

HCV CHIMERIC VACCINE WITH INFLUENZA VIRUS AS VECTOR AND PREPARATION METHOD THEREOF

Technical Filed

5 The present invention relates to an HCV chimeric vaccine with influenza virus as vector and a preparation method thereof.

Background Art

10 Hepatitis C is a fatal killer which threatens human health and lives, and there is a particular critical situation for preventing and managing the Hepatitis C in developing countries. Hepatitis C virus (HCV) is a primary pathogen causing chronic liver diseases, and spread generally through routes of blood and sexual mucosa. It is reported by a Chinese state authority that there are about 17 – 20 billions of people infected with HCV worldwide, and above about 4 billions of HCV infectors in China. approximately 50% - 85% of HCV infections transform into chronic liver diseases, wherein 20%-30% of which develop to cirrhosis, and a portion of which develop to
15 hepatocellular carcinoma (HCC). Currently, a combinational application of polyethylene glycol, interferon-alpha and ribavirin may be a standard regimen for treating Hepatitis C, but with an efficiency of only 50% and disadvantages such as large side effects, high expense, high recurrence rate after discontinuation of medication, for which many of the patients with Hepatitis C lost chances to be cured. This has become a global, serious public health issue.

20 To date, vaccination may be the most effective means for the prevention and control of infectious diseases.

 In past decades, recombinant Hepatitis B vaccine has been used widely so that infection and incidence rates of Hepatitis B have been greatly reduced, providing a great contribution to human health. However, there is still no vaccine for Hepatitis C approved for use nowadays, but the study
25 thereof has been always a hot issue concerned by the whole world. At present, studies on HCV vaccines mainly focus on synthetic polypeptide vaccines, recombinant multi-epitope vaccines, nucleic acid vaccines, viral vector vaccines, and the like, and several experimental vaccines have been brought into a pre-clinical or clinical stage. Accumulating study results show that the vaccine studies are still stagnated with no breakthrough progress, and have a plurality of technically critical
30 bottlenecks to be overcome. As seen from the studies, since HCVs greatly vary in genome and have poor antigens for cross-immunoprotection, there are still no effective susceptible cells and

animal infection models thereof, and the effect of cellular and humoral immune responses there to in the recovery from HCV infection is still unclear, it is difficult to proceed the studies on a preventive and/or therapeutic vaccine for inducing the production of a protective antibody. In addition, those existing HCV vaccines under study exhibit poor induction of immunity in a body after vaccination, incomplete immunoprotection, failure of producing effective mucosal and cellular immunities by injection, presenting challenges in the study of Hepatitis C vaccines. Therefore, the development of a safe, effective Hepatitis C vaccine has become a major scientific issue to solve in the current life sciences.

HCVs have a genomic constitution like that of *Flavivirus* and *Pestivirus*, and were classified into the family of *flaviviridae*, the genera of *hepacivirus*, by the International Committee on Taxonomy of Viruses (ICTV) in 1991. HCVs has a genome of an RNA virus of a single positive strand, a full length of about 9.6 kb, with a 319 - 341 nt relatively conservative non-coding region (5'-NCR) at 5'-end, followed by a continuous open reading frame (ORF) which can encode a polyprotein precursor, which is cleaved into 3 structural proteins (Core, E1, and E2) and 7 non-structural proteins (P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by signal peptidases from the host and from the virus. HCV protein C is composed of 191 amino acid residues, which is a relatively conservative structural region in the genome of HCV. 5'-NCR region is most conservative, while 3'-NCR is essential for normal replication of HCV RNA. Due to the complex characteristics of HCV pathogen in infection and pathogenesis, it is difficult to conduct immunization or prevention and control to HCVs.

Due to the great variations in HCV's gene sequence, the development of an Hepatitis C vaccine may face various challenges such as: ① great variations in HCV RNA and in viral protein, there are at least 6 genotypes and about 70 subgenotypes in HCV, wherein infections with subtypes 1b and 2a mainly prevail in China, but other subtypes also have been reported; ② the existence of HCV quasispecies may be also one of the mechanisms giving rise to immune escape and susceptibility to chronic infection in a host, which severely impacts on the development of HCV vaccines and the success rate in HCV prevention and treatment; ③ incapability of HCVs to massively and effectively propagate in vitro, lack of susceptible cells and animal models, except for chimpanzee which is the only laboratory animal model of HCV infection, but generally unusable because of being protected; ④ incompletely clear immunological mechanism of HCV pathogenicity, and insensitivity of sexually mucosal infection to injected vaccination to produce

cross-immunoprotection. All of these are severe restrictions for development of HCV vaccine.

In past decades, studies on HCV vaccines have always been a focus concerned by the scientists. Early studies mainly relied on eukaryotic expression of E1/E2 immunogens. Ralston et al. transfected mammalian cells with a vector of a recombinant vaccinia virus to express a HCV full-length protein, and found that, despite of high and effective expression, the expressed envelope glycoproteins E1 and E2 tended to form a complex, and immunization of a chimpanzee with a purified complex of envelope glycoproteins E1/E2 could produce high titers of anti- E1 and E2 antibodies, but use of envelope glycoprotein E1 could only stimulate the animals to produce a weak immune response, which indicates that a heterologous dimer formed of E1/E2 envelope glycoproteins may be essential to activate a strong immune response and protection. Moreover, Lagging et al. constructed a gene C (first 1—191aa) recombinant plasmid pcDNA HCV Core. In BALB /c mice which were intramuscularly injected with 0.2 mg of the plasmids 2 - 3 times for immunization and sacrificed 6 weeks thereafter, higher levels of an anti-C antibody were detectable, and the second immunization showed a similar antibody titer as that in the third immunization. The above experiments indicate that all the recombinant plasmids constructed with HCV region C were capable of being expressed in eukaryotes and inducing specific humoral and cellular immune responses, but incapable of eliciting mucosal immunoprotection in sexual transmission route. Also, Duenas-Carrera, et al. immunized rabbits and rhesus monkeys with a DNA vaccine encoding HCV-C, E proteins. 28 weeks after the primary immunization, stronger anti-C, E responses were induced in all rabbits, and 52 weeks after the immunization, a high titer of an antibody against the antigen of HCV structural region occurred in rhesus monkeys. Recently, there are also some achievements in clinical studies. Leroux-Roels et al. conducted phase I clinical experiments where 20 volunteers were intramuscularly injected with a product obtained from the common sequence of 1b E1 (aa192 - 326) which was expressed in a recombinant vaccinia virus and purified. The results indicate that a significant E1 antibody response could be induced after 3 immunizations, and enhanced after the fourth enforcement immunization. A strong cellular immune response against E1 was induced in all volunteers, suggesting that E1 therapeutic vaccine candidate might be good in clinical use in terms of safety and immunization effect. Baryluk et al. studied therapeutic vaccines using a plasmid containing HCV E1 and a recombinant adenovirus, which has entered phase II clinical studies in view of the safety thereof as demonstrated in phase I clinical studies. In the phase II clinical studies, recombinant E1 vaccine could activate a strong T cell response to E1, and

part of patients exhibited significantly decreased ALT levels in serum. Moreover, the researchers from the University of Oxford, etc. reported, in *Sci. Transl. Med.*, 2012, the results from clinical trials of a HCV vaccine they developed, which used a chimpanzee adenovirus as a vector to mimic the relatively long-term stable and constant structure therein and was proved to be preliminary effectiveness in the clinical trials. 41 volunteers were vaccinated with the vaccine, and then tracked during the study, it was found that the effect of the vaccine could last for at least one year, with significantly reduced side effects, but the safety and complete response and production as a DNA vaccine were restricted. Although these HCV vaccine candidates described above undergoing pre-clinical and clinical studies have been made significant progress, these vaccines induce poor immunization effect and incomplete cross immunoprotection in organisms after vaccination, are incapable of effectively inducing an immune effect of mucosal immunocytes as injected immunization, and have problems such as potential safety hazard as a DNA vaccine with adenovirus as vector.

Recently, reverse genetics (RG) has been widely used in the field of influenza viruses. Medimmune corporation, US represents the successful example of applying RG techniques for vaccine development, and live-attenuated influenza vaccines (LAIVs) an influenza trivalent live attenuated vaccine, Flumist, produced thereby was approved by FDA and put into use in June 2003, which is applicable to people of 2-49 years of age, in a nasal route for vaccination, and has the advantages of easiness in use, wide applicability, and safety, effectiveness, and cross-immunoprotection effect as demonstrated in clinical experimental results, bringing about a new opportunity to the development of other vaccines through the expression of a exogenous gene using a cold-adapted live attenuated influenza vaccine as the delivery system. As compared with other vectors such as retrovirus of adenovirus, vaccinia virus, etc. as a vector, influenza viruses produce no DNA intermediate in the replication period, and thus cannot be integrated into the chromosomes of a host, resulting in better safety in use. Strategies used in genomic manipulation of an influenza virus include: embedding a heterogenous protein into surface glycoproteins NA and HA, generating additional gene fragments, engineering non-structural protein NS1, deleting gene PB2, etc. Presently, there are already various reports of successful preparation of recombinant chimeric vaccine candidates allowing for effective immunoprotection, using influenza viruses as viral vaccine vector. For example, chimeric influenza virus (A/B), parainfluenza, respiratory syncytial virus (RSV), chlamydia, *Mycobacterium tuberculosis*, malaria, AIDS, *B. anthracis*,

tumors, etc. all have been reported, widening more space for the study on a multivalent vaccine using influenza virus as vector. Noted that, with an HIV virus which also spread mainly through routes of blood and sex, Anna L. de Goede et al. successfully constructed a replication-deficient rFlu-p17 vaccine candidate with the influenza virus as vector by inserting gene HIV-1 p17Gag into influenza virus gene NA, and the results indicated that rFlu-p17 was capable of inducing stronger specific humoral and cellular immune responses. However, an HCV vaccine produced using an influenza virus as vector has not been reported yet. Thus, the production of an HCV vaccine candidate using reverse genetics with an influenza virus as vector will be a novel strategy for the development of HCV vaccines.

Disclosure of the Invention

The present invention aims to provide an HCV chimeric vaccine with an influenza virus as a vector and a preparation method thereof.

The present invention provides a DNA molecule shown in SEQ ID No.1.

A recombinant virus is also included in the protection scope of the present invention, which is prepared in accordance with following method:

co-transfecting host cells with expression vectors respectively containing a PB2 gene, a PB1 gene, a PA gene, a NP gene, a M gene, and a NS gene from a cold-adapted, attenuated influenza virus, and expression vectors respectively containing an HA gene and a NA gene from a target influenza virus, and incubating the cells, to obtain a recombinant influenza virus;

inserting, or substituting with, a gene of a structural protein of HCV at any position in the open reading frame (ORF) of at least one of following genes:

- (1) the PB2 gene from a cold-adapted, attenuated influenza virus;
- (2) the PB1 gene from a cold-adapted, attenuated influenza virus ;
- (3) the PA gene from a cold-adapted, attenuated influenza virus;
- (4) the NP gene from a cold-adapted, attenuated influenza virus;
- (5) the M gene from a cold-adapted, attenuated influenza virus;
- (6) the NS gene from a cold-adapted, attenuated influenza virus;
- (7) the HA gene from a target influenza virus; and
- (8) the NA gene from a target influenza virus;

wherein the PB2 gene, PB1 gene, PA gene, NP gene, M gene and NS gene from the

cold-adapted, attenuated influenza virus as well as HA and NA gene from the target influenza virus are positioned in a different expression vector; and

wherein a gene of the structural protein of HCV is the whole gene of core protein C, the whole gene of envelope protein E1/E2, a dominant epitope gene of core protein C, and/or a dominant epitope gene of envelope protein E1/E2, from HCV.

For above viruses, the host cells are MDCK, Vero, 293T, COS cells, or co-incubated cells of MDCK/293T, MDCK/COS; and

a gene of the structural protein of HCV is a fusion gene of a dominant epitope gene of core protein C with a dominant epitope gene of envelope protein E1/E2, from HCV.

For any one of the viruses described above, the HCV is HCV type 1, HCV type 2, HCV type 3, HCV type 4, HCV type 5, or HCV type 6;

wherein HCV type 1 is particularly HCV subtype 1b.

For any one of the viruses described above, the fusion gene of the dominant epitope gene of the core protein C and the dominant epitope gene of the envelope protein E1/E2 of HCV has a nucleotide sequence as shown in SEQ ID No.1.

For any one of the viruses described above, the gene of the structural protein of HCV is inserted into the ORF of the NS gene of the cold-adapted, attenuated influenza virus between nucleotides at positions 375 and 376 from 5'-end;

the gene of the structural protein of HCV is linked with the nucleotides at position 375 from 5'-end in the ORF of the NS gene of the cold-adapted, attenuated influenza virus via a sequence of 5'- UAAUG -3';

and/or,

the nucleotides of positions 223 to 760 from 5'-end in the ORF of the M gene of the cold-adapted, attenuated influenza virus are replaced with the gene of the structural protein of HCV;

and/or,

the nucleotides from position 184 to position 1253 from 5'-end in the ORF of the NA gene of the target influenza virus are replaced with the gene of the structural protein of HCV.

For any one of the viruses described above, the cold-adapted, attenuated influenza virus is cold-adapted, attenuated influenza virus type A, or cold-adapted, attenuated influenza virus type B;

wherein the cold-adapted, attenuated influenza virus type A is particularly cold-adapted, attenuated influenza virus subtype H2N2, more particularly cold-adapted, attenuated influenza virus

strain A/Ann Arbor/6/60 (H2N2);

the target influenza virus is influenza virus type A or influenza virus type B, wherein influenza virus type A is particularly any of subtypes H1 - H16, more particularly influenza virus subtype H1N1, even more particularly influenza virus strain A/California/07/2009 (H1N1);

5 the target influenza virus is a common wild-type influenza virus without any treatment (for example, without attenuation or cold-adaption).

For any one of the viruses described above, the gene of the structural protein of HCV is inserted into the ORF of the NS gene of the cold-adapted, attenuated influenza virus between the nucleotides at positions 375 and 376 from 5'-end, to obtain a sequence as represented by the
10 nucleotides from position 51 to position 1466 of SEQ ID No.2;

the nucleotides from position 223 to position 760 from 5'-end in the ORF of the M gene of the cold-adapted, attenuated influenza virus are replaced with the gene of the structural protein of HCV, to obtain a sequence as represented by the nucleotides from position 40 to position 1053 of SEQ ID No.3; and

15 the nucleotides from position 184 to position 1253 from 5'-end in the ORF of the NA gene of the target influenza virus are replaced with the gene of the structural protein of HCV, to obtain a sequence as represented by the nucleotides at positions 35-944 of SEQ ID No.4.

When an expression vector is constructed, each of the PB2 gene, PB1 gene, PA gene, NP gene, M gene, NS gene, HA gene, or NA gene as described above has a 3'NCR sequence linked to 5'-end
20 thereof, and a 5'NCR sequence linked to 3'-end thereof;

a starting plasmid of the expression vector is a bidirectional transcription, expression vector pAD3000;

each of the genes is inserted at *Bsm*BI site of the bidirectionally transcriptional expression vector pAD3000 when the expression vector is constructed.

25 A chimeric vaccine produced from any of above viruses is also included in the protection scope of the present invention.

Use of the DNA molecules described above in the manufacture of a product for preventing and/or treating a disease caused by an influenza virus and/or HCV is also included in the protection scope of the present invention;

30 and/or,

use of any of the viruses described above in the manufacture of a product for preventing

and/or treating a disease caused by an influenza virus and/or HCV is also included in the protection scope of the present invention;

and/or,

use of the chimeric vaccines described above in the manufacture of a product for preventing and/or treating a disease caused by an influenza virus and/or HCV is also included in the protection scope of the present invention;

wherein the influenza virus is Influenza virus type A or Influenza virus type B, and the Influenza virus type A is particularly any of subtypes H1 - H16;

the HCV is HCV type 1 , HCV type 2 , HCV type 3 , HCV type 4 ,HCV type 5, or HCV type 6;

and

the HCV type 1 is particularly HCV subtype 1b.

Description of the Drawing

Figure 1 shows a schematic recombination strategy for constructing an HCV chimeric vaccine with an influenza virus as a vector.

Best Mode to Implement the Invention

Unless otherwise indicated, all the experimental methods used in following examples are conventional methods.

Unless otherwise indicated, all the materials, agents, etc. used in following examples are commercially available.

The plasmid pAD3000 was disclosed in "Hoffmann E, Mahmood K, Yang CF, Webster RG, Greenberg HB, Kemble G. Rescue of influenza B virus from eight plasmids. Proc Natl Acad Sci.2002;99(17):11411-6.", and is available from the Academy of Military Medical and Science, CN.

Mice BALB/c were purchased from Beijing Laboratory Animal Research Center of the Academy of Military Medical and Science, CN.

Ferrets of 10 - 12 years of age were purchased from Angora Corporation, WuXi, CN.

2012-2013 epidemic strain A/California/07/2009 (H1N1) of influenza virus was supplied by Chinese National Influenza Center, and is publicly available from the Academy of Military Medical and Science, CN, from which HA and NA genes were amplified, and inserted into the bidirectional

transcription/expression vector pAD3000 at *Bsm*BI site, respectively, to construct and obtain recombinant plasmids pD-HA and pD-NA of the virus strain.

Cold-adapted, attenuated influenza virus strain, A/Ann Arbor/6/60 (H2N2) (abbreviated as A/AA/6/60) was disclosed in the literature “Yang P, Duan Y, Wang C, Xing L, Gao X, Tang C, Luo D, Zhao Z, Jia W, Peng D, Liu X, Wang X. Immunogenicity and protective efficacy of a live attenuated vaccine against the 2009 pandemic A H1N1 in mice and ferrets. *Vaccine*. 2011 Jan 17;29(4):698-705.”, and is publicly available from the Academy of Military Medical and Science, CN. 6 internal genes of this virus strain, PB2, PB1, PA, NP, NS, M, were inserted into the bidirectional transcription/expression vector pAD3000 at *Bsm*BI site, respectively, to construct and obtain recombinant plasmids, pD-PB2, pD-PB1, pD-PA, pD-NP, pD-NS, and pD-M of the virus.

In the following examples, NCR represents the non-coding region.

Example 1. Preparation of HCV chimeric vaccine rFLU-HCV/NS1 chimed with HCV-C/E1/E2 dominant epitope with influenza virus as vector

I. HCV-C/E1/E2 dominant epitope from HCV subtype 1b with a sequence as shown in SEQ ID No.1 was subjected to animal experiments and clinical validation with serum from patients, and validated to fit with the next experiments.

II. Construction of recombinant plasmid pC/E1/E2-NS1

With the NS1 gene fragment from cold-adapted, attenuated influenza virus strain A/AA/6/60 as a target site at which HCV-C/ E1/E2 dominant epitope gene was to be inserted, a recombinant plasmid pC/E1/E2-NS1 was constructed by molecularly biological methods. Specific strategy is shown in Figure 1A.

Particularly, the HCV-C/E1/E2 dominant epitope gene obtained as above was inserted into the open reading frame (ORF) of NS gene of the cold-adapted, attenuated influenza virus strain A/AA/6/60, behind the nucleotide at position 375 from 5'-end, with a linker (5'-UAAUG-3') serving as both a terminator and a promoter added therebetween for linking, before the nucleotides of positions 376 to 838 in the ORF of the NS gene, to construct a recombinant plasmid, designated as pC/E1/E2-NS1.

Above was performed by specific steps of:

(I) synthesizing a DNA molecule as shown in SEQ ID No.2, having: from 5'-end of SEQ ID

No.2, an enzyme cleavage site at positions 1 to 24, 3'NCR of NS gene at positions 25 to 51, 1st-375th nucleotides from 5'-end of NS gene open reading frame(ORF) of a cold-adapted, attenuated influenza virus strain A/AA/6/60 at positions 51 to 425, a linker at positions 426 to 430, a HCV-C/E1/E2 dominant epitope gene at positions 431 to 1003, 376th-838th nucleotide from 5'-end of NS gene open reading frame (ORF) at positions 1004 to 1466, 5'NCR of NS gene at positions 1467 to 1495, and an enzyme cleavage site at positions 1496 to 1517;

(II) cleaving the DNA molecule as shown in SEQ ID No.2 with *AarI* to obtain a target gene fragment; cleaving pAD3000 with *BsmBI* to obtain a large fragment of the vector; and linking the gene fragment with the large fragment of the vector to obtain a recombinant plasmid, designated as pC/E1/E2-NS1, which was confirmed to be correct by sequencing.

III. 0.2 µg of each of totally 8 plasmids of the recombinant plasmid pC/E1/E2-NS1, recombinant plasmids pD-PB2, pD-PB1, pD-PA, pD-NP and pD-M respectively constructed with viral internal gene backbones PB2, PB1, PA, NP, and M from cold-adapted, attenuated influenza virus strain A/AA/6/60, and recombinant plasmids pD-HA and pD-NA respectively constructed with HA and NA genes from currently epidemic influenza virus strain A/California/07/2009 (H1N1) was mixed with 10 µL of a transfection reagent (Effectene, purchased from Qiagen Corp.), and stood at room temperature for 10min, followed by co-transfecting MDCK cells at 33°C under 5% CO₂ for 48-60h, cell suspensions were obtained and then inoculated into a 9-day aged SPF chicken embryo, which cultured at 33°C for 72 h. Allantoic fluid of the chicken embryo was harvested and measured for a hemagglutination titer of HA. The measurement of the HA hemagglutination titer was 1:256-512. A HCV chimeric vaccine strain (rFLU-HCV/NS1) chimed with HCV-C/E1/E2 dominant epitope was obtained.

IV. The vaccine strain obtained from step III was identified, and observed via electron microscopy for viral morphology. The results showed that the vaccine strain had typical morphological characteristics of an influenza virus, with a viral particle size between 80 and 120nm.

V. The 9 – 11 day aged SPF chicken embryo (purchased from Beijing Laboratory Animal Research Center) inoculated with rFLU-HCV/NS1 was passaged for generations. The allantoic fluid from second generation of the chicken embryo was taken for extracting viral RNA, which was subjected to RT-PCR to amplify PB2, PB1, PA, NP, HA, NA, M and NS1, totally 8 gene fragments. These gene fragments were sent for sequencing. The results were consistent with the expected

gene sequence.

VI. The rFLU-HCV/NS1 was subjected to mass incubation by chicken embryo, ultrafiltration and concentration, purification with sucrose gradient centrifugation, and then SDS-PAGE electrophoresis and gel staining. After destaining the gel, corresponding sizes of NP, HA1, HA2, NEP proteins were detected, suggesting that the main antigenic ingredients were not lost.

VII. Detection of temperature-sensitive (ts), cold-adapted (ca) and attenuated (att) phenotypes of rFLU-HCV/NS1

The rFLU-HCV/NS1 was inoculated to MDCK cells or 9-11 day aged chicken embryos, and cultured at 25, 33, 37, and 39 °C for 3 days, respectively. Supernatants of the cells or allantoic fluid from the chicken embryos were collected and measured for viral titer. The results indicated that the vaccine strain had ts and ca phenotypes. Meanwhile, the vaccine strain exhibited an attenuated phenotype in BALB/c mice and ferrets.

VIII. In vivo Immunization effect of rFLU-HCV/NS1 in mice

The rFLU-HCV/NS1 was subjected to ultrafiltration, concentration, purification with sucrose density gradient centrifugation, and then used to prepare an attenuated, live vaccine form of the vaccine.

BALB/c mice of 6-8 weeks were selected and divided into a vaccine group and a control group, 20 mice per group.

The vaccine group : the BALB/c mice were intranasally administered with the vaccine for immunization, for 2 times with an interval of 2 weeks, in a dose of $10^{5.5}$ - $10^{6.5}$ TCID₅₀ and a volume of 20 µl for each of the administration.

The control group: the same as the vaccine group in terms of the route, volume and time for immunization, except that the vaccine was replaced with PBS.

Blood was collected from each of the mice of the vaccine and control groups by bleeding the tail vein thereof 2 weeks after the first and second immunizations, respectively, and centrifuged to collect the serums of the mice of respective groups.

Titers of Antibodies against wild-type influenza virus A/California/07/2009 (H1N1) were measured in each of the serums by an HI method, and meanwhile the production of the antibodies specific to HCV was determined in each of the serums by a microneutralization method. The results are shown in Table 1.

Table 1. Detection of Immunization Effect of rFLU-HCV/NS1

Groups		Influenza virus (HI titer)	HCV (NT titer)
Vaccine	1 st dose	80.00	30.00
	2 nd dose	320.00	120.00
Control	1 st dose	<10	<10
	2 nd dose	<10	<10

As shown in Table 1 that, after immunization of animals with the HCV chimeric vaccine (rFLU-HCV/NS1) with influenza virus as vector, titers of antibodies against HCV and influenza virus were detectable in the serums thereof, suggesting that immunization with the vaccine allowed for inducing dual immune responses against HCV and influenza virus in vivo.

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Example 2. Preparation of HCV chimeric vaccine rFLU-HCV/M carrying HCV-C/E1/E2 dominant epitope using influenza virus as vector

I. HCV-C/E1/E2 dominant epitope in HCV subtype 1b with a sequence as shown in SEQ ID No.1 was subjected to validation by immunization of an animal according to the method of step I in Example 1, and validated to fit with the next experiments.

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II. Construction of recombinant plasmid pC/E1/E2-M

With M gene fragment from cold-adapted, attenuated influenza virus strain A/AA/6/60 serving as a target site at which HCV-C/ E1/E2 dominant epitope gene was to be inserted, a recombinant plasmid pC/E1/E2-M was constructed by a traditional molecular biological method. Specific strategy is shown in Figure 1B.

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Particularly, the HCV-C/E1/E2 dominant epitope gene obtained as above was inserted into the open reading frame(ORF) of M gene of the cold-adapted, attenuated influenza virus strain A/AA/6/60 behind the first 222 nucleotides and before the last 222 nucleotides, to construct a recombinant plasmid, designated as pC/E1/E2-M.

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(I) A DNA molecule was synthesized as shown in SEQ ID No.3, comprising, from 5'-end of SEQ ID No.3, an enzyme cleavage site at positions 1 to 14, 3'NCR of M gene at positions 15 to 39, 1st to 222nd nucleotides from 5'-end of M gene open reading frame(ORF) of cold-adapted, attenuated influenza virus strain A/AA/6/60 at positions 40 to 261, HCV-C/E1/E2 dominant epitope gene at positions 262 to 831, 761st to 982nd nucleotides from 5'-end of M gene open reading

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frame(ORF) of cold-adapted, attenuated influenza virus strain A/AA/6/60 at positions 832 to 1053, 5'NCR of M gene at positions 1054 to 1074, and an enzyme cleavage site at positions 1075 to 1088.

(II) The DNA molecule as shown in SEQ ID No.3 was cleaved with *Bsm*BI to obtain a target gene fragment; pAD3000 was cleaved with *Bsm*BI to obtain a large fragment of the vector; and the gene fragment was linked with the large fragment of the vector to obtain a recombinant plasmid, designated as pC/E1/E2-M, which was identified as correct by sequencing.

III. MDCK cells were co-transfected with 8 plasmids of recombinant plasmid pC/E1/E2-M, recombinant plasmids pD-PB2, pD-PB1, pD-PA, pD-NP and pD-NS respectively constructed with viral internal gene backbones PB2, PB1, PA, NP and NS from cold-adapted, attenuated influenza virus strain A/AA/6/60, and recombinant plasmids pD-HA and pD-NA respectively constructed with HA and NA genes from currently epidemic influenza virus strain A/California/07/2009 (H1N1), according to the method of step III in Example 1, and cultured at 37°C under 5% CO₂ for 48-60h, cell suspensions were obtained and inoculated to 9-day aged SPF chicken embryos, which incubated at 35°C for 72h. The allantoic fluid of the chicken embryos was collected. An HCV chimeric vaccine strain (rFLU-HCV/M) chimed with HCV-C/E1/E2 dominant epitope was obtained.

IV. The vaccine strain obtained from step III was identified, and observed via electron microscopy for viral morphology. The results showed that the vaccine strain had typical morphological characteristics of an influenza virus.

V. The 9 – 11 day aged SPF chicken embryo (purchased from Beijing Laboratory Animal Research Center) inoculated with the rFLU-HCV/M was passaged for generations. The allantoic fluid from the second generation of the chicken embryo was taken for extracting viral RNA, which was subjected to RT-PCR to amplify PB2, PB1, PA, NP, HA, NA, M and NS1, totally 8 gene fragments. These gene fragments were sent for sequencing. The results were consistent with the expected gene sequence.

VI. The rFLU-HCV/M was subjected to mass incubation by chicken embryo, ultrafiltration and concentration, purification with sucrose gradient centrifugation, and then SDS-PAGE electrophoresis. As a result, main antigenic ingredients were found.

VII. Detection of temperature-sensitive (ts), cold-adapted (ca) and attenuated (att) phenotypes of rFLU-HCV/M

The rFLU-HCV/M was inoculated to MDCK cells or 9-11 day aged chicken embryos, and

incubated at 25, 33, 37, and 39 °C for 3 days, respectively. Supernatants from the cells or allantoic fluid from the chicken embryos were measured for viral titer. The results indicated that the vaccine strain had ts and ca phenotypes. Meanwhile, the vaccine strain exhibited an attenuated phenotype in BALB/c mice and ferrets.

VIII. In vivo immunization effect of rFLU-HCV/M in mice

The rFLU-HCV/M was subjected to ultrafiltration and concentration, purification with sucrose density gradient centrifugation, and then used to prepare an attenuated, live vaccine form of the vaccine.

BALB/c mice of 6-8 weeks were selected and divided into a vaccine group and a control group, each with 20 mice.

The mice of the vaccine group were immunized with rFLU-HCV/M according to the method of step VIII in Example 1, with the control group set up as that in Example 1.

Blood was collected from each of the mice of the vaccine and control groups by bleeding the tail vein thereof 2 weeks after the first and second administrations, respectively, and centrifuged to collect the serums of the mice of respective groups.

Antibody titers against wild-type influenza virus A/California/07/2009 (H1N1) were measured in each of the serums by an HI method, and meanwhile the production of the antibody specific to HCV was determined in each of the serums by the method of step VIII in Example 1. The results are shown in Table 2.

Table 2 Detection of Immunization Effect of rFLU-HCV/M

Groups		Influenza virus (HI titer)	HCV (NT titer)
Vaccine	1 st dose	160.00	32.00
	2 nd dose	640.00	320.00
Control	1 st dose	<10	<10
	2 nd dose	<10	<10

As shown in Table 2 that, after immunization of animals with the HCV chimeric vaccine (rFLU-HCV/M) with influenza virus as vector, titers of antibodies against HCV and influenza virus were detectable in the serums thereof, suggesting that the immunization with the vaccine allowed for inducing dual immune responses to HCV and influenza virus in vivo.

Example 3. Preparation of HCV chimeric vaccine rFLU-HCV/NA chimed with HCV-C/E1/E2 dominant epitope using influenza virus as vector

I. HCV-C/E1/E2 dominant epitope in HCV subtype 1b with a sequence as shown in SEQ ID No.1 was subjected to validation by immunization of an animal according to the method of step I in Example 1, and validated to fit with the next experiments.

II. Construction of recombinant plasmid pC/E1/E2-NA

With NA gene fragment from A/California/07/2009 (H1N1) serving as a target site at which HCV-C/ E1/E2 dominant epitope gene was to be inserted, a recombinant plasmid pC/E1/E2-NA was constructed by a molecularly biological method. Specific strategy is shown in Figure 1C.

Particularly, the HCV-C/E1/E2 dominant epitope gene obtained as above was inserted into the open reading frame(ORF) of NA gene of the currently epidemic influenza virus strain, A/California/07/2009 subtype H1N1, behind the first 183 nucleotides and before the last 157 nucleotides, a recombinant plasmid was constructed and designated as pC/E1/E2-NA.

(I) A DNA molecule was synthesized as shown in SEQ ID No.4, having: from 5'-end of SEQ ID No.4, an enzyme cleavage site at positions 1 to 14, 3'NCR of NA gene at positions 15 to 35, 1st to 183rd nucleotides from 5'-end of NA gene open reading frame(ORF) of currently epidemic influenza virus strain subtype H1N1 at positions 35 to 217, HCV-C/E1/E2 dominant epitope gene at positions 218 to 787, 1254th to 1410th nucleotides from 5'-end of NA gene open reading frame(ORF) at positions 788 to 944, 5'NCR of NA gene at positions 945 to 975, and an enzyme cleavage site at positions 976 to 987.

(II) The DNA molecule as shown in SEQ ID No.4 was cleaved with *Bsm*BI to obtain a target gene fragment; pAD3000 was cleaved with *Bsm*BI to obtain a large fragment of the vector; and the gene fragment was linked with the large fragment of the vector to obtain a recombinant plasmid, designated as pC/E1/E2-NA, which was identified as correct by sequencing.

III. MDCK cells were co-transfected with 8 plasmids of recombinant plasmid pC/E1/E2-NA, recombinant plasmids pD-PB2, pD-PB1, pD-PA, pD-NP, pD-M and pD-NS respectively constructed with viral internal gene backbones PB2, PB1, PA, NP, M and NS from cold-adapted, attenuated influenza virus strain A/AA/6/60, and recombinant plasmid pD-HA constructed with HA gene from currently epidemic influenza virus strain A/California/07/2009 (H1N1), respectively, according to the method of step III in Example 1, and cultured at 37°C under 5% CO₂ for 48-60h.

Cell suspensions were obtained and inoculated to 9-day aged SPF chicken embryos, which are incubated at 33°C for 72h. The allantoic fluid of the chicken embryos was collected. An HCV chimeric vaccine strain (rFLU-HCV/NA) chimed with HCV-C/E1/E2 dominant epitope was obtained.

5 IV. The vaccine strain obtained from step III was identified, and observed via electron microscopy for viral morphology. The results showed that the vaccine strain had typical morphological characteristics of an influenza virus.

V. A 9 – 11 day aged SPF chicken embryo (purchased from Beijing Laboratory Animal Research Center) inoculated with rFLU-HCV/NA was passaged for generations. The allantoic
10 fluid from the second generation of the chicken embryo was taken for extracting viral RNA, which was subjected to RT-PCR to amplify PB2, PB1, PA, NP, HA, NA, M and NS1, totally 8 gene fragments. These gene fragments were sent for sequencing. The results were consistent with the expected gene sequence.

15 VI. The rFLU-HCV/NA was subjected to mass incubation by chicken embryo, ultrafiltration and concentration, purification with sucrose gradient centrifugation, and then SDS-PAGE electrophoresis. As a result, main antigenic ingredients were found.

VII. Detection of temperature-sensitive (ts), cold-adapted (ca) and attenuated (att) phenotypes of rFLU-HCV/NA

20 The rFLU-HCV/NA was inoculated to MDCK cells or 9-11 day aged chicken embryos, and incubated at 25, 33, 37, and 39 °C for 3 days, respectively. Supernatants of the cells or allantoic fluid from the chicken embryos were collected and measured for viral titer. The results indicated that the vaccine strain had ts and ca phenotypes. Meanwhile, the vaccine strain exhibited an attenuated phenotype in BALB/c mice and ferrets.

VIII. In vivo immunization effect of rFLU-HCV/NA in mice

25 The rFLU-HCV/NA was subjected to ultrafiltration and concentration, purification with sucrose density gradient centrifugation, and then used to prepare an attenuated, live vaccine form of the vaccine.

BALB/c mice of 6-8 weeks were selected and divided into a vaccine group and a control group,
20 mice each group.

30 The mice of the vaccine group were immunized with rFLU-HCV/NA according to the method of step VIII in Example 1, with the control group set up as that in Example 1.

Blood was collected from each of the mice of the vaccine and control groups by bleeding the tail vein thereof 2 weeks after the first and second immunizations, respectively, and centrifuged to collect the serums of the mice of respective groups.

Antibody titers against wild-type influenza virus A/California/07/2009 (H1N1) were measured in each of the serums by an HI method, and meanwhile the production of the antibody specific to HCV was determined in each of the serums by the method of step VIII in Example 1. The results are shown in Table 3.

Table 3. Detection of Immunization Effect of rFLU-HCV/NA			
Groups		Influenza virus (HI titer)	HCV (IgG NT titer)
Vaccine	1 st dose	120.00	30.00
	2 nd dose	640.00	320.00
Control	1 st dose	<10	<10
	2 nd dose	<10	<10

As shown in Table 3 that, after immunization of animals with the HCV chimeric vaccine (rFLU-HCV/NA) with influenza virus as vector, titers of antibodies against HCV and influenza virus were detectable in the serums thereof, suggesting that the immunization with the vaccine allowed for inducing dual immune responses to HCV and influenza virus in the animals.

Industrial Use

The HCV chimeric vaccine of the present invention is capable of covering the pathogens of diseases caused by HCV-infection, protecting people against influenza viruses and HCV infection, and can build up a basis for “a dual-purpose vaccine” or “a multi-purpose vaccine”.

Claims

What is claimed:

1. A DNA molecule as shown in SEQ ID No.1.

2. A recombinant virus, prepared by a method of:

co-transfecting host cells with expression vectors respectively containing a PB2 gene, a PB1 gene, a PA gene, a NP gene, a M gene and a NS gene from a cold-adapted, attenuated influenza virus, and expression vectors respectively containing an HA gene and a NA gene from a target influenza virus, and incubating the cells, to obtain a recombinant influenza virus;

inserting, or substituting with, a gene of a structural protein of Hepatitis C virus (HCV) at any position in the open reading frame (ORF) of at least one of following genes:

(1) the PB2 gene from the cold-adapted, attenuated influenza virus;

(2) the PB1 gene from the cold-adapted, attenuated influenza virus ;

(3) the PA gene from the cold-adapted, attenuated influenza virus;

(4) the NP gene from the cold-adapted, attenuated influenza virus;

(5) the M gene from the cold-adapted, attenuated influenza virus;

(6) the NS gene from the cold-adapted, attenuated influenza virus;

(7) the HA gene from the target influenza virus; and

(8) the NA gene from the target influenza virus;

wherein the gene of the structural protein of HCV is the whole gene of core protein C, the whole gene of envelope protein E1/E2, a dominant epitope gene of core protein C, and/or a dominant epitope gene of envelope protein E1/E2, from HCV.

3. The virus according to claim 2, characterized in that: the host cells are MDCK, Vero, 293T, COS cells, or co-incubated cells of MDCK/293T, MDCK/COS; and

the gene of the structural protein of HCV is a fusion gene of the dominant epitope gene of the core protein C and the dominant epitope gene of the envelope protein E1/E2, from HCV.

4. The virus according to any one of claim 2-3, characterized in that: the HCV is HCV type 1 , HCV type 2 , HCV type 3 , HCV type 4 ,HCV type 5, or HCV type 6;

the HCV type 1 is particularly HCV subtype 1b.

5. The virus according to any one of claim 2-4, characterized in that: the fusion gene of the dominant epitope gene of the core protein C and the dominant epitope gene of the envelope protein

E1/E2 from HCV has a nucleotide sequence as shown in SEQ ID No.1.

6. The virus according to any one of claim 2-5, characterized in that: the gene of the structural protein of HCV is inserted into the open reading frame of the NS gene of the cold-adapted, attenuated influenza virus between the nucleotides at positions 375 and 376 from 5'-end;

5 the gene of the structural protein of HCV is linked with the nucleotide at position 375 from 5'-end in the open reading frame of the NS gene of the cold-adapted, attenuated influenza virus via a sequence of 5'- UAAUG -3';

and/or,

10 the nucleotides from position 223 to position 760 from 5'-end in the open reading frame of the M gene of the cold-adapted, attenuated influenza virus are replaced with the gene of the structural protein of HCV;

and/or,

15 the nucleotides from position 184 to position 1253 from 5'-end in the open reading frame of the NA gene of the target influenza virus are replaced with the gene of the structural protein of HCV.

7. The virus according to any one of claim 2-6, characterized in that: the cold-adapted, attenuated influenza virus is cold-adapted, attenuated influenza virus type A, or cold-adapted, attenuated Influenza virus type B;

20 the cold-adapted, attenuated influenza virus type A is particularly cold-adapted, attenuated influenza virus subtype H2N2, more particularly cold-adapted, attenuated influenza virus strain A/Ann Arbor/6/60 (H2N2); and

the target influenza virus is Influenza virus type A, or Influenza virus type B, and the Influenza virus type A is particularly any of subtypes H1 - H16, more particularly influenza virus subtype H1N1, even more particularly influenza virus strain A/California/07/2009 (H1N1).

25 8. The virus according to any one of claim 2-7, characterized in that: the gene of the structural protein of HCV is inserted into the open reading frame of the NS gene of the cold-adapted, attenuated influenza virus between the nucleotides at positions 375 and 376 from 5'-end to obtain a sequence as represented by the nucleotides from position 51 to position 1466 of SEQ ID No.2;

30 the nucleotides from position 223 to position 760 from 5'-end in the open reading frame of the M gene of the cold-adapted, attenuated influenza virus are replaced with the gene of the structural protein of HCV to obtain a sequence as represented by nucleotides from position from 40 to

position 1053 of SEQ ID No.3;

the nucleotides from position 184 to position 1253 from 5'-end in the open reading frame of the NA gene of the target influenza virus are replaced with the gene of the structural protein of HCV to obtain a sequence as represented by the nucleotides from position from 35 to position 944 of
5 SEQ ID No.4.

9. A chimeric vaccine prepared by the virus of any of claims 2-8.

10. Use of the DNA molecule of claim 1 in the manufacture of a product for preventing and/or treating a disease caused by an influenza virus and/or HCV;
and/or,

10 use of the virus of any of claims 2-8 in the manufacture of a product for preventing and/or treating a disease caused by an influenza virus and/or HCV;
and/or,

use of the chimeric vaccine of claim 9 in the manufacture of a product for preventing and/or treating a disease caused by an influenza virus and/or HCV;

15 wherein the influenza virus is Influenza virus type A or Influenza virus type B, and the Influenza virus type A is particularly any of subtypes H1 - H16;

the HCV is HCV type 1 , HCV type 2 , HCV type 3 , HCV type 4 ,HCV type 5 or HCV type 6;
and

the HCV type 1 is particularly HCV subtype 1b.

Z1606597CPSG-English translation of Sequence Listing

Sequence Listing

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Z1606597CPSG-English translation of Sequence Listing

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