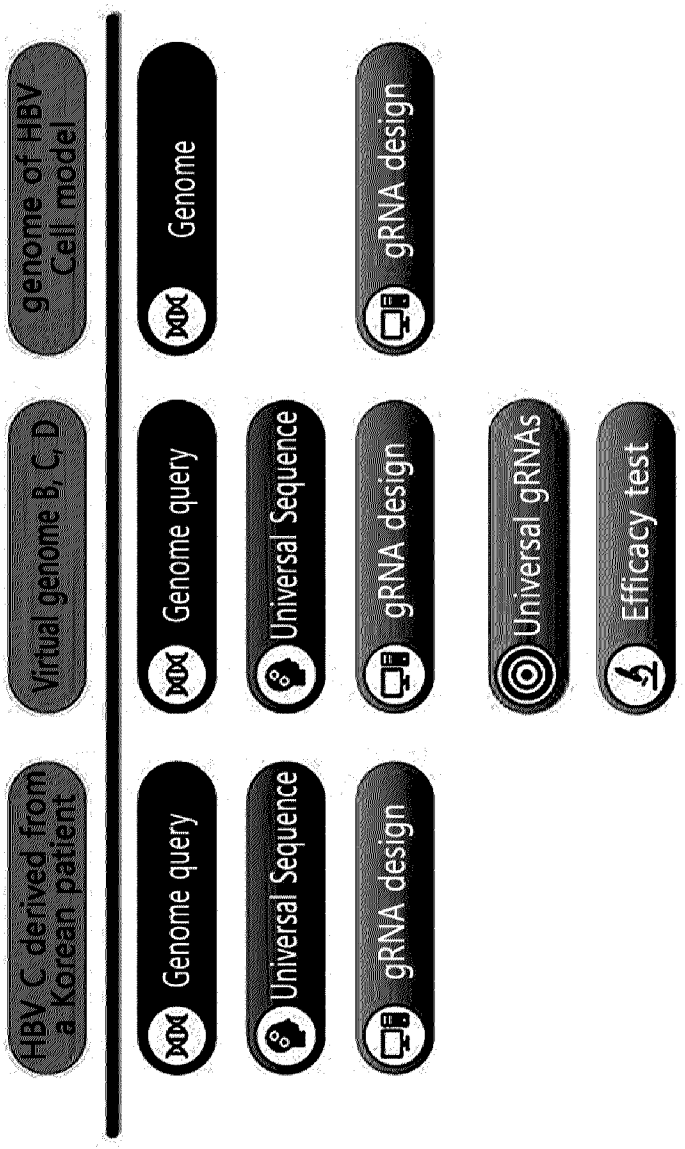
【Claim 17】

The composition of claim 11,

wherein the composition comprises a guide nucleic acid comprising SEQ ID NO.51, 73 and 76.

[DRAWINGS]

[Figure 1]



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[Figure 2]

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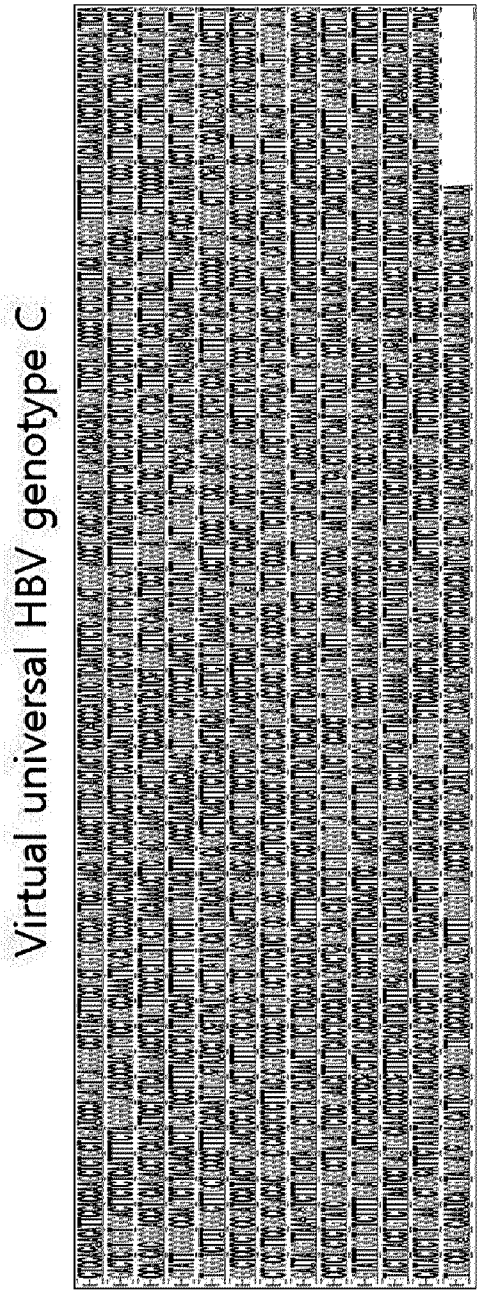
[Figure 3]



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[Figure 4]

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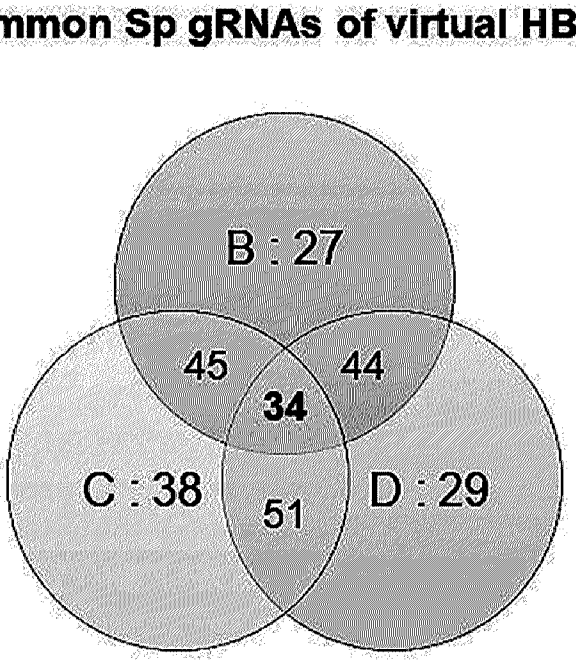
[Figure 5]



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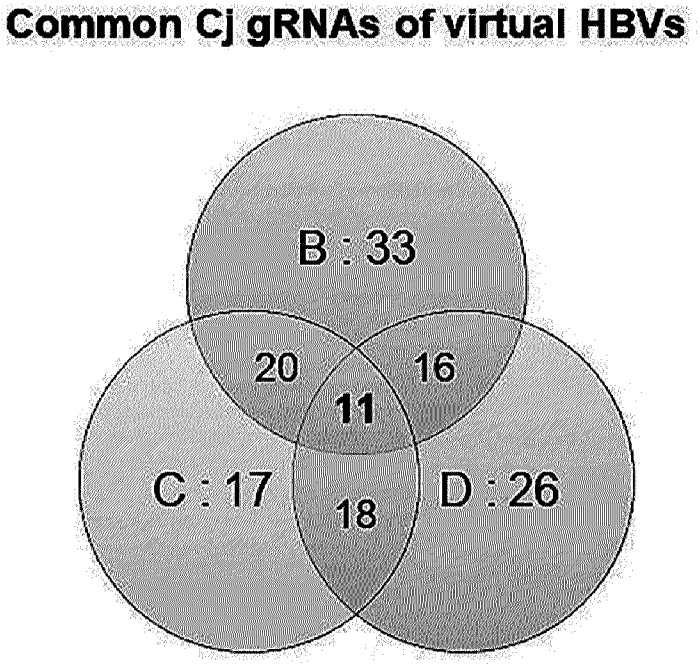
[Figure 6]



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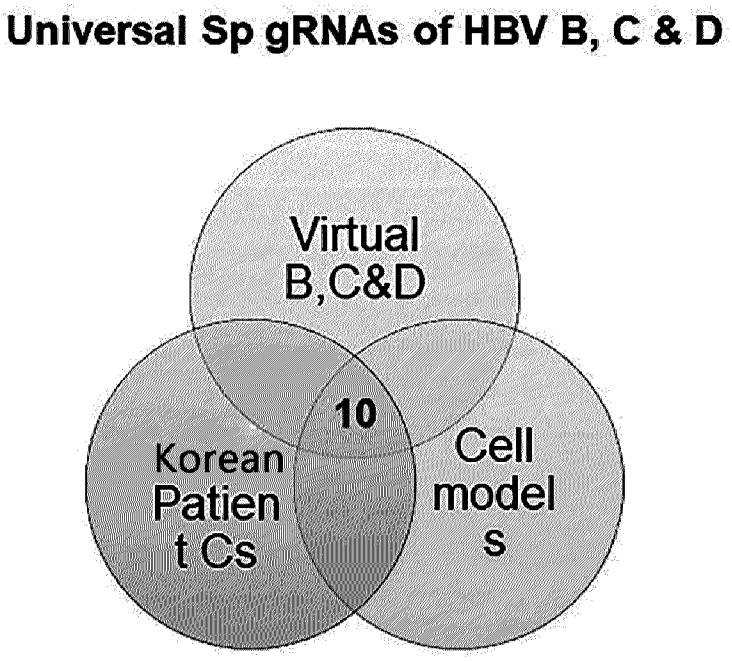
[Figure 7]



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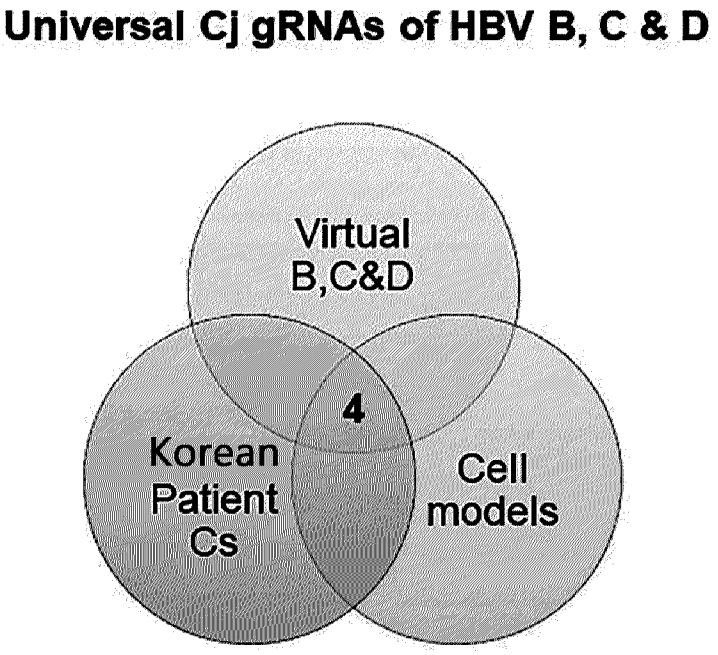
[Figure 8]



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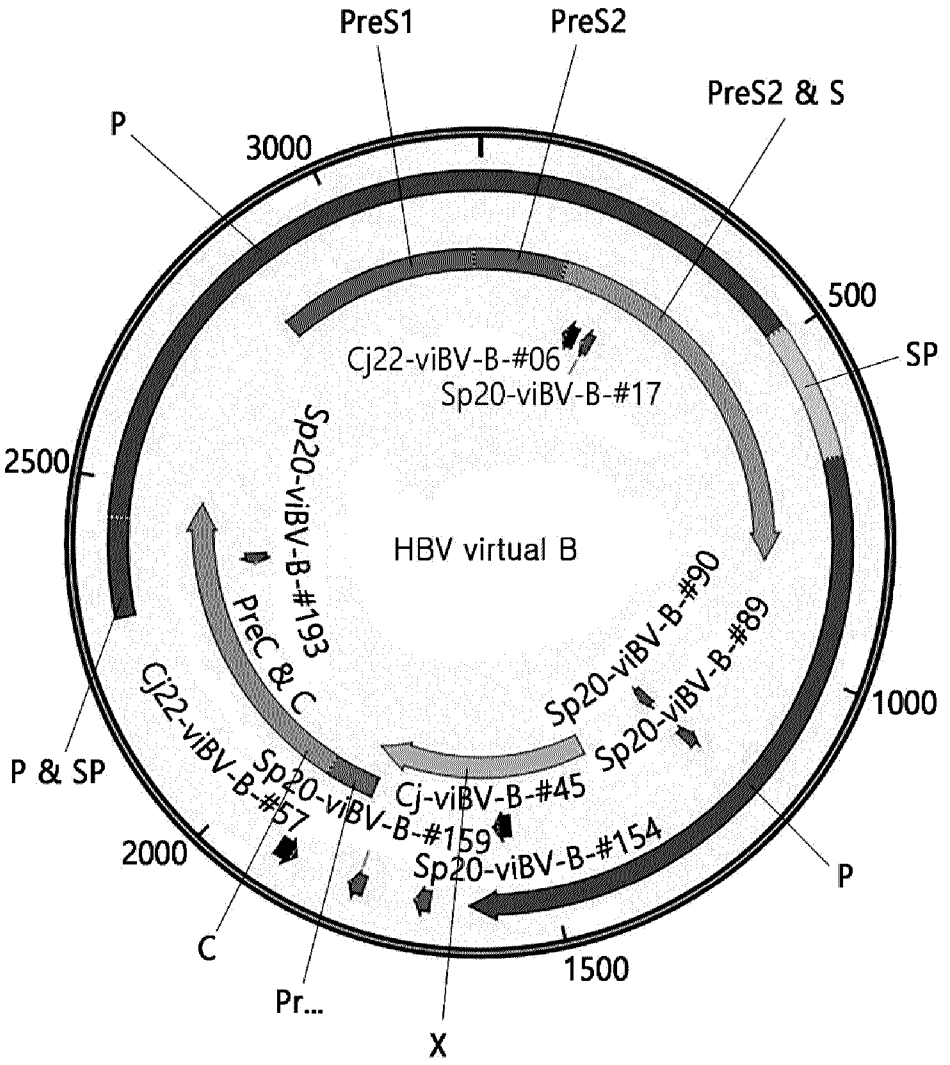
[Figure 9]



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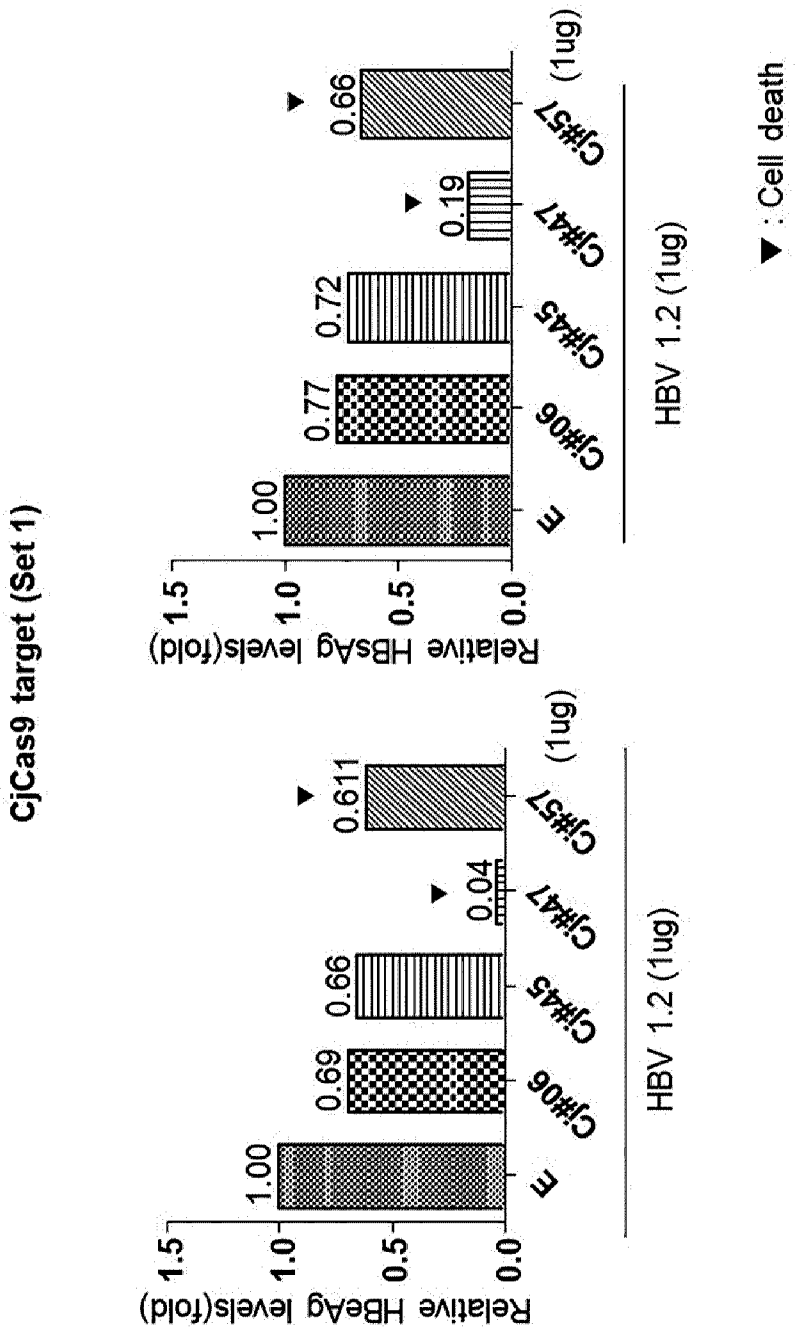
[Figure 10]



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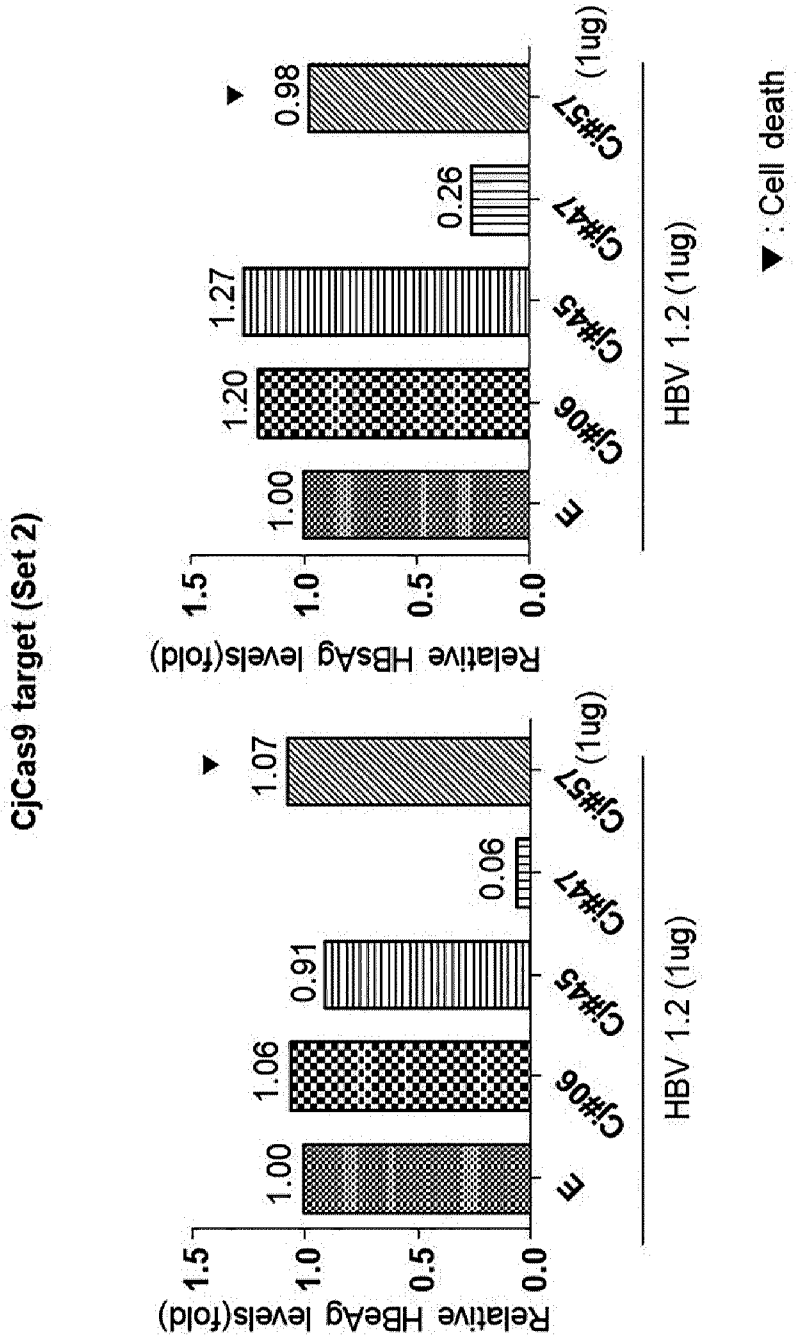
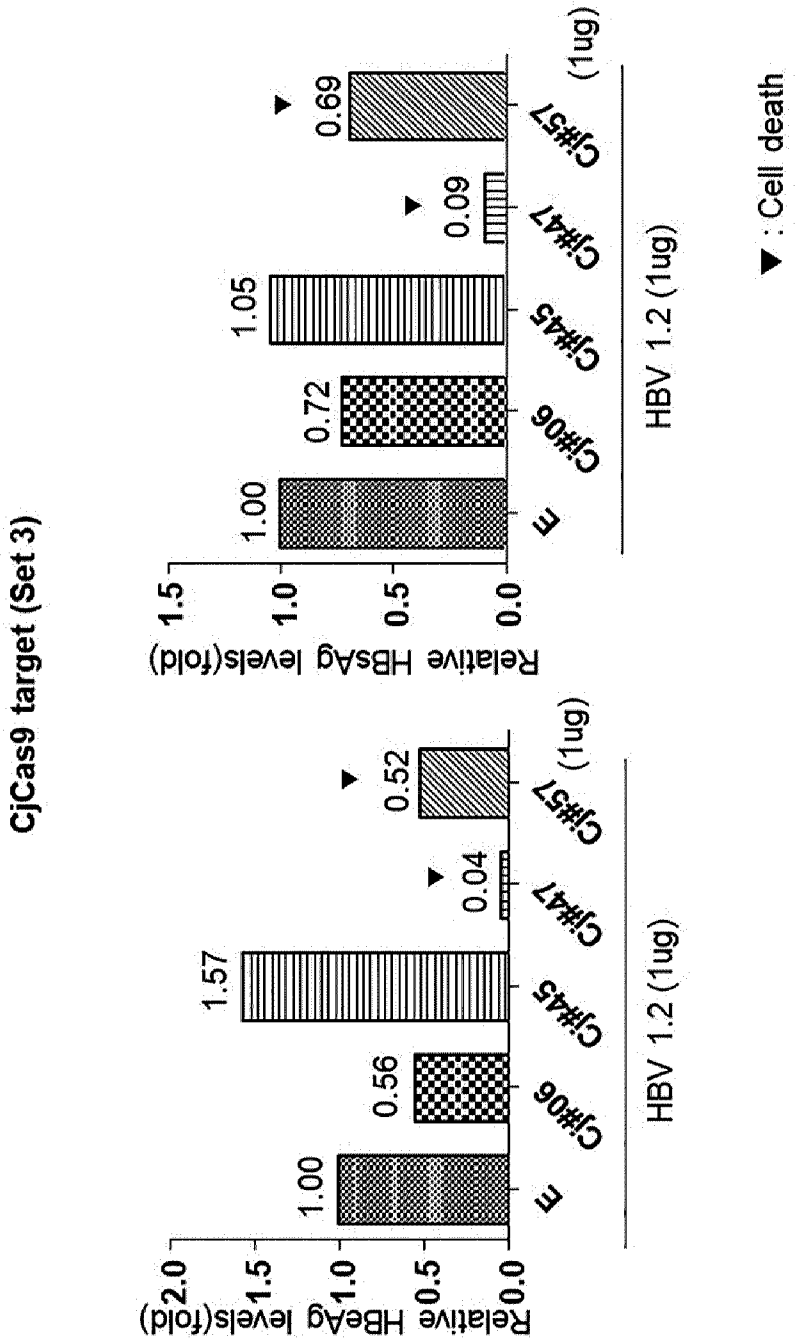
[Figure 11]


CjCas9 target (Set 2)
Relative HBeAg levels(fold)
1.5
1.0
0.5
0.0
1.00
1.06
0.91
0.06
1.07
E
Cjh06
Cjh45
Cjh47
Cjh57
HBV 1.2 (1ug)
HBV 1.2 (1ug)
Relative HBsAg levels(fold)
1.00
1.20
1.27
0.26
0.98
▼ : Cell death

[Figure 12]



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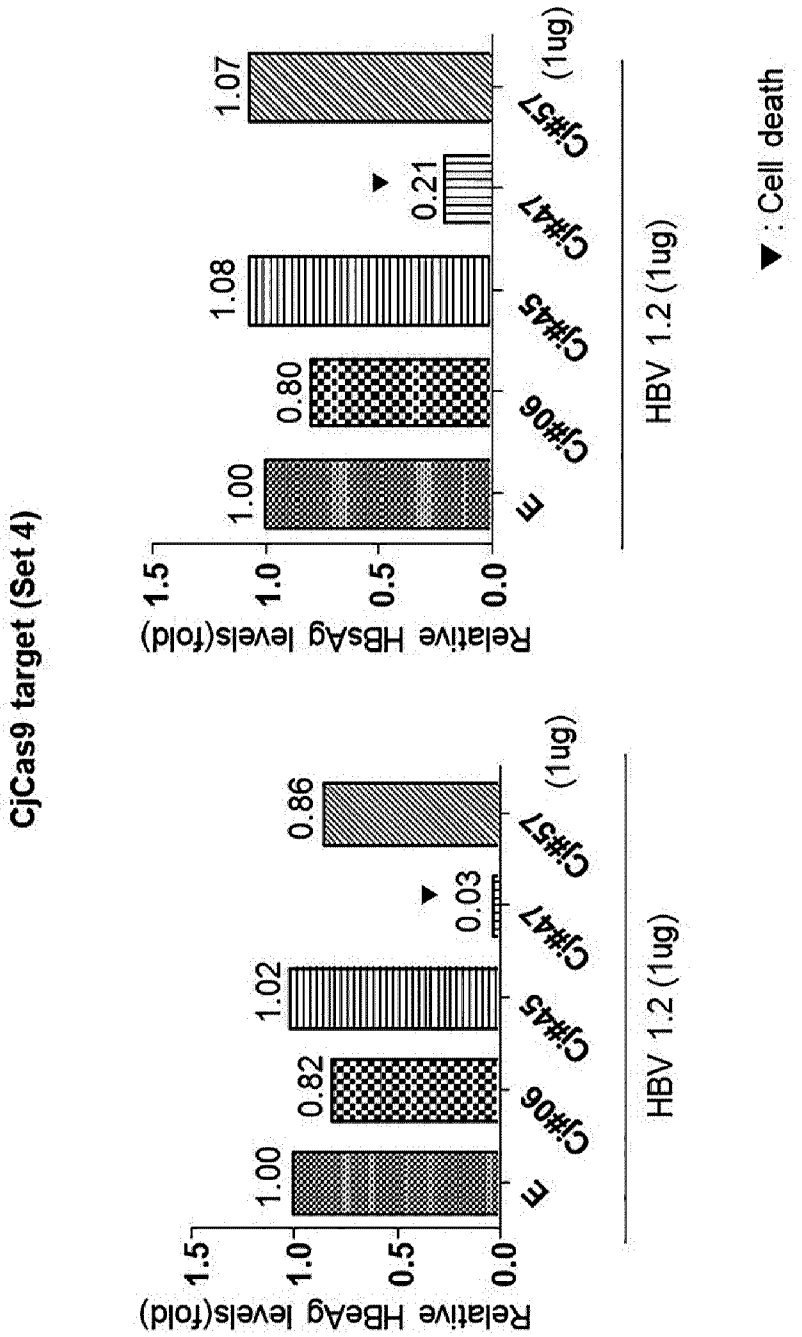
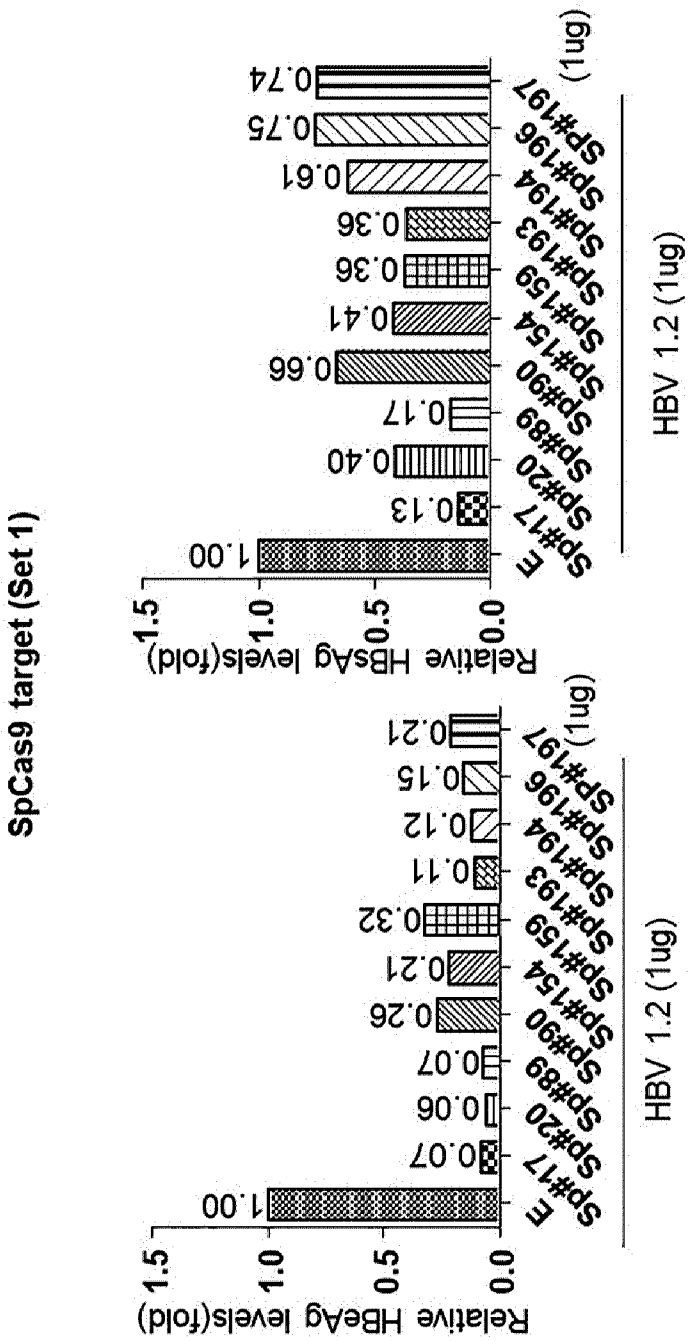
[Figure 13]


CjCas9 target (Set 4)
Relative HBeAg levels(fold)
1.5
1.0
0.5
0.0
1.00
0.82
1.02
0.03
0.86
E
Cj#06
Cj#45
Cj#47
Cj#57
HBV 1.2 (1ug)
(1ug)
Relative HBsAg levels(fold)
1.00
0.80
1.08
0.21
1.07
▼ : Cell death

[Figure 14]



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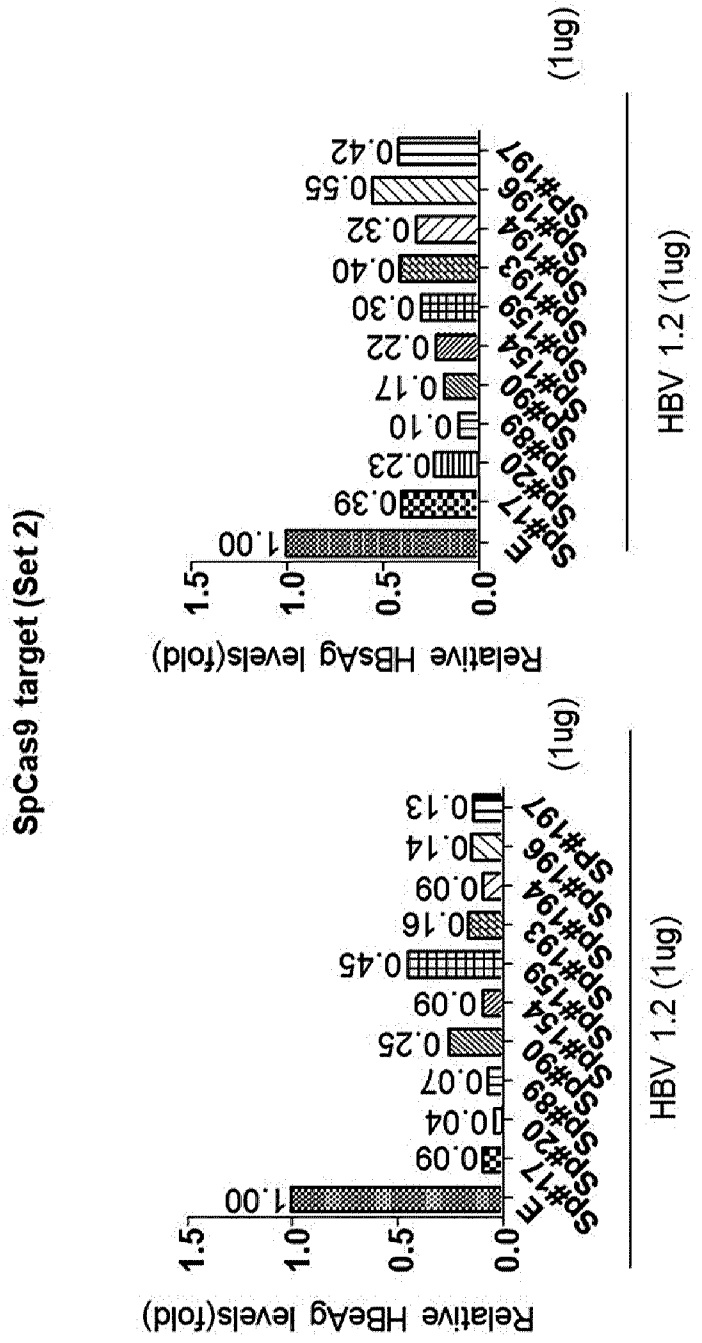
[Figure 15]



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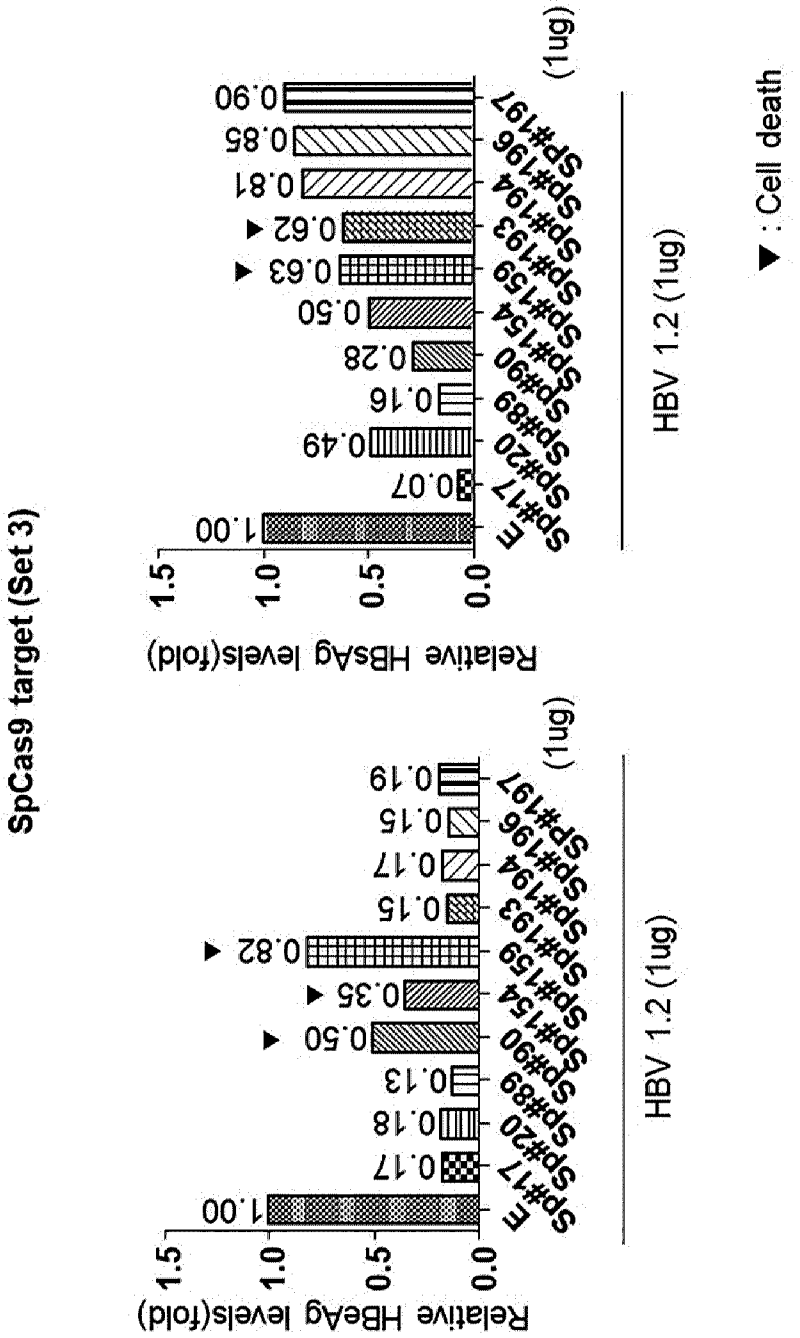
[Figure 16]



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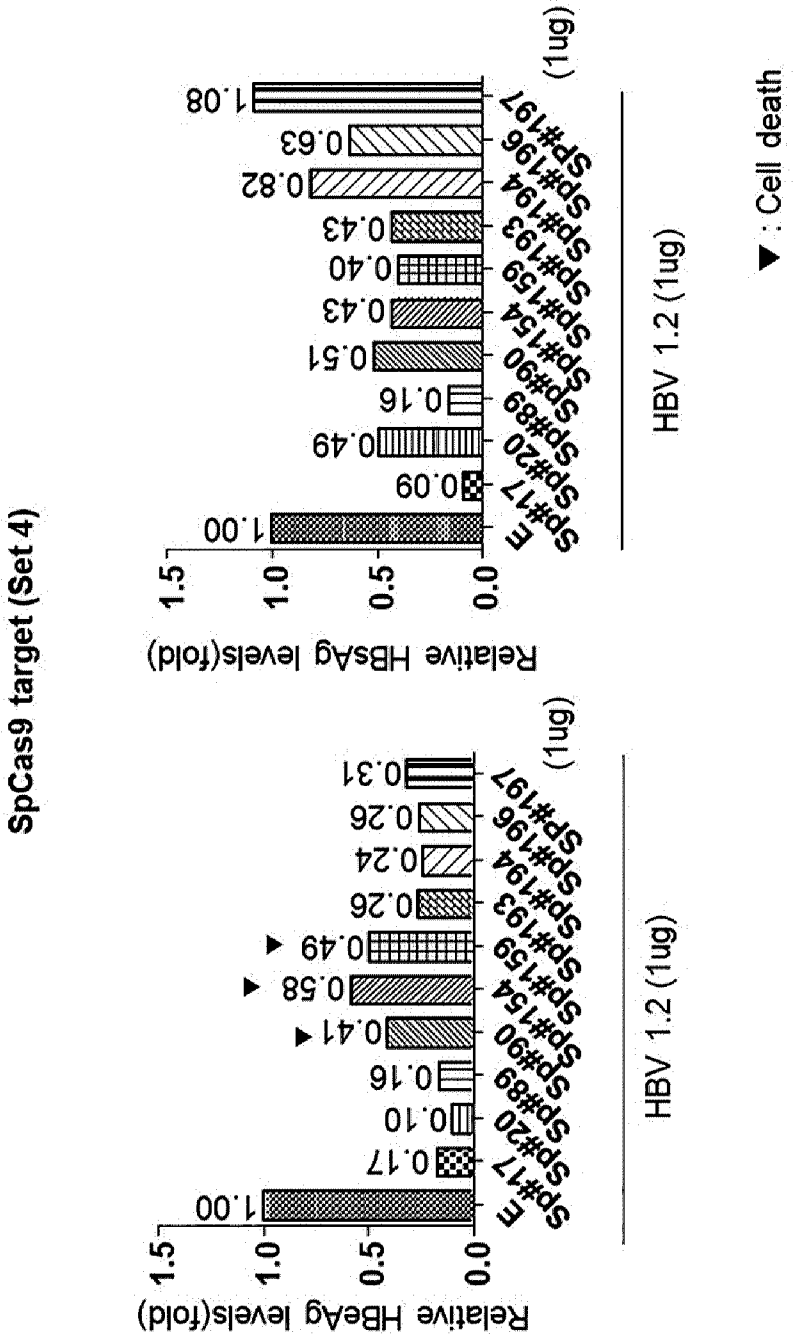
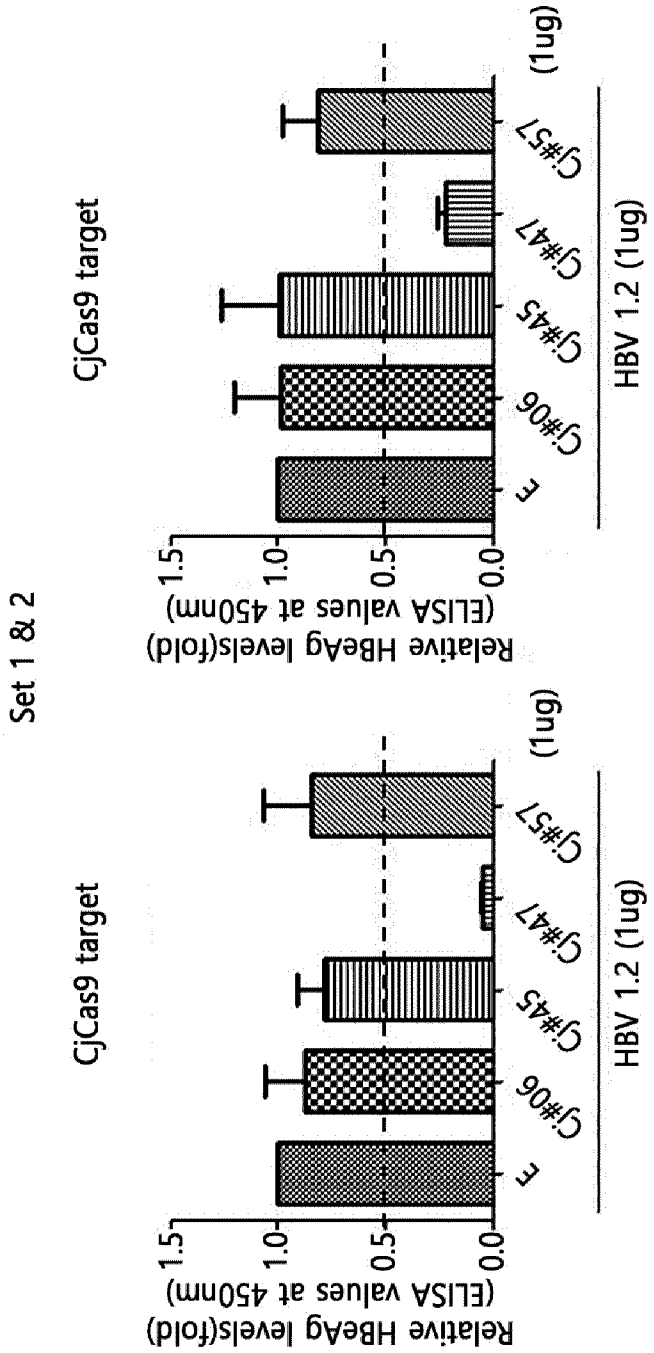
[Figure 17]


SpCas9 target (Set 4)
Relative HBeAg levels(fold)
1.5
1.0
0.5
0.0
EV
Sp#17
Sp#20
Sp#89
Sp#90
Sp#154
Sp#159
Sp#193
Sp#194
Sp#196
Sp#197
1.00
0.17
0.10
0.16
0.41
0.58
0.49
0.26
0.24
0.26
0.31
HBV 1.2 (1ug)
(1ug)
Relative HBsAg levels(fold)
1.00
0.09
0.49
0.16
0.51
0.43
0.40
0.43
0.82
0.63
1.08
▼ : Cell death

[Figure 18]



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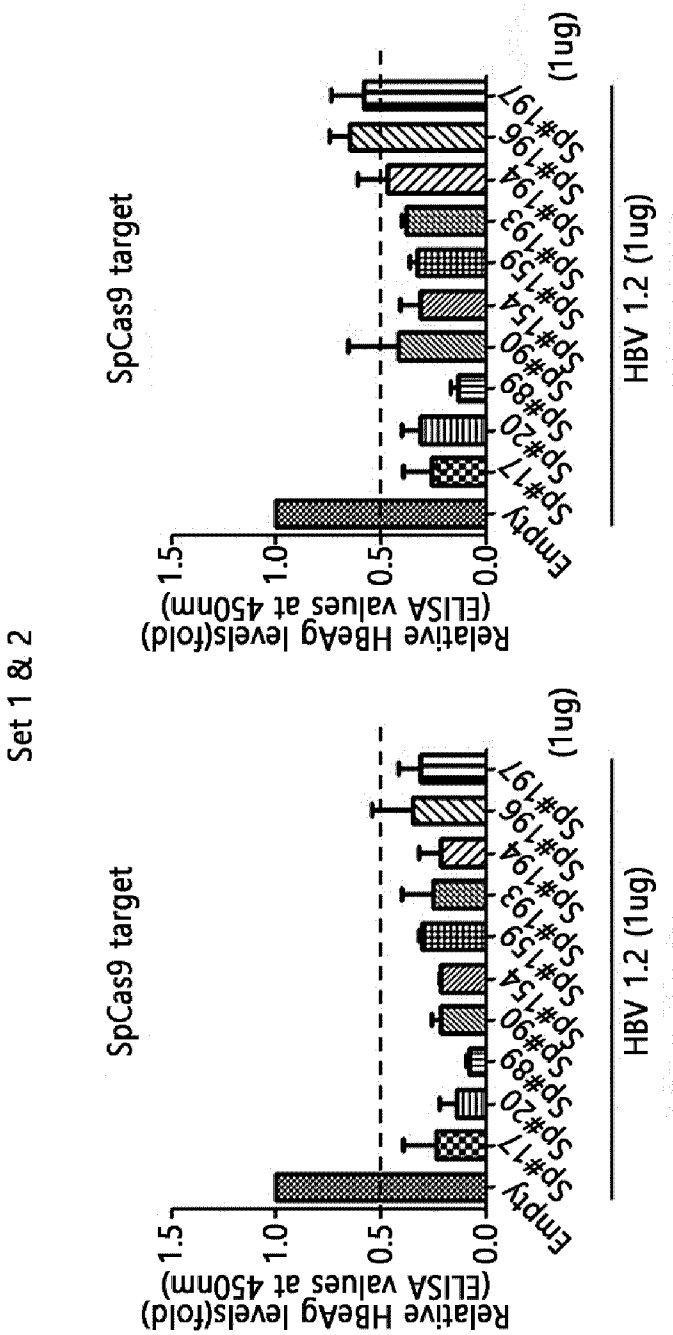
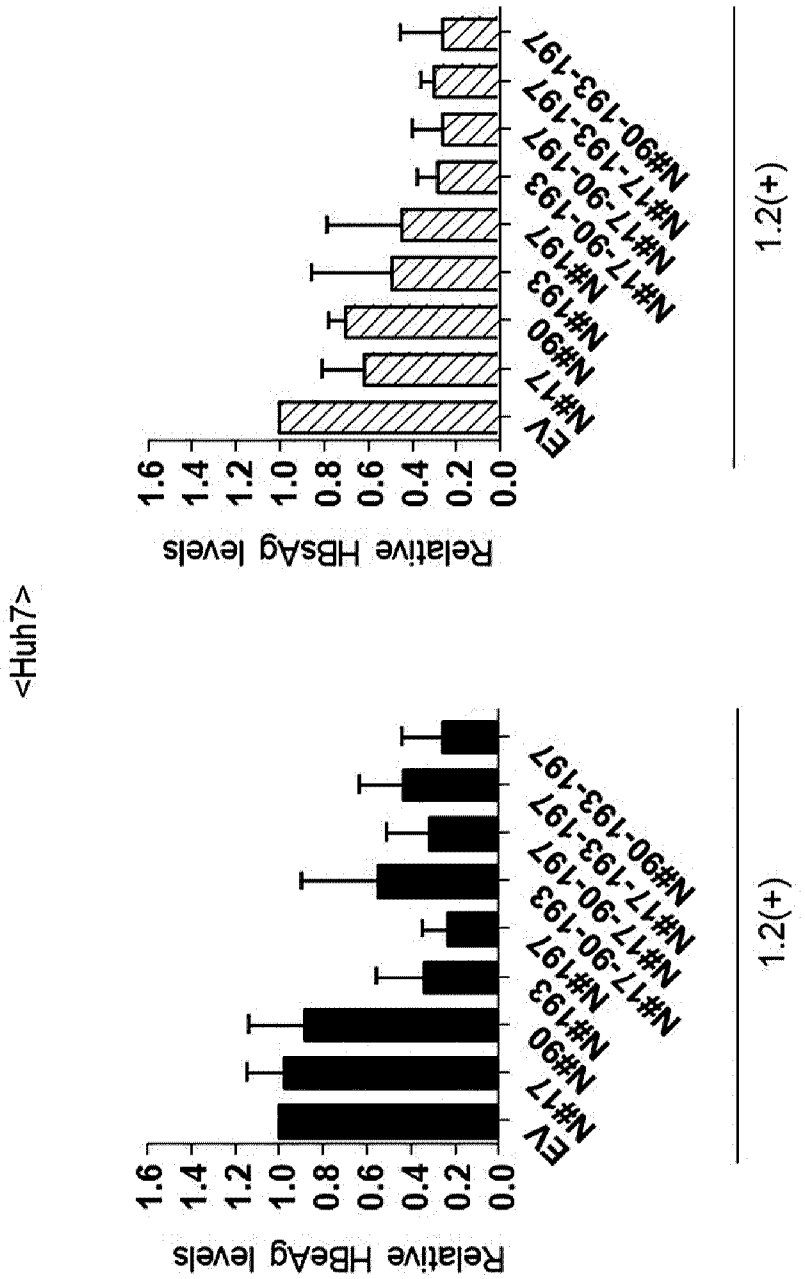
[Figure 19]



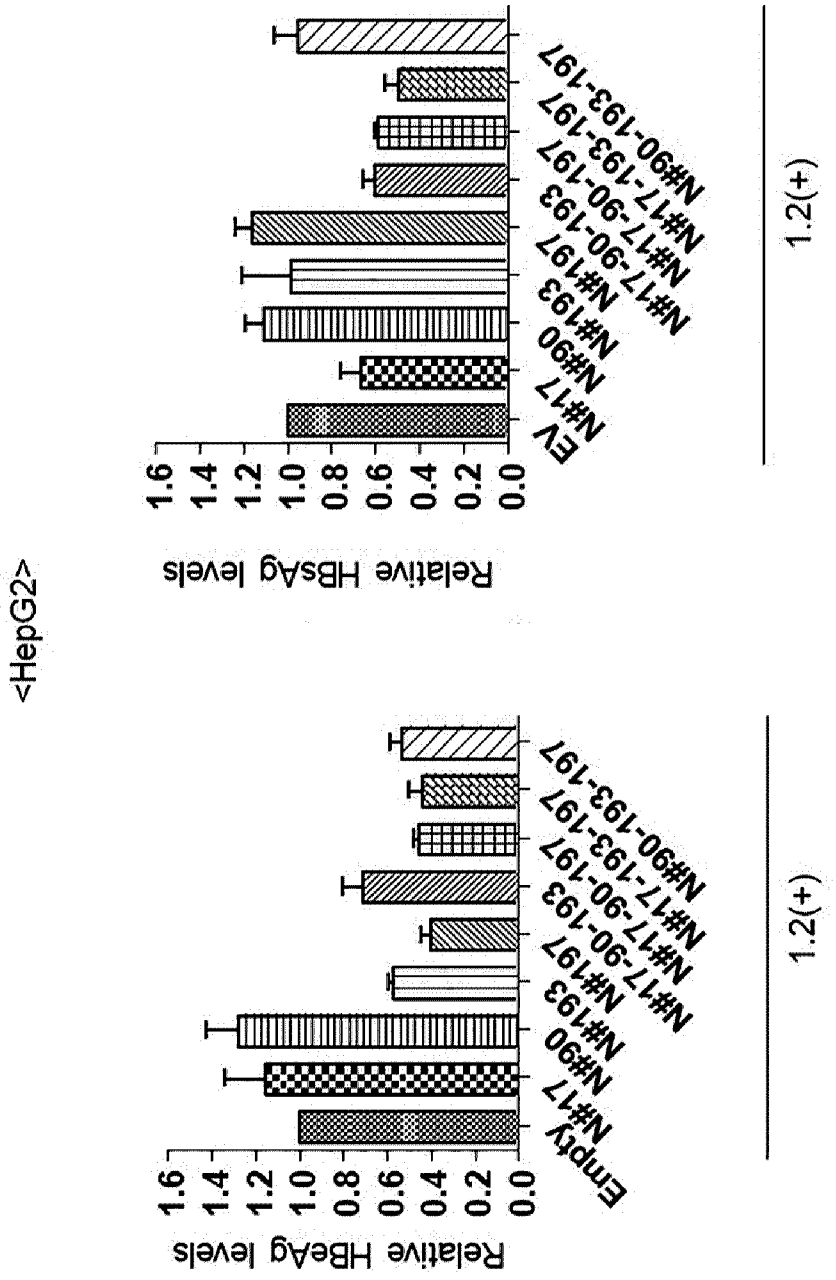
Date Reçue/Date Received 2021-10-15

[Figure 20]



Date Reçue/Date Received 2021-10-15

[Figure 21]



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[Figure 22]

	Huh7		HepG2	
	HBeAg	HBsAg	HBeAg	HBsAg
N#17	-	+++	-	+
N#90	-	+	-	-
N#193	++	-	++	-
N#197	+++	+	+++	-
N#17-90-193	+	+++	+	++
N#17-90-197	**++**	**+++**	**++**	**++**
N#17-193-197	**++**	**+++**	**++**	**++**
N#90-193-197	+++	+++	++	-

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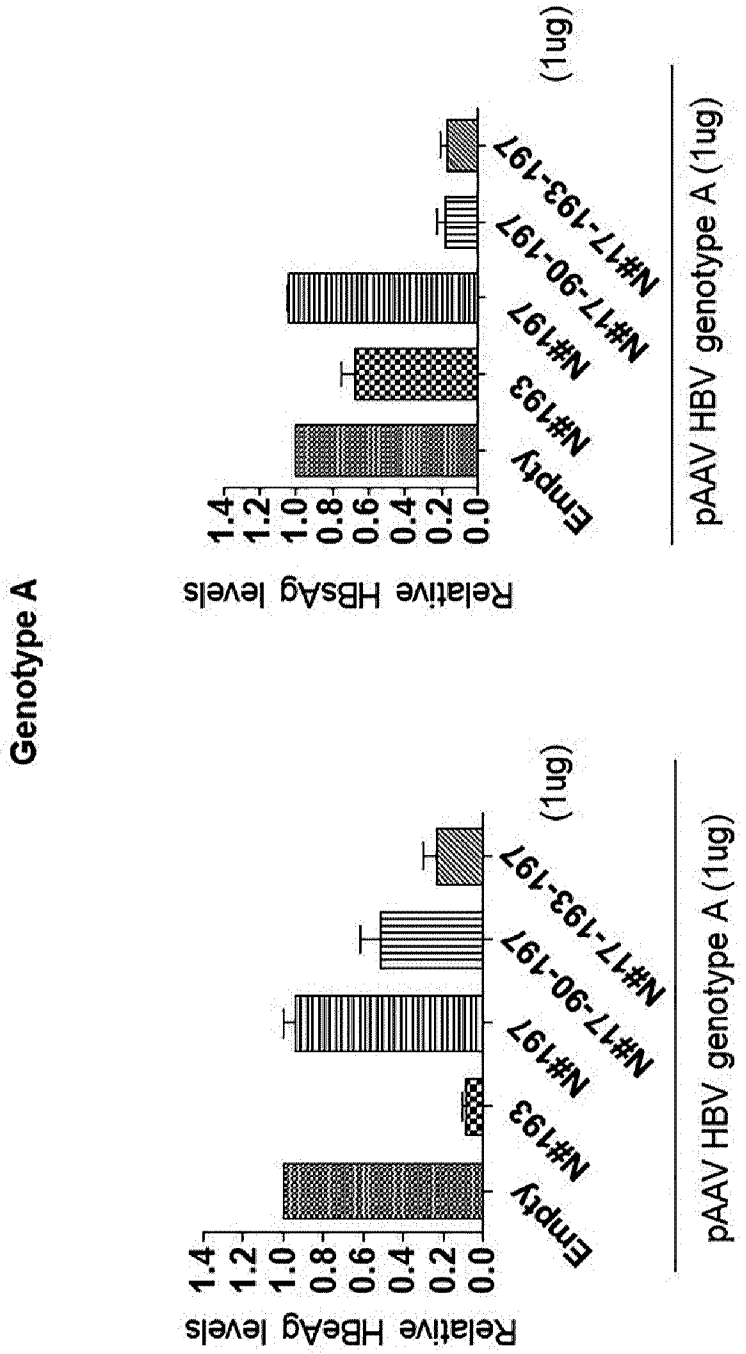
[Figure 23]



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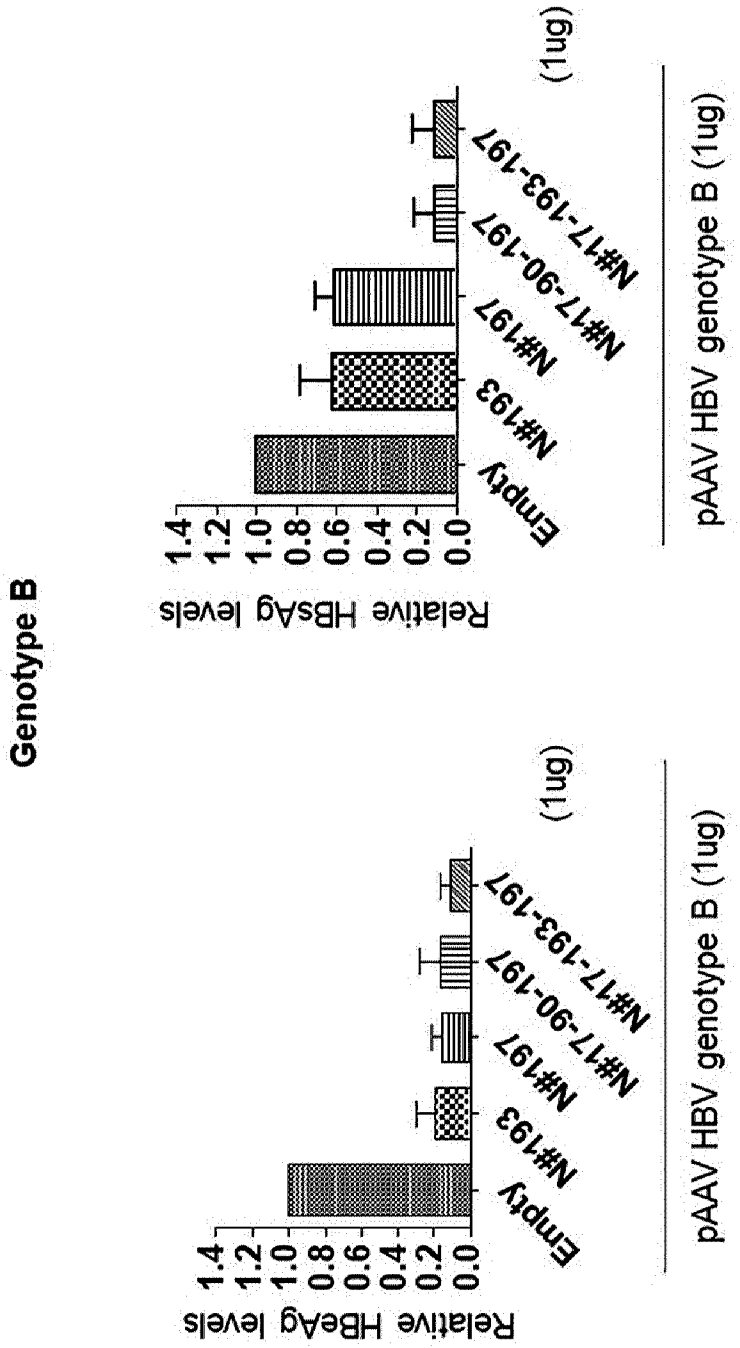
[Figure 24]



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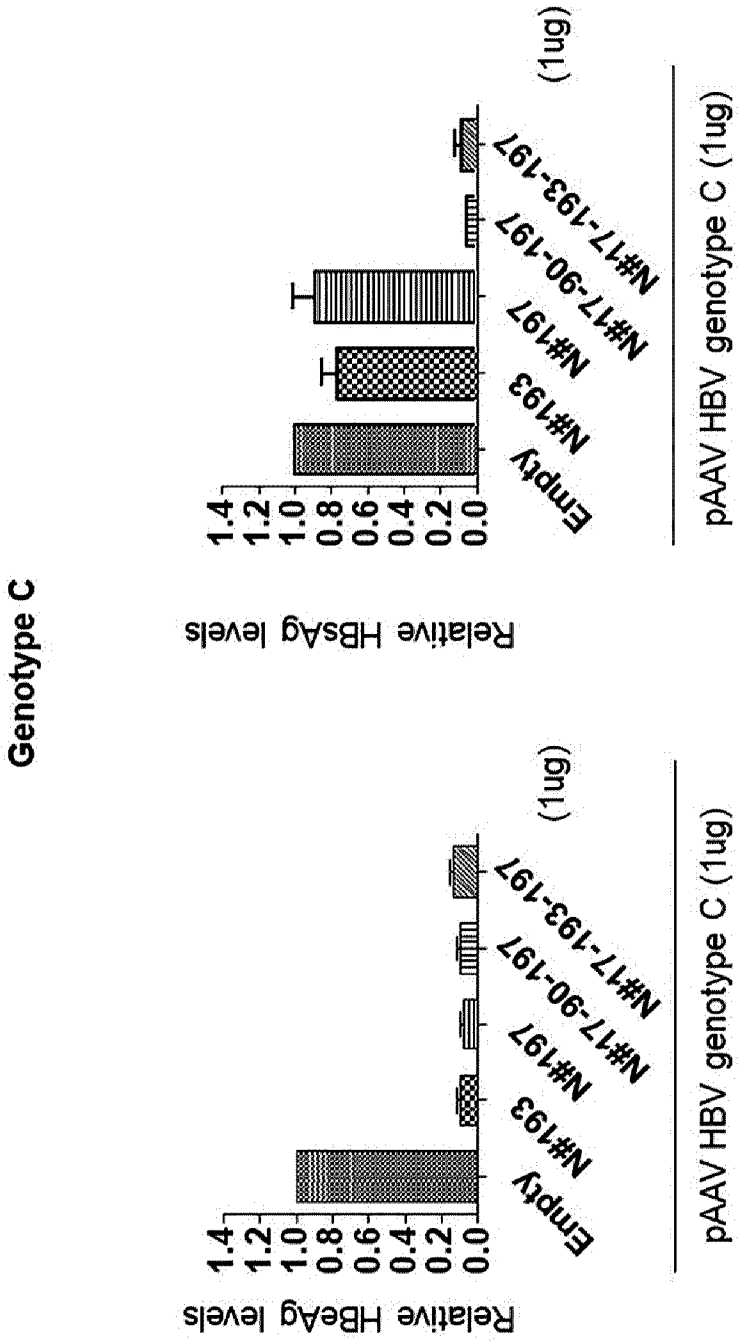
[Figure 25]



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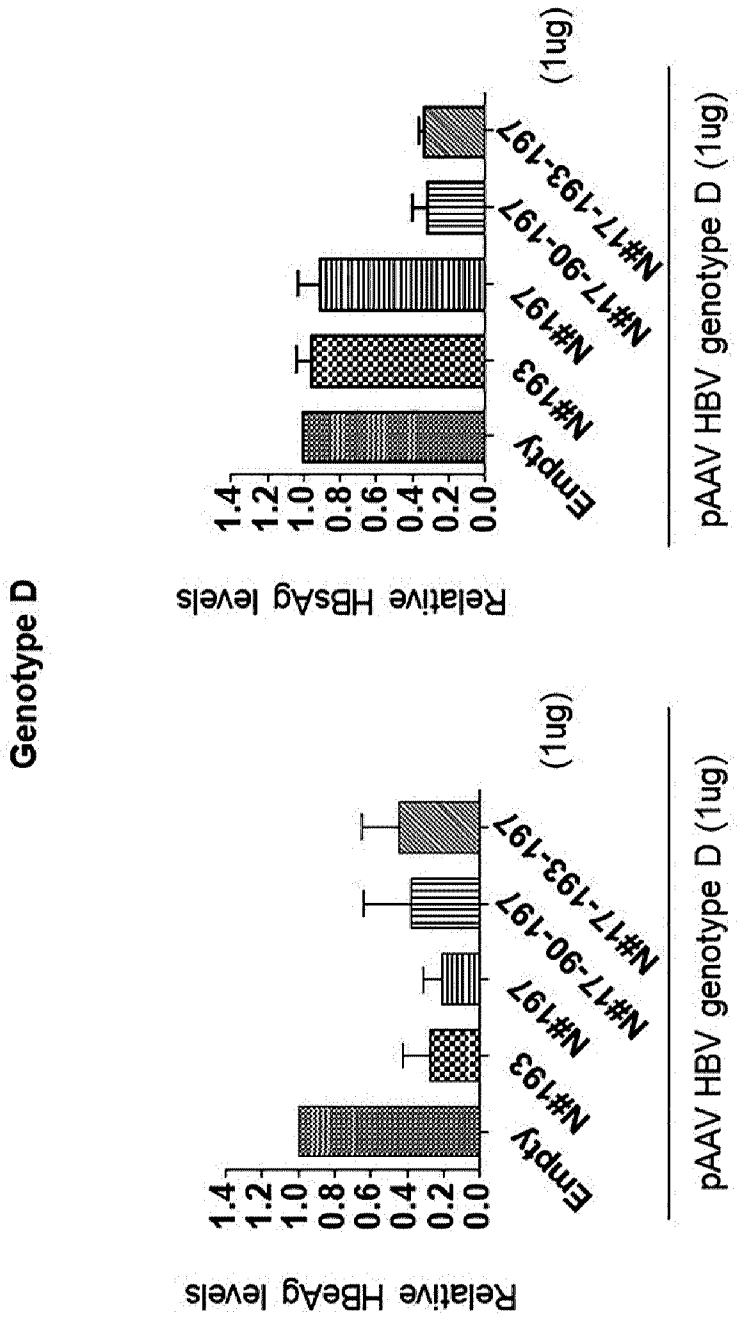
[Figure 26]



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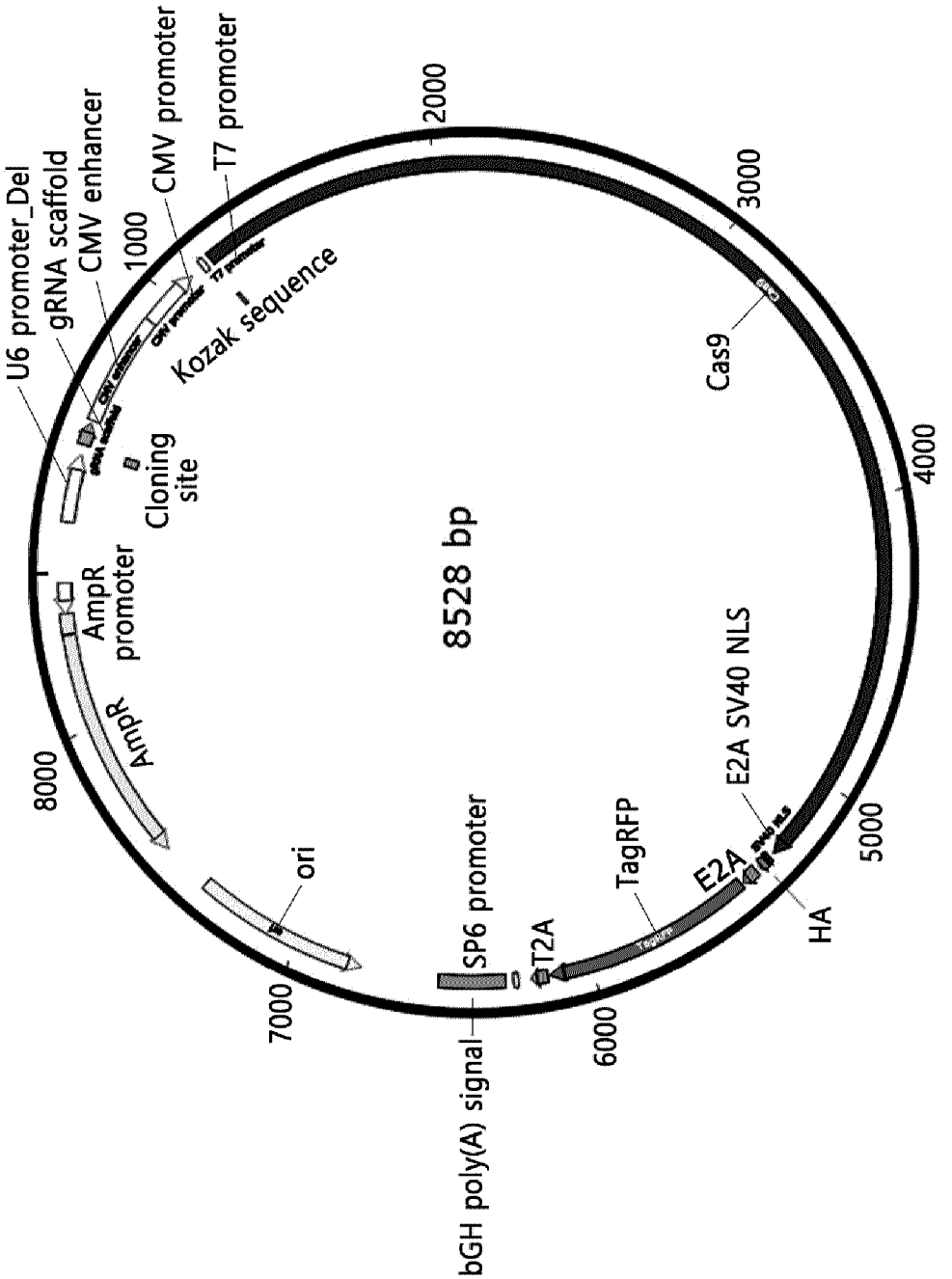
[Figure 27]



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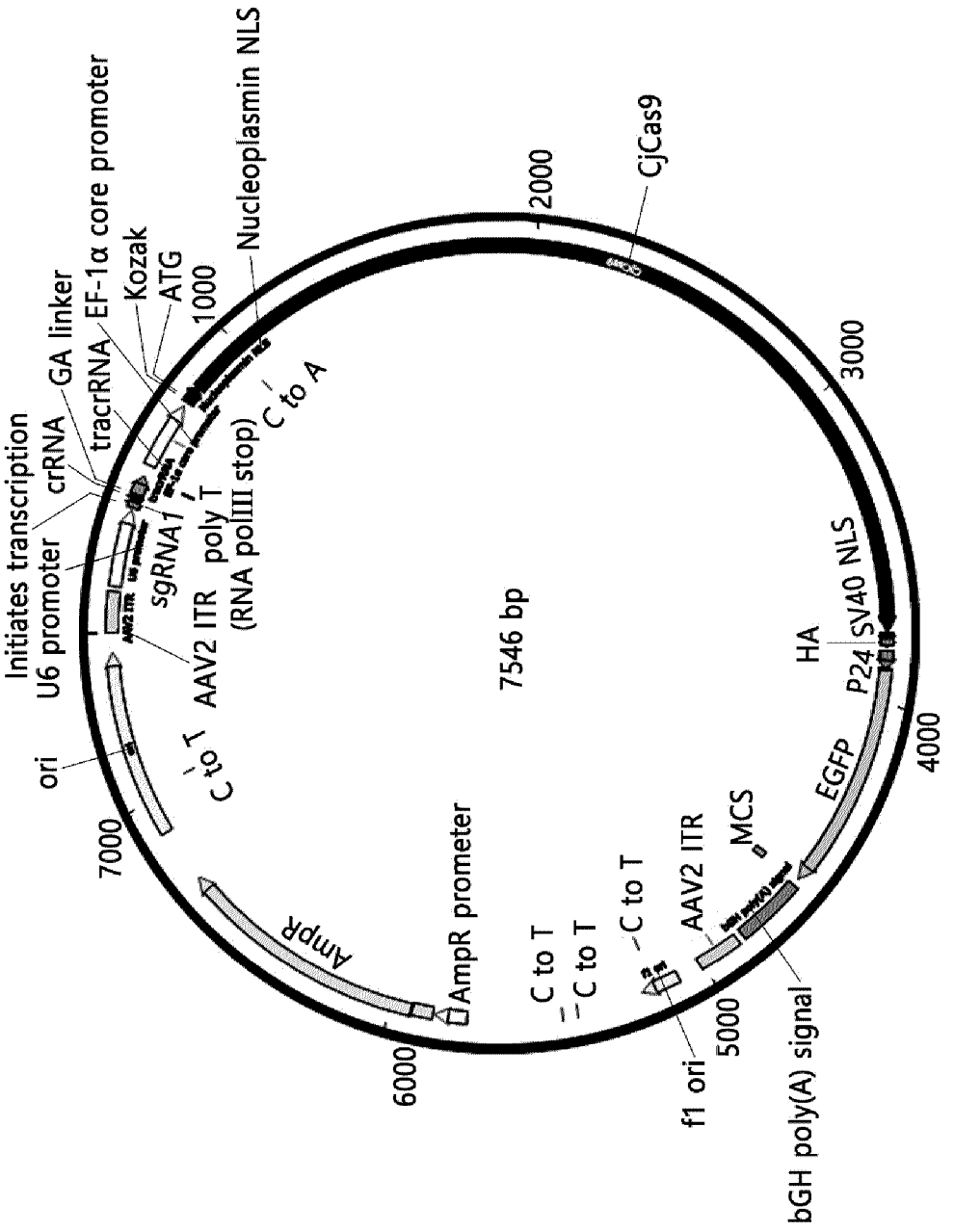
[Figure 28]



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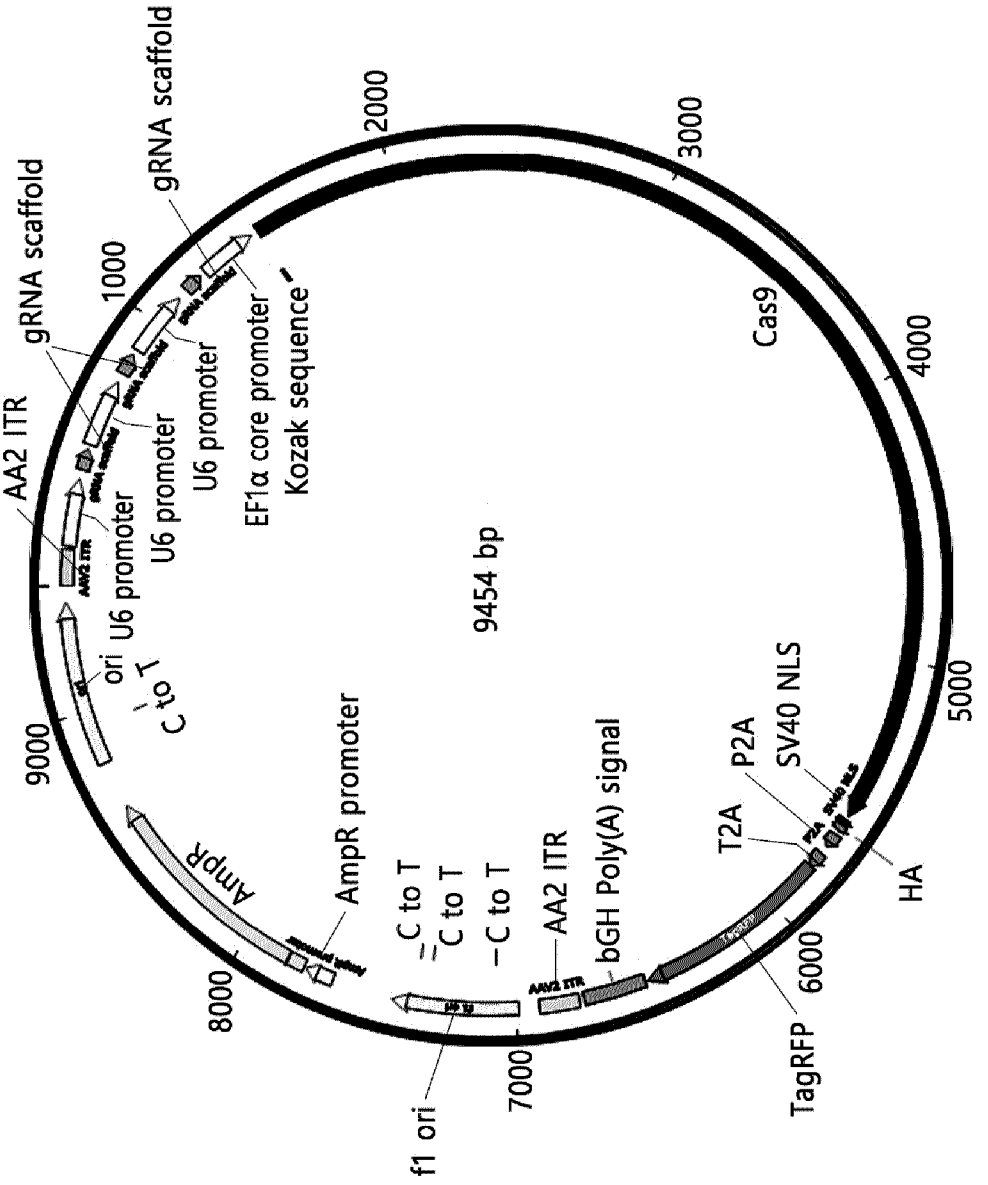
[Figure 29]



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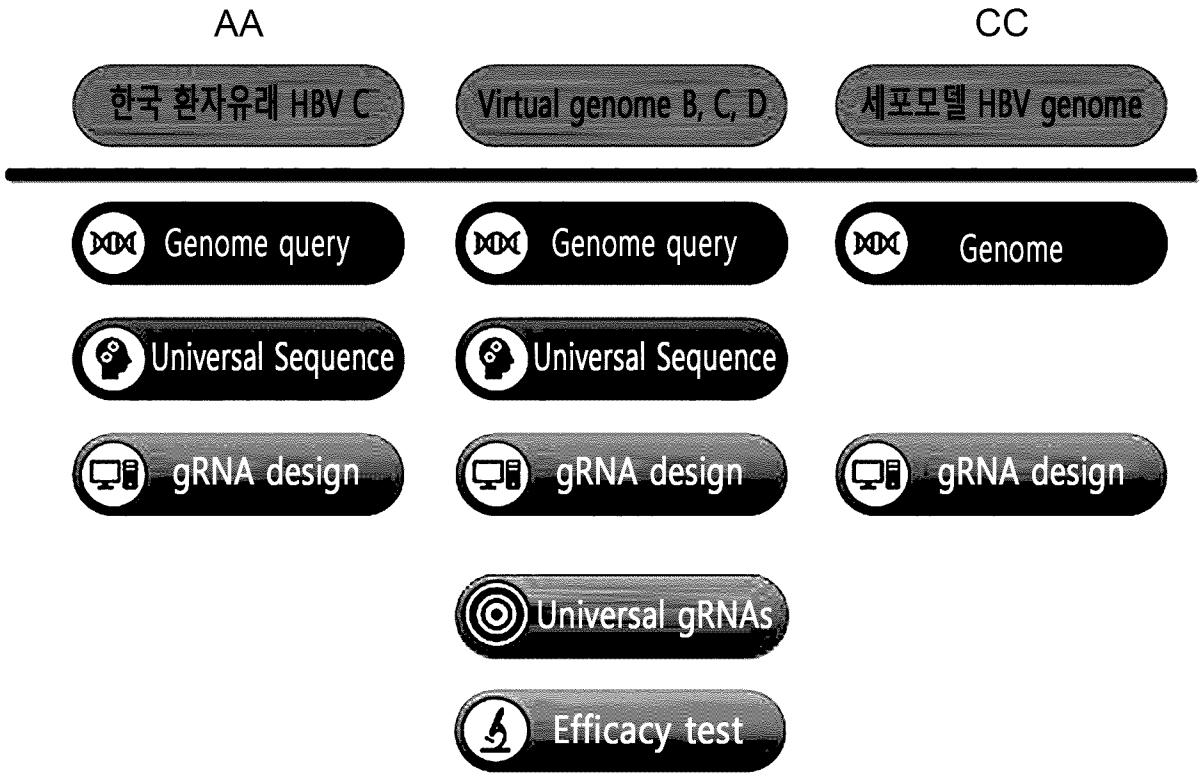

AA ... Korean patient-derived HBV C
CC ... Cell model HBV genome