

## Antiviral effect of hepatitis B virus S/C gene loci antisense locked nucleic acid on transgenic mice *in vivo*

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**ABSTRACT.** We investigated the effects of hepatitis B virus (HBV) S/C double gene loci antisense locked nucleic acid on replication and expression of HBV in hepatitis transgenic mice. HBV mice (N = 30) were randomly divided into five groups of six mice: 5% glucose solution control, empty liposome control, single-target S, single-target C, and dual-target SC groups. An antisense locked nucleic acid fragment was injected into the mice. Serum HBsAg, serum HBV DNA, HBV C-mRNA expression in liver tissue, HbsAg and HbcAg expression in hepatocytes, serum albumin, alanine transaminase (ALT), urea nitrogen, and creatinine were detected. Liver and kidney sections were examined for the effects of antisense locked nucleic acid. The expression of HBsAg was markedly inhibited; the inhibition rates of the S, C, and SC target groups were 36.63, 31.50, and 54.87%, respectively; the replication of HBV DNA was also inhibited: 23.97, 21.13, and 35.83%, respectively. After injection at 1, 3, and 5 days, the corresponding rates for HBsAg inhibition were 14.40, 25.61, and 31.33%, and for HBV DNA inhibition they were 11.04, 19.24, and 24.13%. Compared with the control group, the differences in serum albumin, ALT, urea nitrogen,

and creatinine in each group were not statistically significant, and the number of HbsAg- and HBcAg-positive cells in the mouse liver was significantly reduced. The liver and kidney tissues were normal. The gene therapy had significant inhibitory effects on the replication and expression of HBV in transgenic mice, and double-gene targeting was better than single-gene targeting.

**Key words:** Liposome; Locked nucleic acid; Gene therapy; HBV; Hepatitis B virus; Transgenic mice

## INTRODUCTION

Locked nucleic acid (LNA) is a recently discovered cyclic nucleotide derivative. When oligomers containing it are compared with other oligonucleotides, they have better thermal stability, higher molecular hybridization capability, and stronger resistance to nuclease degradation (Wengel et al., 2003; Inohara et al., 2004; Elmén et al., 2005; Castoldi et al., 2006; Rapozzi et al., 2006; Swayze et al., 2007). Therefore, it has broad application prospects. Our preliminary study results show that LNA can effectively inhibit the replication and expression of hepatitis B virus (HBV) by affecting the HBV S gene (Tang and Wang, 2006; Deng et al., 2009). In this study, we designed and synthesized LNA fragments against the HBV S/C double gene. LNA was imported into the hepatocytes of HBV transgenic mice via the tail vein using the tissue-targeting capability of cationic liposomes. The antiviral effects of double gene loci antisense LNA were then observed.

## MATERIAL AND METHODS

### Materials

#### *Experimental animals*

HBV transgenic mice (N = 30) were purchased from the Guangzhou Military Air Force Hospital of the People's Liberation Army, China. The mice weighed 18-20 g, the sex ratio was 1:1, and all mice were positive for serum HBsAg and HBV DNA.

#### *Main reagents and instruments*

A liposome transfection reagent was purchased from the Invitrogen Company; a HBsAg quantitative detection kit was purchased from Suzhou Xinbo Biological Technology Co., Ltd.; a HBV DNA quantitative detection kit was purchased from the Shenzhen PG Biotechnology Co., Ltd; an RNA extraction kit was purchased from the American Sangon Company; oligodeoxy thymine nucleotide, avian and marrow cell tumor virus reverse transcriptase, RNA enzyme inhibitor, Taq enzyme, buffer, and three phosphate DNA were purchased from the TaKaRa Company in Japan; the primary antibodies of mouse anti-HBs and mouse anti-HBc were bought from the Wuhan Boster Company; a PV-6002 two-step immunohistochemical detection kit was purchased from the Beijing Zhongshan Jinqiao Biological Technology Co. Ltd; albumin, alanine transaminase (ALT), urea nitrogen, and

creatinine assay kits were purchased from Shanghai Rongsheng Biological Technology Co., Ltd.; the time-resolved fluoroimmunoassay instrument was purchased from the Wallac Company, USA; the real-time quantitative polymerase chain reaction (PCR) instrument was purchased from the ABI Company, USA; the thermal cycling PCR instrument was purchased from the Biometra Company, Germany; and the Hitachi 7150 automatic biochemistry analyzer was purchased from the Hitachi Company, Japan.

## Methods

### *Synthesis and modification of antisense LNA*

We separately synthesized the translation initiation region 157-167-nucleotides (nt) S fragment (5'-TaCcTctTgTa-3', wherein the uppercase letters represent LNA and lowercase letters represent DNA), and the translation initiation region 1813-1823-nt C fragment (5'-TgGtAcgTtGa-3'), which were complementary to the HBV (ayw subtype) S gene and the HBV C gene, respectively. The syntheses, modifications, and purification were carried out by the GeneLink Company, USA.

### *Preparation of liposome-encapsulated LNA*

Liposomes were mixed with 5% glucose solution in a ratio of 2:3, and an equal volume of LNA (200 mg/L) solution was quickly added. The liposome-LNA mixture was kept at room temperature for 1 h.

### *Treatment of transgenic mice*

The 30 HBV transgenic mice were randomly divided into five groups with six mice in each group as follows: a blank control group (injection of 5% glucose solution); an empty liposomes control group; a single-target area S group; a single-target area C group; and a dual-target SC group. Each mouse in the target groups was injected with the liposome-LNA mixture (200  $\mu$ L) via the tail vein at 1, 3, and 5 days. The same volume of 5% glucose solution was injected into the blank control (or empty liposome) group mice. Blood samples were collected from the orbital venous before and after injection on days 1, 3, and 5, and centrifuged at 5000 revolutions per minute for 5 min. The upper serum was removed and put into sterile microcentrifuge tubes that were labelled appropriately. The serum was stored at -20°C until required for inspection. The mice were sacrificed after the final injection on day 5, and the liver and kidneys were fixed by 4% formalin.

### *Detection of serum HBsAg*

The serum HbsAg was detected by time-resolved fluoroimmunoassay analysis, according to the manufacturer instructions, and the concentration of HBsAg was expressed in ng/mL.

### *Detection of HBV DNA*

A total of 25  $\mu$ L reaction solution and sample DNA were added to the PCR tube, and

each reaction tube was placed in the fluorescence quantitative PCR instrument. The amplification conditions were: heat preservation at 37°C for 2 min, pre-degeneration at 94°C for 3 min, 94°C for 5 s, 60°C for 40 s, for a total of 40 cycles. The collected fluorescence signal was analyzed and quantified automatically by the computer software. The HBV DNA average copy number was calculated using mean logarithmic values.

### ***Detection of HBV C-mRNA***

RNA extraction was conducted follows: after the injection on day 5, the mice were killed. The fresh liver tissue samples of approximately 1.0 x 0.5 x 0.5 cm and 1 mL iced RNA extraction reagent were quickly homogenized, and 0.2 mL chloroform was added. The mixture was kept at room temperature for 15 min and centrifuged at 12,000 g at 4°C for 15 min. Isopropyl alcohol (0.5 mL) was then added to the supernatant, which was kept at room temperature for 10 min and centrifuged at 12,000 g for 10 min. The supernatant was discarded and 1 mL 75% focal carbonate ethyl ester two ethanol solution was added. The mixture was centrifuged at 7500 g at 4°C for 5 min, the supernatant was discarded, and the mixture was dried at room temperature for 5 min and dissolved in sterile two ethyl carbonate water.

For the RNA reverse transcription, we designed and evaluated the primers using software according to the base sequence of the objective gene fragment; the synthesis and purification were carried out by the Shanghai Invitrogen Biotechnology Co., Ltd. The template RNA and primer mixture were prepared (taking the upstream primer in each group) in the micro reaction tube to a total volume of 12 µL. The reaction mixture was kept at 65°C for 5 min and rapidly cooled on ice. We then added 8 µL 2X PCR pre-reaction solution (Premix EX Taq), mixed gently, kept the mixture at room temperature for 10 min, maintained at 42°C using a thermostat for 60 min, increased the temperature to 70°C for 10 min, and cooled in ice for 2 min.

For PCR amplification of C gene DNA, we placed the reaction tube described above into the PCR amplification instrument, and used the following designed C region primers: upstream 5'-CTGGGTGGGTGTTAATTTGG-3'; downstream 5'-TAAGCTGGAGGAGTGCGAAT-3'; the product length was 186 bp. The reaction conditions were: pre-degeneration at 94°C for 5 min; 94°C for 30 s, 58°C for 30 s, 72°C for 1 min, for a total of 35 cycles, then terminal extension at 72°C for 10 min. According to the electrophoresis strip of the HBV PCR product and the relative intensity ratio of the β-actin inner control (550 bp) band, we determined the degree of inhibition of the antisense locked nucleic acid on HBV C gene mRNA.

### ***Detection of liver and kidney function***

The serum albumin was detected using bromocresol green; ALT levels were compared with the International Clinical Chemistry Federation recommended levels; urea nitrogen was detected using the continuous monitoring method; creatinine detection was by the picric acid method. The above markers were all detected using an automatic biochemistry analyzer. Serum determinations were carried out at an isotonic saline dilution of 1:20.

### ***Detection of HBsAg and HbcAg in liver tissue***

The liver tissue was embedded in paraffin and sectioned. It was investigated using a two-step immunohistochemical method, dyed with two amino benzidine reagents, and re-

stained with hematoxylin. The mouse anti-HBs was diluted to 1:100, and the mouse anti-HBc was diluted to 1:40, as specified in the literature. The staining of HBsAg and HBcAg in the liver tissues was observed by electron microscopy to determine the inhibitory effect of LNA.

### ***Pathology of liver and kidney tissues of mice***

After embedding the mouse liver and kidney tissues in paraffin, slicing, and staining with hematoxylin and eosin, the organizational structure of the mouse organs was observed by electron microscope to determine the damage inflicted by the liposomes.

### **Statistical analysis**

All data are reported as means  $\pm$  SD. We used the SPSS 12.0 statistical software. The groups were compared using the Student-Newman-Keuls test, and the Kruskal-Wallis H-test was used for repeated analysis of variance. The inhibition rate (%) = (before treatment N - after administration N) / before treatment N  $\times$  100%.

## **RESULTS**

### **Inhibitory effect of antisense LNA on HBsAg**

After injection of LNA into the mice, all target groups exhibited strong inhibitory effects on the expression of HBsAg ( $F = 276.02$ ,  $P < 0.05$ ); the average inhibition rates of the single-target S group, the single-target C group, and the dual-target SC group were 36.63, 31.50, and 54.87%, respectively. Compared with before injection, the secretion of HbsAg also decreased significantly ( $F = 210.14$ ,  $P < 0.05$ ); the average rates of decrease after injection on days 1, 3, and 5 were 14.40, 25.61, and 31.33%, respectively. This suggests that the inhibitory effect of LNA on the HBsAg in animals is time-dependent, and the dual-target area has a stronger inhibitory effect than the single-target area (see Table 1).

**Table 1.** Detection of serum HBsAg in mice (N = 6, means  $\pm$  SD, ng/mL).

Group	Before injection	After injection		
		1 day	3 days	5 days
5% glucose control group	106.96 $\pm$ 8.12	108.23 $\pm$ 8.31 <sup>h</sup>	107.45 $\pm$ 6.83 <sup>i</sup>	107.15 $\pm$ 6.57 <sup>j</sup>
Empty liposome group	106.35 $\pm$ 7.22	107.45 $\pm$ 7.52 <sup>h</sup>	106.98 $\pm$ 6.91 <sup>i</sup>	107.16 $\pm$ 7.16 <sup>i</sup>
Single-target S group	107.80 $\pm$ 4.11 <sup>a</sup>	82.66 $\pm$ 1.29 <sup>ah</sup>	66.88 $\pm$ 2.66 <sup>ai</sup>	55.28 $\pm$ 2.56 <sup>aj</sup>
Single-target C group	105.90 $\pm$ 5.52 <sup>b</sup>	83.42 $\pm$ 2.94 <sup>bh</sup>	71.42 $\pm$ 4.22 <sup>bi</sup>	62.83 $\pm$ 6.38 <sup>bj</sup>
Dual-target SC group	106.90 $\pm$ 8.37 <sup>c</sup>	74.18 $\pm$ 3.47 <sup>ch</sup>	41.53 $\pm$ 2.17 <sup>ci</sup>	29.13 $\pm$ 2.75 <sup>cj</sup>

Repeated analysis of variance using the Student-Newman-Keuls test. Compared with the control group:  $F = 276.02$ ,  $P < 0.05$ ; compared with before injection:  $F = 210.14$ ,  $P < 0.05$ .

### **Inhibitory effect of antisense LNA on HBV DNA**

The investigation of the inhibitory effect of LNA on the replication of HBV DNA revealed that the average inhibition rates in the single-target S group, the single-target C group, and the dual-target SC group were 23.97, 21.13, and 35.83%, respectively. Compared with

before injection, the replication of HBV DNA also decreased significantly. The average rates of decrease were 11.04, 19.24, and 24.13%, respectively, after injection at days 1, 3, and 5 (see Table 2).

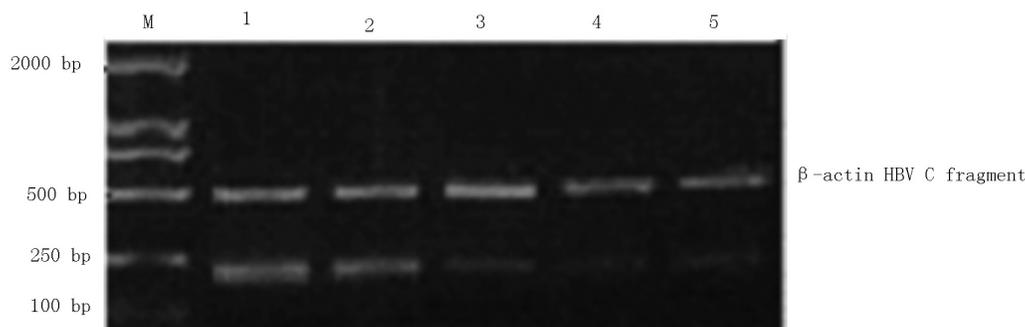
**Table 2.** Detection of serum HBV DNA in mice (N = 6, means  $\pm$  SD,  $\times 10$  copies/mL).

Group	Before injection	After injection		
		1 day	3 days	5 days
5% glucose control group	5.96 $\pm$ 0.17	5.29 $\pm$ 0.27 <sup>h</sup>	5.40 $\pm$ 0.17 <sup>i</sup>	5.38 $\pm$ 0.19 <sup>j</sup>
Empty liposome group	5.44 $\pm$ 0.18	5.38 $\pm$ 0.32 <sup>h</sup>	5.42 $\pm$ 0.34 <sup>i</sup>	5.51 $\pm$ 0.37 <sup>j</sup>
Single-target S group	5.48 $\pm$ 0.31 <sup>a</sup>	4.79 $\pm$ 0.26 <sup>ab</sup>	4.03 $\pm$ 0.16 <sup>ai</sup>	3.68 $\pm$ 0.17 <sup>aj</sup>
Single-target C group	5.42 $\pm$ 0.32 <sup>b</sup>	4.79 $\pm$ 0.18 <sup>bb</sup>	4.09 $\pm$ 0.13 <sup>bi</sup>	3.94 $\pm$ 0.15 <sup>bj</sup>
Dual-target SC group	5.51 $\pm$ 0.24 <sup>c</sup>	4.49 $\pm$ 0.37 <sup>cb</sup>	3.52 $\pm$ 0.33 <sup>ci</sup>	2.59 $\pm$ 0.23 <sup>cj</sup>

Repeated analysis of variance by Student-Newman-Keuls test. Compared with the control group: F = 141.12, P < 0.05; compared with before injection: F = 141.41, P < 0.05.

### Inhibitory effect of antisense LNA on HBV C-mRNA

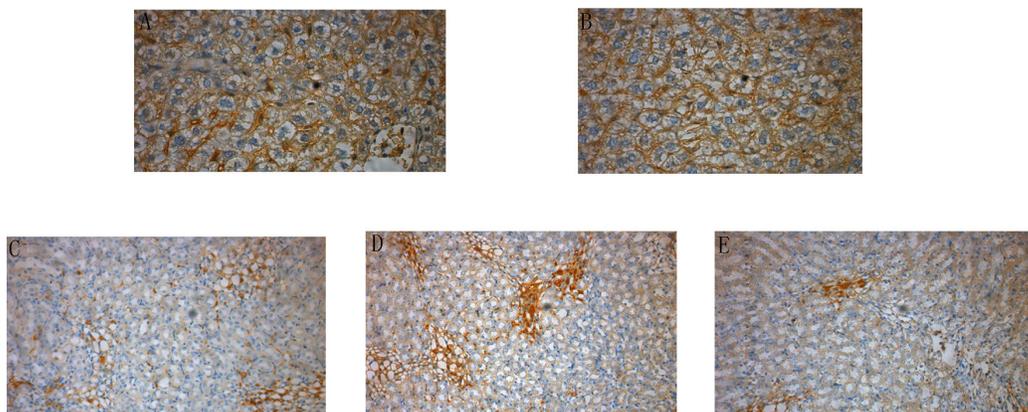
LNA had an inhibitory effect on the expression of hepatitis B virus C gene mRNA. The average grayscale values of lanes 1-5 for HBV C-DNA (with  $\beta$ -actin set to 1) were 1.006, 0.998, 0.412, 0.404, and 0.401, respectively (see Figure 1). This suggests that the target groups all experienced decreased HBV C-mRNA expression levels in hepatic cells, but the inhibitory effect on the single-target group S was relatively low, and there was no significant difference in inhibitory effects between the single-target C group and the dual-target SC group.



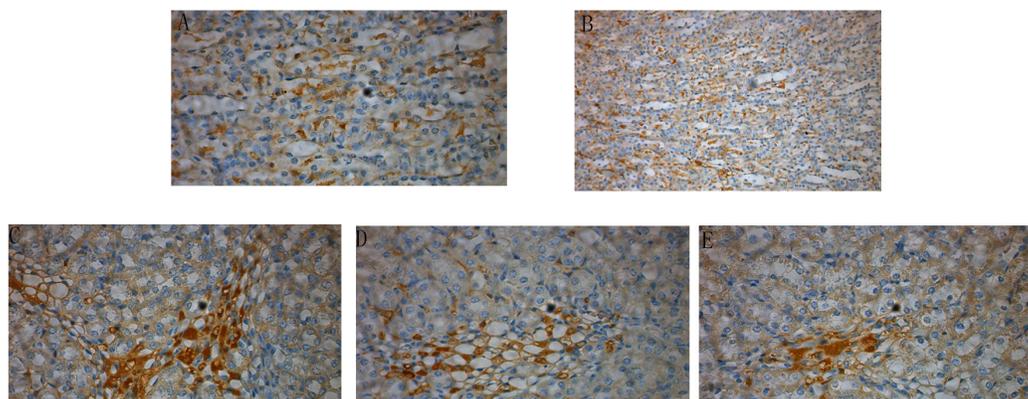
**Figure 1.** Results of electrophoresis showing expression levels of hepatitis B virus (HBV) C-mRNA in the mice. Lane M = DNA ladder. Lane 1 = 5% glucose control group; lane 2 = empty liposome control group; lane 3 = single-target S group; lane 4 = single-target C group; lane 5 = dual-target SC group.

### Expression of HBsAg and HBcAg in liver tissue detected by immunohistochemistry

The immunohistochemical detection results using an electron microscope showed that the number of HbsAg-positive cells (Figure 2A-E) and the number of HBcAg-positive cells (Figure 3A-E) in the target group liver tissue sections were all lower than those in the control group. This suggests that antisense LNA can inhibit the expression of HBsAg and HBcAg in hepatic cells.



**Figure 2.** Distribution of HBsAg in each group of mice livers using 3,3'-diaminobenzidine (DAB) staining (100X magnification). **A.** 5% glucose control group. **B.** Empty liposome control group. **C.** Single-target S group. **D.** Single-target C group. **E.** Dual-target SC group.



**Figure 3.** Distribution of HBcAg in each group of mice livers using 3,3'-diaminobenzidine (DAB) staining (100X magnification). **A.** 5% glucose control group. **B.** Empty liposome control group. **C.** Single-target S group. **D.** Single-target C group. **E.** Dual-target SC group.

### Toxic effect of LNA on liver and kidney tissue

Compared with the control group, the differences in serum albumin, ALT, urea nitrogen, creatinine, and other liver and kidney function indices in the mice from each group were not statistically significant. There were no significant differences in the liver and renal tissue structures among groups, suggesting that antisense LNA had no obvious toxicity effect on liver and kidney function or structure organization.

### DISCUSSION

In this study, we used liposomes as an antisense LNA drug delivery system; conju-

gates were formed through the interaction between the positively charged (cationic) liposomes and the negatively charged lipophilic LNA series. When lipophilic liposomes are introduced to the liver cells, the LNA sequence is released slowly and combines with the intracellular HBV S and HBV C gene complementary regions, which affects its antiviral role. The results showed that the expression of HBsAg and the replication of HBV DNA in the target groups were significantly reduced after injection of LNA ( $P < 0.05$ ), and the dual-target group showed the greatest reductions ( $P < 0.05$ ). After injection on day 5, the rates of decrease in HBsAg and HBV DNA in the dual-target SC group were 72.8 and 52.9%, respectively (see Tables 1 and 2). The immunohistochemical results also showed that the number of HbsAg- and HbcAg-positive cells in liver cells were significantly reduced when compared with the control group (Figures 2A-E and 3A-E), which suggests that antisense LNA mediated by cationic liposomes can effectively enter the liver cells via tail vein injection and play a role in anti-HBV replication and expression. Moreover, the antiviral effect was time-dependent. Because the HBV DNA polymerase lacks proofreading functionality, the production of mutations, which confer resistance, is possible in the replication process. Therefore, it is necessary to study multilocus combined drugs. Our results showed that the double gene target area has a stronger antiviral effect than the single gene target area, which is consistent with previous literature (Zhao et al., 2005). We also found that the antisense LNA of the S gene can also significantly reduce HBV C-mRNA levels in hepatic cells, but there was no significant difference in the inhibitory effect of LNA between the C gene and the S/C double gene (see Figure 1). Because LNA inhibition experiments on HBV S-mRNA levels were not conducted in this study, the mechanism by which the LNA of the S gene reduces C-mRNA levels still requires investigation.

In addition, the serological markers such as serum albumin, ALT, urea nitrogen, creatinine, and other liver and kidney function indicators were also measured. We carried out hematoxylin and eosin staining of liver kidney pathological tissue sections to investigate the changes in organ tissue cell morphology, and the toxic effects of LNA liposomes were also assessed. The results showed that the antisense LNA had no obvious toxic or injurious effects on hepatic or renal function and tissue structure.

In summary, the *in vivo* application of the antisense LNA fragment complementary to the HBV S/C gene can significantly inhibit the replication and expression of the hepatitis B virus, and the dual-target area is better than the single-target area. Therefore, LNA is expected to become a new type of nucleic acid drug against the hepatitis B virus in gene therapy.

### Conflicts of interest

The authors declare no conflict of interest.

### ACKNOWLEDGMENTS

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### REFERENCES

- Castoldi M, Schmidt S, Benes V, Noerholm M, et al. (2006). A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). *RNA* 12: 913-920.
- Deng YB, Nong LG and Wang YF (2009). Hepatitis B virus (HBV) S gene-specific antisense-locked nucleic acid

- significantly inhibits HBV replication and expression in HBV transgenic mice. *Shijie Huaren Xiaohua Zazhi* 17: 2338-2445.
- Elmén J, Thonberg H, Ljungberg K, Frieden M, et al. (2005). Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Res.* 33: 439-447.
- Inohara H, Obika S and Imanishi T (2004). 2',4'-BNA derivatives bearing an unnatural nucleobase: synthesis and application to triplex-forming oligonucleotides. *Nucleic Acids Symp. Ser.* 48: 63-64.
- Rapozzi V, Cogoi S and Xodo LE (2006). Antisense locked nucleic acids efficiently suppress BCR/ABL and induce cell growth decline and apoptosis in leukemic cells. *Mol. Cancer Ther.* 5: 1683-1692.
- Swayze EE, Siwkowski AM, Wancewicz EV, Migawa MT, et al. (2007). Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity in animals. *Nucleic Acids Res.* 35: 687-700.
- Tang Y and Wang YF (2006). Initial study on inhibition of HBV expression by Locked nucleic acid directed at HBV S gene. *Jiangxi Yiyao* 41: 205-208.
- Wengel J, Petersen M, Frieden M and Koch T (2003). Chemistry of locked nucleic acids (LNA): Design, synthesis, and bio-physical properties. *Lett. Peptide Sci.* 10: 237-253.
- Zhao W, Chen H, Peng ZY, Li WG, et al. (2005). Antiviral effects of dual-target antisense rna: an experimental study with hepatitis B virus transgenic mice. *Zhonghua Yi Xue Za Zhi.* 85: 3486-3490.