

Original Article

Adeno-associated virus vector-mediated sirna inhibition of hepatitis b virus gene expression in vitro

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Abstract: Up to date, there is no adeno-associated virus (AAV) vector-mediated gene therapy targeting Hepatitis B virus (HBV). In this study, adeno-associated virus 2 vector, AAV2, targeting Hepatitis B surface antigen (HBsAg) was constructed, and its effect of mediating the short hairpin RNA (shRNA) inhibition of HBV gene expression was investigated. Phage display technology was used to screen polypeptides that bind to HBsAg. Using PCR and cloning, a specific polypeptide was inserted at aa587 of nucleocapsid protein of AAV2, and AAV2 containing shRNA of HBsAg was also constructed. Using these two plasmids and a helper plasmid, a recombinant virus, rAAVssyU6-shRNA-hrGFP, with both specific polypeptide and shRNA was constructed. Flow cytometry was employed to evaluate the specificity of HepG2.215 cells infection by rAAVssyU6-shRNA-hrGFP and ELISA to detect its inhibitory effect to HBsAg and Hepatitis B e antigen (HBeAg) expression. The specific HBsAg polypeptide sequence is SSYAPYVWQPIA, and rAAVssyU6-shRNA-hrGFP containing both specific polypeptide and shRNA was successfully constructed and the produced virus titer was more than 10⁹ v.g./ml. The infection rate of rAAVssyU6-shRNA-hrGFP virus was higher than that of AAV2 control virus in both HepG2 and HepG2.215. Compared to HepG2, HepG2.215 obtained higher infection rate. Both HBsAg and HBeAg secretions were inhibited in HepG2.215 infected by rAAVssyU6-shRNA-hrGFP virus. Furthermore, blocking with Hepatitis B surface antibody (HBsAb) significantly inhibited the infection rate of rAAVssyU6-shRNA-hrGFP virus in HepG2.215 cells. It is promising to develop AAV-mediated gene therapy targeting HBV.

Keywords: Hepatitis B virus, adeno-associated virus vector, gene therapy, RNAi

Introduction

Viral Hepatitis B is one of intractable liver diseases that is a threat to human health [1, 2]. Up to date, interferon and nucleoside analogs are the main drugs for treating chronic Hepatitis B. Only 1/3 of the patients can tolerate the therapy with interferon. Although nucleoside analogs can inhibit Hepatitis B virus (HBV) replication, drug-resistant HBV mutants are induced after long-term use [3]. Therefore, it is crucial to develop new and effective drugs for HBV therapy. In vitro and in vivo studies showed that RNAi-mediated gene therapy could effectively inhibit HBV replication and gene expression,

indicating that it is a promising therapy strategy for Hepatitis B [4, 5]. However, how to efficiently deliver siRNA to HBV-infected liver cells and maintain its inhibitory effect is an obstacle.

Viral vectors had advantage on delivering target gene for the therapy. Among them, adeno-associated virus (AAV) vectors have been widely used in the field of gene therapy for its characters including wide range of host, no pathogenicity, and lower immunogenicity [6-8]. However, the wide range of host is also a disadvantage of AAV, which limits its use on targeting specific organs. Although some types of AAV show liver tropism, it is still impossible to target only at

AAV vector-mediated siRNA inhibited HBV

Table 1. Primers sequence

Primers	Nucleotide sequence	Note
siHBs-to p	5'-GATCC GAA TCC TCA CAA TAC CAC A-3'	shRNA expression
siHBs-bt m	5'-AGTCC TGC GGT ATT GTG AGG ATT C-3'	shRNA expression
P1	5'-GGGCGTGGATAGCGGTTT-3'	AAV titer detection
P2	5'-GGCGGAGTTGTTACGACATTT-3'	AAV titer detection
Pa	5'-TCCCGTACGT CCTCGGCTCG-3'	AAV nucleocapsid modification
Pc-12	5'-AGCAATAGGCTGCCACACATAAGGCGCATACTAGAGTTGCCTCTGGAGGTTGG TAG-3'	AAV nucleocapsid modification
Pd-12	5'-AGCAATAGGCTGCCACACATAAGGCGCATACTAGAGTTGCCTCTGGAGGTTGG TAG-3'	AAV nucleocapsid modification
Pb	5'-TCCCCCGGGCTGTAGTTAATGATTAACCCGCCA-3'	AAV nucleocapsid modification

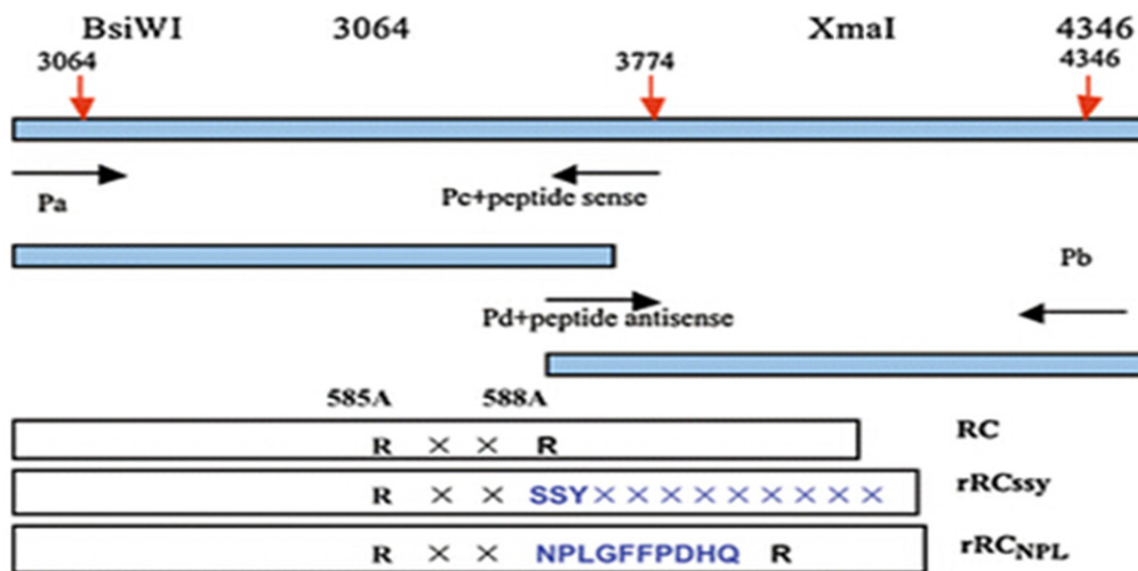


Figure 1. Recombinant RC plasmid maps.

HBV-infected liver cells. Therefore, it is necessary to construct an AAV vector targeting infected liver cells specifically.

Until now, the strategy to construct a targeting vector is to find a specific protein, which could be targeted by specific binding and help to guide siRNA into cells. As we all know that Hepatitis B surface antigen (HBsAg) is only expressed on the surface of infected liver cells, therefore HBsAg can be the targeted protein. Micromolecular polypeptides can act as a bridge due to their advantages such as simple structure, lower molecular weight, good penetration, and lower immunogenicity, and phage display technology can help to screen the polypeptides that specifically bind to HBsAg.

In this study, a polypeptide targeting HBsAg was screened by phage display technology and inserted into the nucleocapsid gene of AAV2 vector. A short hairpin RNA (shRNA) against

HBsAg was also carried in the same AAV2 vector. The new virus packaged by constructed AAV2 vector was rAAV_{ssy}U6-shRNA-hrGFP. The specificity and effectiveness of rAAV_{ssy}U6-shRNA-hrGFP was further studied in vitro. This work will shed light on the development of new gene therapy drugs for Hepatitis B.

Materials and methods

Phage display technology for screening of polypeptides specifically binding to hbsag

HBsAg produced in yeast (a gift from Wuhan institute of biologic products) was diluted to 70 µg/mL in 0.1 mol/L NaHCO₃ (pH 8.6) and added into 96-well plate at 100 µL/well for incubation at 4°C overnight. After blocking with 5% BSA in 0.1 mol/L NaHCO₃ for 2 h at 4°C, 1.0 × 10¹¹ pfu of phage was added into each well and the cells were incubated at room temperature for 1 h with gentle shaking. After washing with Tris-

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Table 2. Selected clone sequencing result of phage display technology

Clone no.	Sample no.	Amino acid sequence	Frequency
1	1, 8, 11	AHRHPISFLSTL	3/15
2	3, 7, 12	ATWSHHLSSAGL	3/15
3	5, 9, 13	SSYAPYVWQPIA	3/15
4	2	LPSPPRIPGHKL	1/15
5	4	MTKGSYSPSPWR	1/15
6	6	TRPAPPYQSQW	1/15
7	10	GHLLQYTDVMF	1/15
8	14	SWPLYSRDSSLG	1/15
9	15	YQLRPNAESLRF	1/15

buffered saline containing 0.05% Tween 20 for 10 times, the specifically bound phages were harvested by 100 μ L of elution (0.2 mol/L Glycine-HCl, pH 2.2, 1% BSA) buffer and neutralized by 1 mol/L Tris-HCl (pH 9.1). The eluate was then amplified and titrated for the next turn of screening. Three more screenings were done using the same methods described above to enrich the phage clones that were binding HBsAg. Finally, phage clones were randomly selected for DNA isolation and sequencing. Phage display peptide library used in this study was Ph.D-12TM (New England Biolabs) with *E. coli* 2738 as host bacteria at 1.0×10^{13} pfu/mL.

Construction of AAV2 Vector targeting HBsAg

Construction of recombinant rc plasmid: The HBsAg-targeting polypeptide gene sequence was amplified by PCR and inserted into RC plasmid at 3774 bp (aa 587 of nucleocapsid protein) using BsiWI and XmaI enzymes. This plasmid was rRCSSY. (Table 1) By the same method, control plasmid, rRCNPL, was constructed by the insertion of PreS1 20-29AA (NPLGFFPDHQ). All plasmids were sequenced to further confirm the correct insertion. The plasmid maps are shown in Figure 1.

Virus production: HBsAg-targeting virus package was performed as previously reported [12]. In this experiment, rRCSSY, pHelper, and pAAVU6-shHBs-hrGFP were used to pack the virus targeting HBsAg, named as rAAVssyU6-shRNA-hrGFP. RCNPL, pHelper, and pAAVU6-shHBs-hrGFP were used to pack control virus, named as rAAVNPLU6-shRNA-hrGFP. In addition, p-RC, pHelper, and pAAVU6-hrGFP were used to pack the wild control virus named as AAV-hrGFP. The

virus purification and titration were done as previously reported [12].

Virus infection and flow cytometry analysis

HepG2.215 cells were plated into 24-well plate at the density of 2×10^5 cells/well and cultured for 24 h until it reached 70-90% confluence. Cells were then infected for 2 h with rAAVssyU6-shHBs-hrGFP virus at different doses

including 2×10^9 , 2×10^8 , and 2×10^7 V.g/mL and then cultured in complete medium for 72 h. After trypsin digestion, cells were analyzed by flow cytometry (BD, USA) and GFP-positive cells were considered as infected cells. The best multiplicity of infection (MOI) was then determined. Based the optimized MOI, AAV-hrGFP, rAAVssyU6-shHBs-hrGFP, and rAAVNPLU6-shHBs-hrGFP viruses were used to infect HepG2.215 cells. Infection rates were analyzed by flow cytometry. To further confirm the specificity of HBsAg, cells were first blocked with the Hepatitis B surface antibody (HBsAb) (diluted in PBS at 1:200) (Dako).

HBsAg and hepatitis B e antigen (HBeAg) ELISA

HepG2.215 cells were plated into 24-well plate at the density of 1.5×10^5 cells/well. After being cultured for 24 h, cells were then infected with AAV-hrGFP, rAAVssyU6-shHBs-hrGFP, and rAAVNPLU6-shHBs-hrGFP viruses at 1.5×10^9 V.g/mL. After 6 h, the infected cells were washed 2 times with serum-free DMEM and cultured in normal culture medium for 4 more days. Every day, the supernatant was collected and fresh culture medium was added. All supernatant was kept at -80°C until use. HBsAg and HBeAg ELISA kits (Kehua, Shanghai, China) were then employed to detect the HBsAg and HBeAg levels in all supernatant.

Results

Screening and identification of specific binding peptide to HBsAg

After four rounds of phage peptide library screening, we randomly selected 15 clones for identification by sequencing and ELISA. In 9

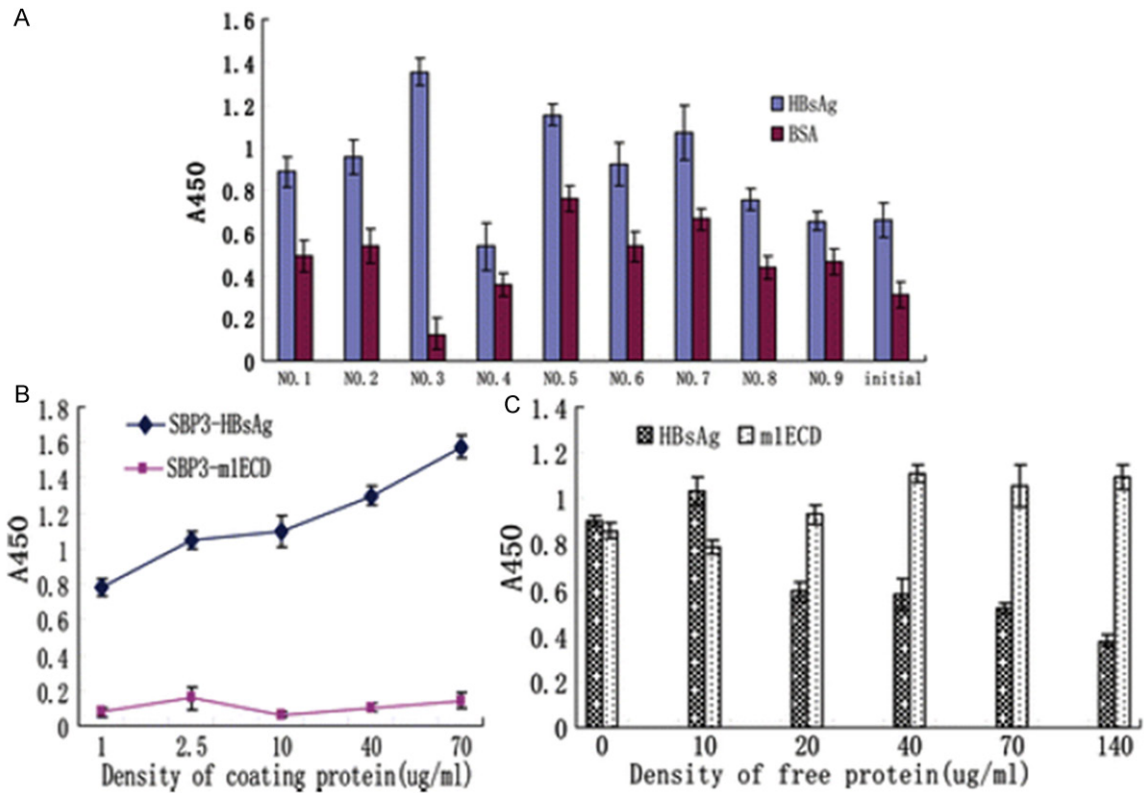


Figure 2. Specificity of polypeptide binding. A. ELISA identification of No. 1-No. 9 polypeptide; B. Binding of No. 3 polypeptide to different dose of HBsAg; C. Inhibition of unbound HBsAg to the binding of SBP3 and coated HBsAg.

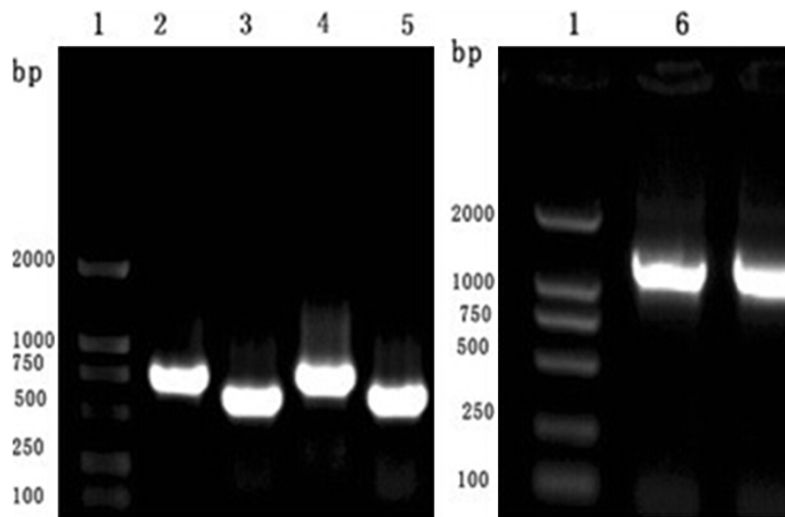


Figure 3. RCSSY PCR. 1. DL2000; 2. PCR product carrying both SBP3 and BsiWI (746 bp); 3. PCR product carrying both SBP3 and XmaI (608 bp); 4. PCR product carrying control polypeptide and BsiWI (740 bp); 5. PCR product carrying control polypeptide and XmaI (602 bp); 6. PCR product carrying the full length of SBP₃ (1318 bp); 7. PCR product carrying the full length of control polypeptide (1312 bp).

identified sequences (Table 2), the No.3 sequence, which was SSYAPYVWQPIA and named SBP3, showed the strongest binding

capacity to HBsAg and weakest binding capacity to non-related protein control, m1ECD (mouse asialoglycoprotein receptor extracellular domain) (Figure 2A). ELISA was performed with different concentrations of HBsAg to determine whether the binding of SBP3 to HBsAg was dose dependent or not. As shown in Figure 2B, by increasing the concentration of coated HBsAg, binding of SBP3 to HBsAg increased; but the binding of SBP3 to m1ECD did not increase. Competitive inhibition experiment was also performed using unbound HBsAg to competitively bind to SBP3 to confirm the binding specificity of SBP3 and HBsAg. As shown in Figure 2C, after applying the unbound HBsAg, the binding of SBP3 to coated SBP3 decreased significantly as the

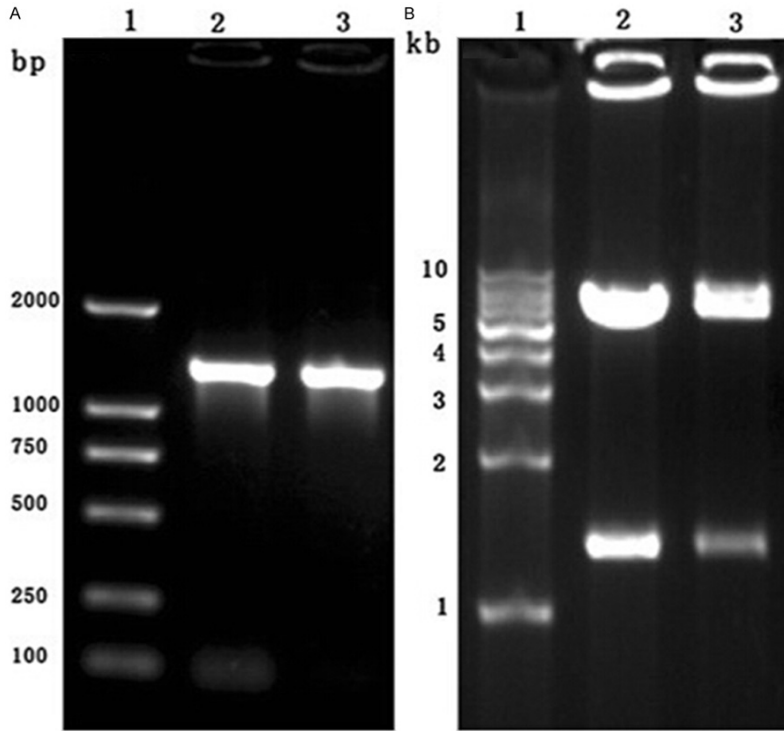


Figure 4. Recombinant RC plasmid PCR and restriction enzyme reaction identification. A. PCR. 1. DL2000; 2. rRCssy; 3. rRCNPL; B. Restriction enzyme reaction identification. 1. 1 kb DNA marker; 2. rRCssy; 3. rRCNPL.

constructed for comparing the tropism of two AAV2 vectors (Figures 3, 4). The result of the detection of virus titer is shown in Table 3.

Identification of HBsAg-targeting AAV2 virus

Confirmation of the best MOI: HepG2.215 cells were infected with three different titers of rAAVssyU6-shHBs-hrGFP virus. After 72 h, cells were digested and analyzed by flow cytometry for infection rates. MOI of 104:1, 103:1, 102:1 corresponded to the infection rate of 56 ± 6.23 , 27 ± 3.56 , $3.6 \pm 1.25\%$, respectively (Figure 5). We therefore selected the MOI of 104:1 for the further study, considering it had the highest infection rate.

Table 3. Virus titer detection

Virus	Titer (v.g/mL)
AAV-hrGFP	4.19×10^9
rAAVssyU6-shHBs-hrGFP	2.82×10^9
rAAVNPLU6-shHBs-hrGFP	3.9×10^9

unbounded HBsAg concentration increased. In addition, m1ECD has no competitive inhibition capacity to the binding of SBP3 and HBsAg. All these results suggested that the binding of SBP3 and HBsAg was specific.

Construction of AAV2 vector targeting HBsAg

The first PCR was performed to amplify polypeptide and single restriction enzyme site using p-RC plasmid as the template, and then another PCR was done with the two products from the first PCR as the templates to obtain a complete sequence containing polypeptide and two restriction enzyme sites. The obtained segments were 1318 bp (insertion of SBP3) and 1312 bp (insertion of control polypeptide, NPLGFFPDHQ). HBV PreS1 20-29AA (NPLGFFPDHQ) has liver tropism. In this experiment, the AAV2 carrying NPLGFFPDHQ was

Specificity of viral infection: The AAV-hrGFP, rAAVssyU6-shHBs-hrGFP, and rAAVNPLU6-shHBs-hrGFP viruses at MOI of 104:1 were employed to infect HepG2 and HepG2.215 cells, respectively. We found that the infection rate of rAAVssyU6-shHBs-hrGFP virus ($61.8 \pm 6.23\%$) to HepG2.215 cells was significantly higher than that of AAV-hrGFP ($3.22 \pm 0.67\%$) and rAAVNPLU6-shHBs-hrGFP ($2.5 \pm 0.51\%$) (Figures 6, 7). Moreover, the infection rate of rAAVssyU6-shHBs-hrGFP virus ($8.7 \pm 0.7\%$) to HepG2 cells was also higher than that of AAV-hrGFP ($0.73 \pm 0.38\%$) and rAAVNPLU6-shHBs-hrGFP ($0.37 \pm 0.35\%$) (Figures 6, 7). When cells were blocked with HBsAb prior to the infection, the infection rate of modified virus decreased significantly, but that of control virus did not change (Figure 8).

Inhibition of HBsAg and HBeAg secretion from HepG2.215 cells by HBsAg-targeting AAV2 virus

HepG2.215 cells were infected by AAV-hrGFP and rAAVssyU6-shHBs-hrGFP viruses at MOI of 104:1, respectively. After 2 h infection, the supernatant was removed and fresh complete

AAV vector-mediated siRNA inhibited HBV

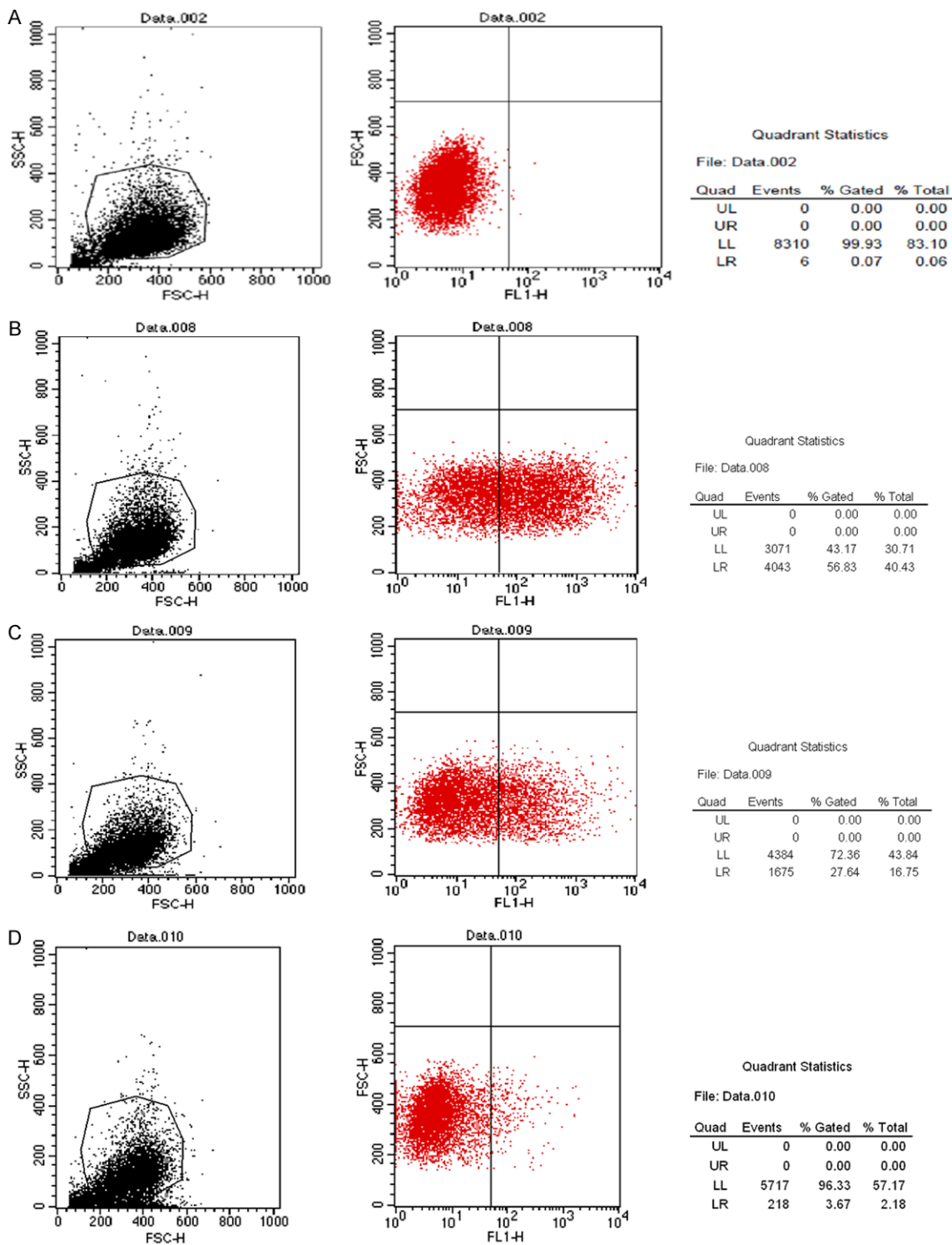
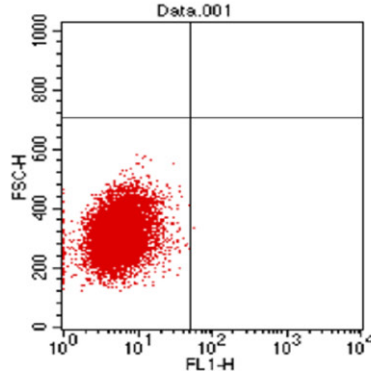
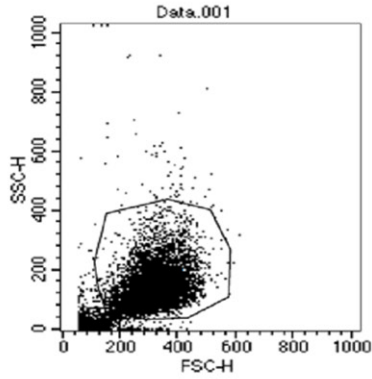


Figure 5. HepG2.215 cells infection analyzed by flow cytometry. A. Without infection; B. MOI of 10^4 :1; C. MOI of 10^3 :1; D. MOI of 10^2 :1.

culture medium was added. The supernatant was collected every 24 h for 4 days. ELISA was

then used to detect the HBsAg and HBeAg in the harvested supernatant. Our result indicat-

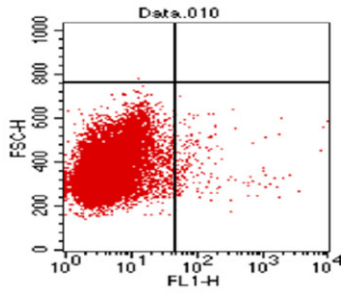
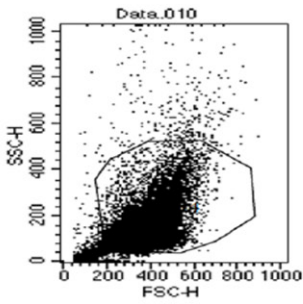
AAV vector-mediated siRNA inhibited HBV



Quadrant Statistics

File: Data.001

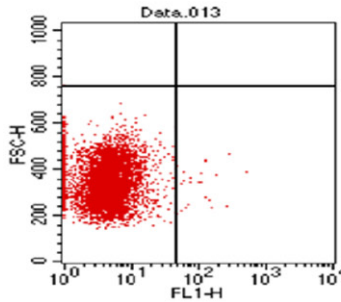
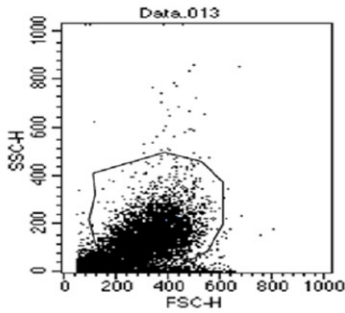
Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	0	0.00	0.00
LL	8194	99.99	81.94
LR	1	0.01	0.01



Quadrant Statistics

File: Data.010

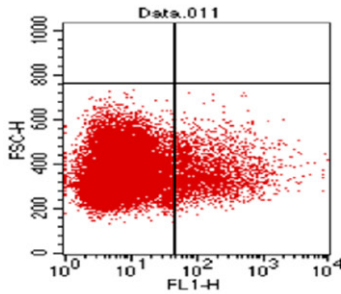
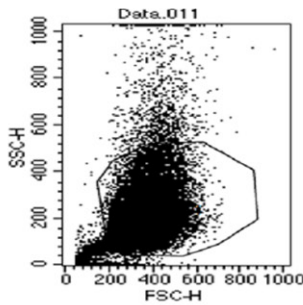
Quad	Events	% Gated	% Total
UL	1	0.00	0.00
UR	0	0.00	0.00
LL	27808	99.27	92.69
LR	204	0.73	0.68



Quadrant Statistics

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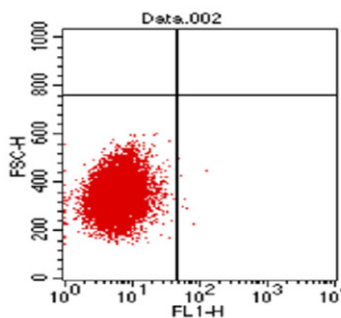
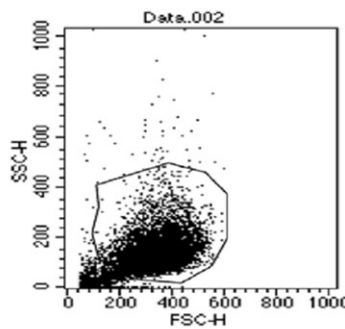
Quad	Events	% Gated	% Total
UL	1	0.02	0.01
UR	0	0.00	0.00
LL	5883	99.61	58.83
LR	22	0.37	0.22



Quadrant Statistics

File: Data.011

Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	0	0.00	0.00
LL	22986	91.25	76.62
LR	2205	8.75	7.35



Quadrant Statistics

File: Data.002

Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	0	0.00	0.00
LL	8178	99.91	81.78
LR	7	0.09	0.07

AAV vector-mediated siRNA inhibited HBV

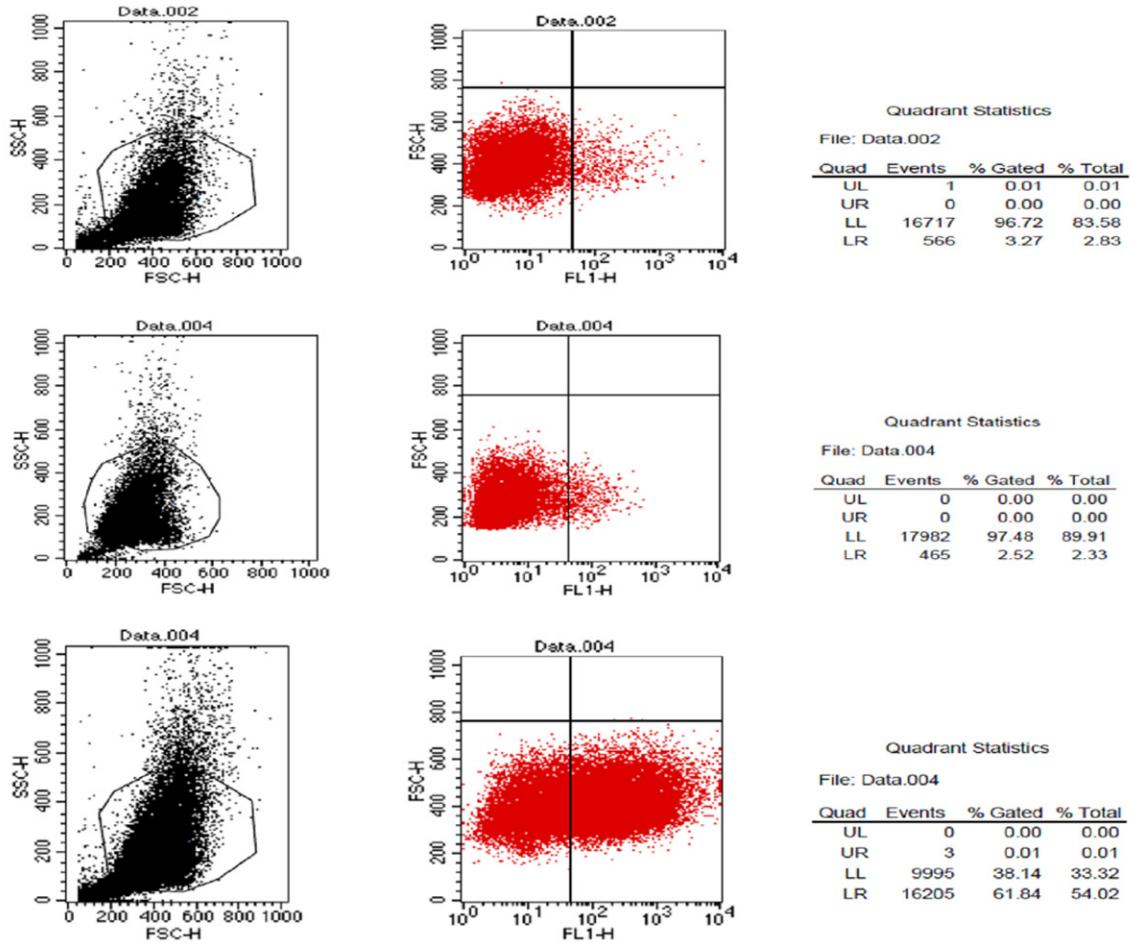


Figure 6. HepG2 and HepG2.215 cells infection analyzed by flow cytometry.

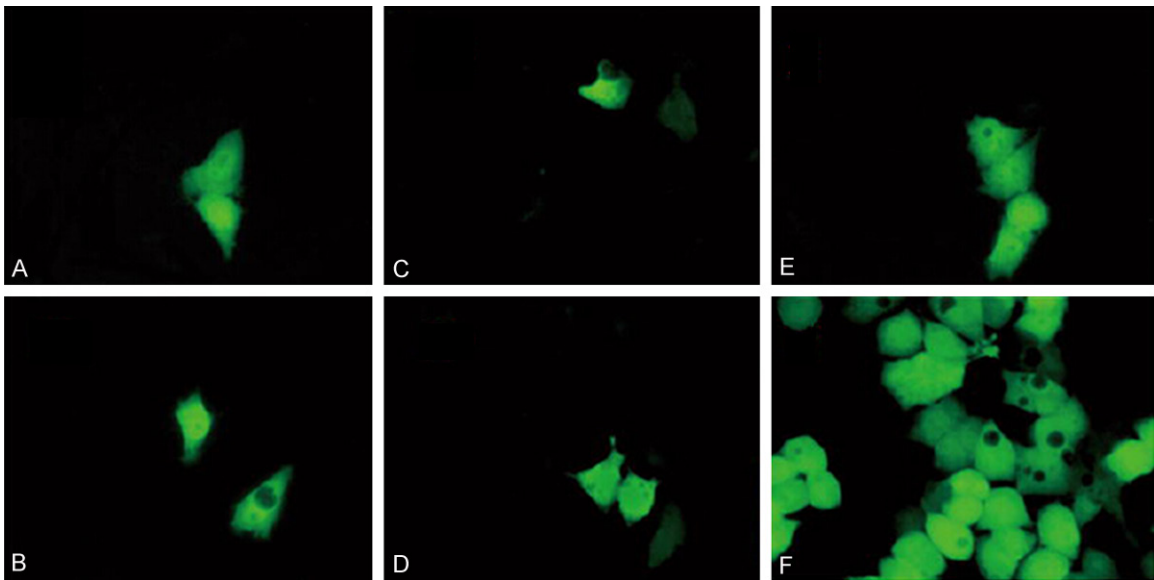


Figure 7. Viral infection detected by immunofluorescence microscope. A. AAV-hrGFP virus-infected HepG2 cells; B. AAV-hrGFP virus-infected HepG2.215 cells; C. rAAVNPLU6-shHBs-hrGFP virus-infected HepG2 cells; D. rAAVNPLU6-shHBs-hrGFP virus-infected HepG2.215 cells; E. rAAVSSYU6-shHBs-hrGFP virus-infected HepG2 cells; F. rAAVSSYU6-shHBs-hrGFP virus-infected HepG2.215 cells.

AAV vector-mediated siRNA inhibited HBV

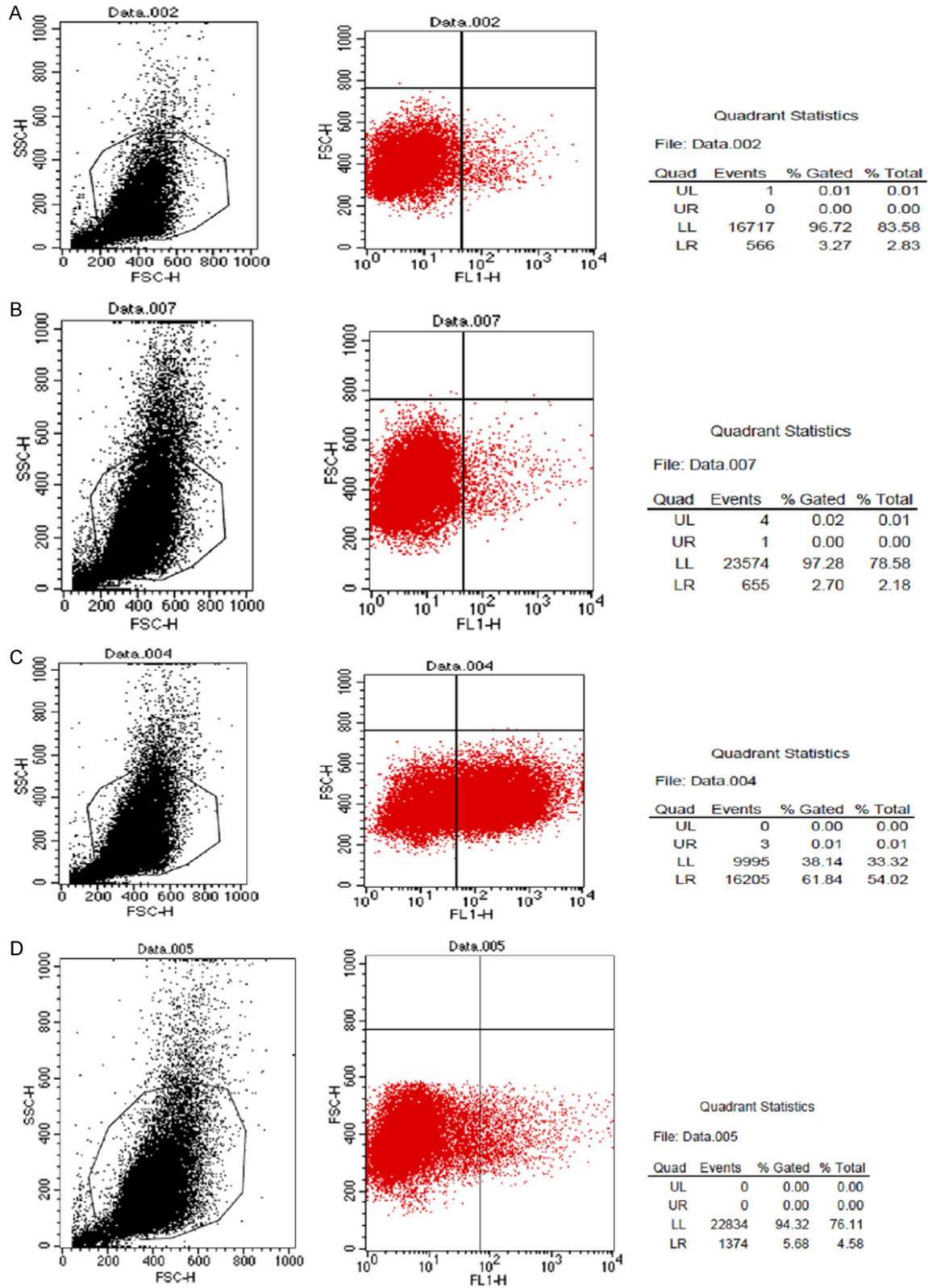


Figure 8. Specificity of viral infection to HepG2.215 cells.

ed that, at the third day after infection, the inhibitory effect of rAAVssyU6-shHBs-hrGFP to

HepG2.215 secretion of HBsAg and HBeAg was the highest (90%) (Figure 9).

AAV vector-mediated siRNA inhibited HBV

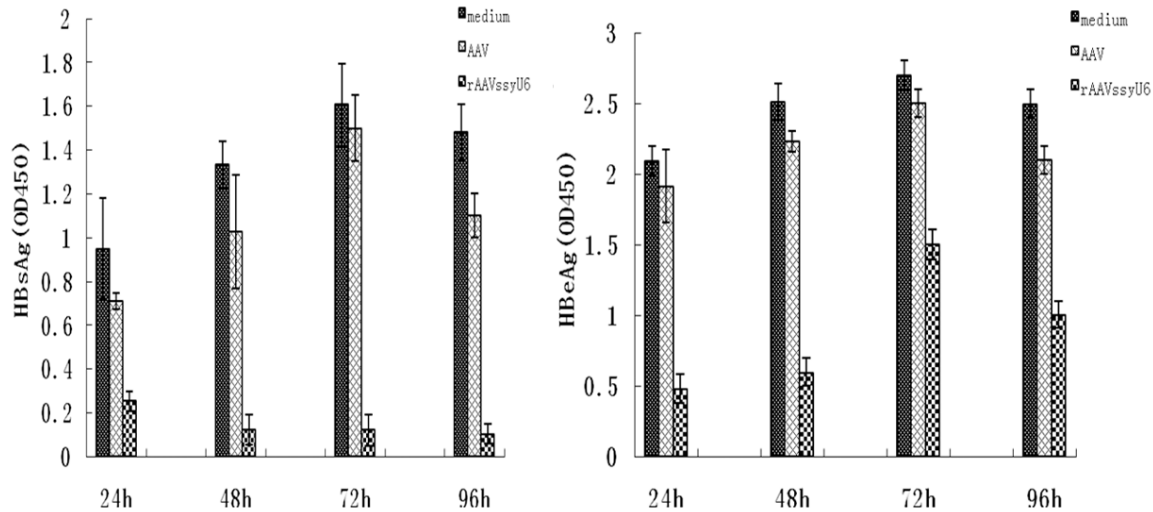


Figure 9. Detection of HBsAg and HBeAg levels by ELISA.

Discussion

Virus carrying shRNA can achieve long-term regulation of target gene in mammals, and is widely used in siRNA-mediated gene therapy. AAV vectors are the most popular vectors used in gene therapy because of their advantages including efficient package, lower immunogenicity, and safety [6-8]. In recent years, researchers successfully performed *in vivo* and *in vitro* studies using AAV carrying HBV siRNA and obtained promising results [13]. AAV2 is, up to date, the safest AAV vector. However, lack of tropism limits its application in gene therapy [14]. Although AAV2 pseudovirus showed strong liver tropism, it still cannot precisely target HBV-infected cells [9-11]. Therefore, we modified the AAV2 nucleocapsid protein to achieve the precise targeting to the HBV-infected liver cells and inserted the HBsAg siRNA into the AAV2 to observe the therapeutic effect on HBV infection.

In this study, the constructed pAAV-shHBs-hrGFP has mammal U6 promoter, which can start the transcription on predesigned site, producing the shRNA with stem-loop structure. The shRNA sequence was derived from the inner of s gene, which was capable of degrading the three mRNA of HBV (3.5 kb, the template of DNA replication and nuclear protein and virus polymerase; 2.4 kb, responsible for synthesis of PreS1 protein; and 2.1 kb, responsible for synthesis of PreS2 and HBsAg proteins) and inhibition of virus replication and protein syn-

thesis for packaging. In a previous study published by our group, we have proved that transfection of pAAV-shHBs-hrGFP into HepG2.215 significantly inhibited its secretion of HBsAg and HBeAg; however, transfection of AAV-hrGFP plasmid with non-related siRNA into HepG2.215 cells did not influence secretion of HBsAg and HBeAg [12], suggesting that shRNA sequence could significantly inhibit HBV replication and protein expressions for packaging virus.

In addition, we modified the p-RC plasmid by inserting HBsAg-specific polypeptide, SBP3 into the AAV nucleocapsid protein. Using the same strategy, we inserted PreS1 20-29AA (NPLGFFPDHQ) into the AAV nucleocapsid protein. The reason we chose PreS120-29AA (NPLGFFPDHQ) in this study was because Urban et al. had reported that this peptide could stop the HBV infection to primary culture liver cells from shrews and it had liver tropism [15, 16]. Therefore, it is a good control for studying the liver tropism. Girod et al. first successfully achieved the AAV2 tropism modification by insertion of polypeptide at aa 587 of AAV nucleocapsid in 1997 [17], and Shi et al. also proved that aa 588 of AAV2 nucleocapsid protein was a suitable insertion site for polypeptide [18]. These two sites later became the widely used insertion sites for small polypeptides [19, 20]. Yu et al. inserted a polypeptide targeting skeletal muscles at aa 587 and proved its tropism to skeletal muscles in both *in vitro* and *in vivo*, indicating the potential of modified AAV2 on both local and systemic applica-

tions [21]. Given these previous findings, we decided to insert the polypeptide at aa 587 of AAV2 nucleocapsid protein.

Virus package was done in AAV293 cells by transfecting the modified p-RC, pAAV-shHBs-hrGFP, and pHelper. The harvested rAAVssyU6-shHBs-hrGFP virus showed significantly higher infection rate ($61.8 \pm 6.23\%$) compared with those of AAV-hrGFP ($3.22 \pm 0.67\%$) and rAAVN-PLU6-shHBs-hrGFP (2.47%).

Although the infection rate of rAAVssyU6-shHBs-hrGFP virus ($8.7 \pm 0.7\%$) to HepG2 cells also increased compared with AAV-hrGFP ($0.73 \pm 0.38\%$), we considered it as non-specific infection since the infection rate was still lower than 10%. This non-specific infection might be mediated by the natural receptor HSPG and also possibly resulted from the modified natural construction by changing of viral nucleocapsid protein. To further confirm the viral specificity, we detected the infection rate after blocking HepG2.215 cells with HBsAb. Blocking with HBsAb significantly decreased the infection rate of rAAVssyU6-shHBs-hrGFP virus, but showed no influence on the AAV-hrGFP virus, indicating the strong infection specificity of rAAVssyU6-shHBs-hrGFP virus.

Finally, the supernatant of infected HepG2.215 cells were harvested for further analysis of HBsAg and HBeAg levels by ELISA. The result suggested that rAAVssyU6-shHBs-hrGFP inhibited secretion of both HBsAg and HBeAg, with stronger effect on HBsAg. However, the specificity and efficacy of this modified AAV2 virus needs to be further investigated in vivo.

Overall, AAV is a good gene therapy vector for achieving targeting and efficacy to specific cells by the insertion of siRNA and specific binding sequence. This study provided a new therapy strategy to HBV infection. We should not only consider higher infection efficacy to inhibit HBV replication and antigen expression, but also the specificity and safety. In the serum samples of the HBV-infected patient, there is high level of HBsAg, which may decrease the targeting effect of modified AAV2 virus to HBV-infected cells. In addition, we should avoid the cytotoxic effect of T cells immune response in HBV-infected patient who has been infected by AAV2 before, and evaluate the risk of AAV virus gene integration into human genome.

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Disclosure of conflict of interest

None.

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AAV vector-mediated siRNA inhibited HBV

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