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(71) Applicant *(for all designated States except US)*: **MOGAM BIOTECHNOLOGY RESEARCH INSTITUTE** [KR/KR]; 341 Pojung-ri Guseong-eup Yongin-si, Kyonggi-do 449-913 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KIM, Meehyein** [KR/KR]; 528-24 Bangbae-2dong Seocho-ku, Seoul 137-823 (KR). **SHIN, Duckhyang** [KR/KR]; #205 Neovillage 1074-3, Pungdoekcheon-2dong Yongin-city, Kyonggi-do 449-555 (KR). **KIM, Soo In** [KR/KR]; #107-804 Hansung Apt. 698-2, Pungdoekcheon-dong Yougin-city, Kyonggi-do 449-768 (KR). **PARK, Mahnhoon** [KR/KR]; 202-501 Samsung Shervill Sinbong-dong Yongin-city, Kyonggi-do 449-150 (KR).

(74) Agent: **LEE, Won-hee**; 8th Fl. Sung-ji Heights Ii 642-16, Yoksam-dong Kangnam-ku, Seoul 135-080 (KR).

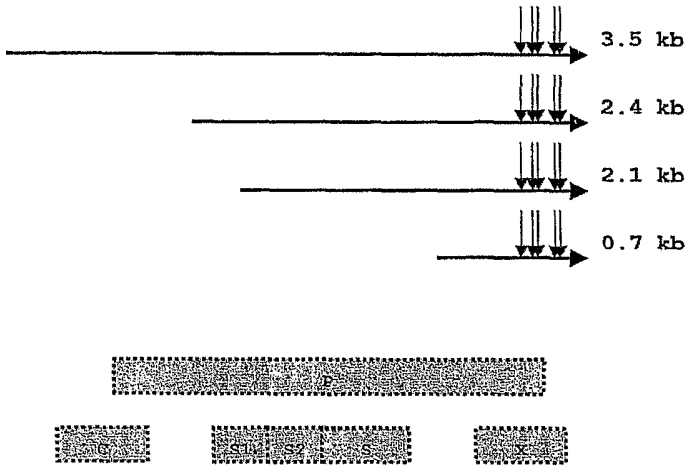
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(54) Title: SMALL INTERFERING RNA AND PHARMACEUTICAL COMPOSITION FOR TREATMENT OF HEPATITIS B COMPRISING THE SAME




(57) Abstract: The present invention relates to RNA interference mediated inhibition of Hepatitis B virus (HBV) by short interfering RNA (siRNA) molecules. Specially, siRNAs of the present invention which are double-stranded RNAs concern directing the sequence-specific degradation of viral RNA in mammalian cells. Disclosed is a DNA vector encoding the RNA molecules and synthesized siRNA molecules as well as method of therapeutic treatment for inhibition of HBV gene expression and viral replication by the administration of RNA molecules of the present invention.

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# Description

# SMALL INTERFERING RNA AND PHARMACEUTICAL COMPOSITION FOR TREATMENT OF HEPATITIS B COMPRISING THE SAME

## Technical Field

[1] The instant patent application claims priority to U.S. Application Ser. No. 60/660,132 filed on March 9, 2005. The instant application claims the benefit of the listed application, which is hereby incorporated by reference herein in its entirety.

[2]

[3] The present invention relates to a small interfering RNA specific for Hepatitis B virus X gene and the pharmaceutical use thereof.

[4]

## Background Art

[5] It is estimated that over 300 million people worldwide are chronically infected with Hepatitis B virus (HBV). Patients with HBV-associated liver failure may develop liver cirrhosis or hepatocellular carcinoma. One of the major anti-HBV therapies is treatment of interferon-alpha or lamivudine, or combination therapy with both of them. However, interferon-alpha as an anti-viral drug shows shortcomings, such as the low efficacy, side effects and high costs. Lamivudine, a nucleoside analogue, is a very potent and specific inhibitor to HBV reverse transcriptase. Nonetheless, it causes the viral genomic mutation resistant to the drug and a reactivation of viral replication by cessation of the treatment in patients. Only about 20% of the HBV patients response to combination therapy with interferon-alpha and lamivudine.

[6] HBV is a small enveloped DNA virus and belongs to hepadnaviridae. Human liver is the primary target organ of HBV. HBV infection usually leads to severe liver failure, such as chronic hepatitis, cirrhosis or hepatocellular carcinoma. HBV genome is a partial double-stranded circular DNA with length of 3.2 kb that contains four open reading frames, called S, C, P and X. Transcription of genomic DNA produces four different viral RNAs that are of size 3.5 (pregenomic RNA), 2.4, 2.1, and 0.7 kb (message RNAs) See Fig. 1. (Ganem and Varmus, *Annu. Rev. Biochem.*, 1987, 56, 651). The pregenomic RNA plays critical roles for not only translation of viral proteins but also reverse-transcription of viral DNA by polymerase protein. The core protein packages partial circular DNA and polymerase protein followed by the nucleocapsid assembly. And then the nucleocapsid particle interacts with viral envelop proteins to form mature infectious virions that are secreted out of the cell at the last step of viral life cycle.

[7] HBV X (HBx) gene is the smallest, with length of 465 nucleotides and encodes HBx protein that is 154 amino acids long with a molecular weight of 17 kDa (Fujiyama *et al.*, *Nucleic Acids Res.*, 1983, 11, 4601). It is a pleiotropic transactivator to stimulate not only the HBV promoters and enhancers, but also a wide range of other viral promoters via protein-protein interaction (Nakatake *et al.*, *Virology*, 1993, 195, 305; Spandau and Lee, *J. Virol.*, 1988, 62, 427). Moreover, the HBx protein is a critical element inducing cellular transformation and liver tumors either through interaction with cellular transcription factors or through a signal transduction pathway (Kekule *et al.*, *Nature*, 1993, 361, 742). As the HBx protein is implicated in HBV-mediated HCC and its coding region is contained in all of the four HBV mRNAs and highly conserved in a wide range of HBV subtypes, HBx gene must be an ideal target to design and develop the anti-HBV siRNAs.

[8] The viral life cycle can be initiated and propagated artificially by transfection of the HBV genomic plasmid (of adr subtype of gene-bank access no. M38636), pcDNA-HBV1.3, to introduce the viral replication system. See Fig. 2. The pcDNA-HBV1.3 clone was developed by modification of the previously reported protocol (Guidotti *et al.*, *J. Virol.*, 1995, 69, 6158). Transfection of the HBV genomic plasmid leads to the expression of viral RNAs and proteins *in vitro*. It can be also applied to construct an *in vivo* mouse model system, in which the complete immune responses and viral replication and assembly of mature viral particles are accompanied by hydrodynamic injection of a naked plasmid DNA bearing the HBV genome into tail veins of mice. This is a powerful tool to mimic and induce the viral replication cycle experimentally and to monitor the efficiency of antiviral drugs by detection of viral proteins or observation of viral nucleic acids. For example, co-injection of the HBV complete plasmid together with siRNA or its expression vector caused a significant inhibition in the level of viral antigens, transcripts and replicative DNA in the livers and sera (Giladi, *Molecular Therapy*, 2003, 8, 769; McCaffrey, *Nat. Biotechnol.*, 2003, 21: 639).

[9] In the meantime, RNA interference (RNAi) is evolutionally conserved process in which (endogenous and exogenous) gene expression is suppressed by introduction of double-stranded RNA (dsRNA) in all eukaryotes. RNAi is initiated by an RNase III-like endonuclease, called Dicer, which promotes consecutive cleavage of long dsRNAs into 21-23 nt short interfering RNAs (siRNAs) (Bernstein *et al.*, *Nature*, 2001, 409, 363). siRNAs are incorporated into an RNA-induced silencing complex (RISC), which unwinds the siRNA in the presence of ATP (Hammond, *et al.*, *Nature*, 2000, 404, 293). The antisense RNAs incorporated into RISC recognize the homologous RNAs and direct their degradation in the cellular cytoplasmic region.

[10] The dsRNA over 30 nt in length induces a nonspecific interferon (IFN) response

that activates protein kinase R (PKR) and RNase L (Balachandran *et al., Immunity*, 2000, 13, 129). The induction of PKR and RNase L activity finally leads to mRNA degradation and represses mRNA translation, nonspecifically, in mammal cells. However, siRNAs are short enough to bypass the interferon pathway and direct gene silencing with sequence specificity (Elbashir *et al., Nature*, 2001, 411, 494). Generation of siRNA is expected to protect against genetic invasion caused by transposons, transgenes and viruses, which partially or completely harbor long dsRNA elements (Plasterk, *Science*, 2002, 296, 1263; Zamore, *Science*, 2002, 296, 1265; Hannon, *Nature*, 2002, 418, 244).

[11] Many trials have been performed to select siRNAs to inhibit the replication of pathogenic RNA viruses, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), poliovirus, and so on (Novina *et al.*, *Nat. Med.*, 2002, 8, 681; Wilson *et al.*, *Proc. Natl. Acad. Sci. U S A*, 2003, 100, 2783; Getlin *et al., Nature*, 2002, 418, 430).

[12] However, there is no known effective anti-viral inhibitor including siRNA molecules to inhibit the replication of hepatitis B viruses upto date.

[13] Thus, it is required to develop urgently an anti-viral inhibitor to treat HBV infected patients.

[14]

[15] As HBV pregenomic RNA is a key intermediate to maintain viral DNA replication via reverse transcription in the virus life cycle, it is a reasonable candidate for RNAi. Consequently, the present inventors invented the present invention by paying attention to an applicability of siRNA specific for the HBV pregenomic RNA and finding that a sereis of siRNAs specific for Hepatitis B virus X gene could inhibit of viral replication and geen expression.

[16]

## Disclosure of Invention

## Technical Problem

[17] The object of the present invention is to provide a pharmaceutical agent effective in treating hepatitis B.

[18]

## Technical Solution

[19] In order to achieve the object, the present invention provides a small interfering RNA molecule (siRNA) specific for Hepatitis B virus X gene. This invention is based on the discovery siRNA molecules by targeting HBV X gene, which induces degradation of HBV pregenomic RNA and message RNAs, and finally inhibits the expression of viral proteins and the viral replication.

## Brief Description of the Drawings

[20] Fig. 1 is a schematic diagram illustrating the location of siRNA target sites specific for HBV X gene. Downward arrows indicate the target sites within the HBV RNA transcripts. The ORFs are drawn below aligned with the HBV mRNAs:

[21] P: polymerase; C: HBcAg;

[22] S1: large pre-surface antigen; S2: middle pre-surface antigen;

[23] S: HBsAg; and X: X protein.

[24]

[25] Fig. 2 is a schematic presentation of HBV1.3:

[26] Enh: enhancer; X: X gene; C: core gene;

[27] S1: preS1 gene; S2: preS2 gene; and S: S gene.

[28]

[29] Fig. 3 is a schematic diagram illustrating a pRNAiDu siRNA expression cassette. To construct the pRNAiDu vector, the human U6 and human H1 promoter sequences were cloned in the opposite direction. Appropriate mutations were induced to define termination signals for siRNA transcription by the RNA polymerase III or facilitate ligation of siRNA-encoding oligomers.

[30]

[31] Fig. 4 is a graph showing relative levels of HBsAg in culture media of siRNA expression vector-transfected cell. The HBsAg levels were measured at day 1, 2 and 3, following standardization of the transfection efficiency via FLuc assay as an internal control.

[32]

[33] Fig. 5 is a graph showing dose-dependant kinetics of inhibition of HBsAg expression by synthetic siRNA. Huh-7 cells ($4X10^5$) were transfected with 0.5 □ of pcDNA-HBV1.3 and the indicated amount of the synthetic HBx-1 siRNA or control siRNA, and assayed for the amount of HBsAg secreted into the media at day 1, 2, and 3 after transfection. The amount HBsAg by HBx-1 siRNA are shown as percentages of the amounts secreted by control siRNA-transfected cells.

[34]

[35] Fig. 6 is a series of photographs showing detection of the synthetic siRNA in the mouse liver. The synthetic double-stranded siRNA labeled with fluorescein was delivered into mice by hydrodynamic tail vein injection. After 20 hour postinjection, liver was dissected via cryosection and exposed on the fluorescence microscopy. Liver cells with fluorescence labeled siRNAs are indicated with arrows.

[36]

[37] Fig 7 is a graph showing serum HBsAg levels in synthetic siRNA-received mice.

HBsAg levels in C57BL/6 mouse sera were measured at day 2 after injection with pcDNA-HBV1.3 and 0.5 nmol synthetic siRNA of HBx-1 or control.

[38]

[39] Fig 8 is a graph showing dose-dependent inhibition of HBsAg expression in mice. Mice were injected with 10 □ of pcDNA-HBV1.3 DNA separately, or together with increasing amounts of synthetic siRNA of HBx-1 or control, and monitored for the levels of HBsAg after 2 days.

[40]

## Best Mode for Carrying Out the Invention

[41] This invention is based on the discovery siRNA molecules by targeting HBV X gene, which induces degradation of HBV pregenomic RNA and message RNAs, and finally inhibits the expression of viral proteins and the viral replication.

[42] In some embodiments, the siRNA is obtained by hybridization of the two complementary synthetic RNAs or transfection of a vector encoding the RNA in the cell. For efficient inhibition of the viral replication, siRNA sequences for the target segments on the HBV X gene were selected from the group of following SEQ. ID. NOs: 1-5, a complement thereof, or a portion thereof:

[43] HBx-1: 5'-GAGGACUCUUGGACUCUCA-3'(SEQ. ID. NO: 1);

[44] HBx-2: 5'-UGUCAACGUCCGACCUUGA-3'(SEQ. ID. NO: 2);

[45] HBx-3: 5'-CGUCCGACCUUGAGGCAUA-3'(SEQ. ID. NO: 3);

[46] HBx-4: 5'-UGAUCUUUGUACUAGGAGG-3'(SEQ. ID. NO: 4); and

[47] HBx-5: 5'-AUUGGUCUGUUCACCAGCA-3'(SEQ. ID. NO: 5).

[48] In an embodiment, the present invention provides an isolated nucleic acid molecule comprising a nucleotide sequence selected from the group of SEQ. ID. NOs: 1 to 5, or a complement thereof, or a portion thereof.

[49] In a preferred embodiment, the isolated nucleic acid molecule is a single stranded nucleic acid molecule.

[50] In another preferred embodiment, the isolated nucleic acid molecule further comprises a complementary strand of said isolated nucleic acid molecule, which can hybridize with the same.

[51] In a preferred embodiment, the isolated nucleic acid molecule is a short interfering RNA (siRNA).

[52] In a more preferred embodiment, the complementary strands of the siRNA are covalently connected via a linker molecule.

[53] In another preferred embodiment, the linker molecule is a polynucleotide linker or a non-nucleotide linker.

[54] In further preferred embodiment, the nucleic acid molecule binds to a HBV X gene.

[55] The present invention provides a method for treatment of an infectious disease related to HBV, comprising administrating to the subject pharmaceutically effective amount of a double-stranded siRNA molecule comprising a nucleotide sequence selected from the group of SEQ. ID. NOs: 1 to 5, or a complement thereof, or a portion thereof.

[56] Also, the present invention provides a DNA vector comprising a DNA sequence corresponding a nucleotide sequence selected from a group of SEQ. ID. NOs: 1 to 5, or a complement thereof, or a portion thereof.

[57] In a preferred embodiment, the DNA vector of the present invention is suitable for expression of siRNA.

[58] In addition, the present invention provides a pharmaceutical composition comprising the isolated nucleic acid molecule decribed above or the DNA vector and pharmaceutically acceptable carriers or excipients, for treating, preventing or diagnosing hepatitis B, liver cirrhosis or liver cancer.

[59]

[60] To increase the stability of siRNA or the specific interaction between viral target RNA region and siRNA fragment, the 3'ends of both of the two strands of siRNA were extended with dTdT or UU, by chemical synthesis. In some embodiments, synthetic s iRNA can be modified by chemical derivatives or tagging molecules for acquiring its physiogical stability and chasing its distribution in the cell.

[61] In some preferred embodiments, each strand of double-stranded siRNA is expressed from the two separated promoters, in opposite or in parallel, and hybridizes with its complement in the living cell. Alternatively, shRNA can be transcribed from a single promoter independently and processed into double-stranded siRNA by cellular Dicer, following induction of degradation of target RNA. A vector expressing siRNA contains not only promoter(s) for initiation of transcription but also enhancer, transcription termination signal, or other expression regulatory sequences. The vector can be delivered into the cellular nucleus as a naked plasmid DNA, a complex with transfection reagent or target-delivery material, or as a form of recombinant viral vector. The construction of the vector is determined by specific situations, such as the cell state or type to be transfected, the time and level of siRNA expression, and so on.

[62] The present invention demonstrates a DNA vector that transcribes double-stranded siRNA from the two convergent promoters. The vector, partially or completely, inhibits HBV gene expression and viral replication in the cell. RNA interference effect is dependant on the detection time and transfected DNA dose and causes over 90% of inhibition of viral RNA accumulation or protein expression. Specially, the siRNA expression cassette, separated from the vector by restriction endonucleases, is an efficient element inducing the RNAi effect.

[63] The invention also demonstrates the RNAi activity induced by synthetic siRNA in which 3' end of each strand RNA in extended with dTdT for its stability. The synthetic RNA efficiently inhibits accumulation of viral RNA and gene expression by 98% in the cell and by 97% in the HBV mouse model, respectively. In the mouse, it is observed that the fluorescein labeled siRNA is delivered into the liver tissue by hydrodynamic injection. It will be a new therapeutic approach for treating a hepatitis viral carrier, infected by HBV, by administration to a subject in need thereof synthetic siRNA or vector.

[64] The present invention demonstrates a therapeutic application of synthetic siRNA or vector encoding double-stranded siRNA and the combination therapy containing siRNA to inhibit HBV replication in its carriers.

[65]

## Mode for the Invention

[66] This invention relates to siRNA molecules specific for Hepatitis B virus X gene and their application for the clinical treatment to hepatitis B virus (HBV) chronic carrier to inhibit viral replication and gene expression.

[67] An siRNA of the present invention can be synthesized chemically or enzymatically (Caruthers *et al.*, *Methods in Enzymology*, 1992, 211, 3; Wincott *et al.*, 1995, *Nucleic Acids Res.*, 23, 2677; Brennan *et al., Biotechnol. Bioeng.*, 1998, 61, 33).

[68] An siRNA or vector of this invention can be delivered to target cells using transfection carriers, such as liposomes, hydrogels, bioadhesive microspheres and the like (Akhtar *et al., Trends Cell Bio.*, 1992, 2, 139).

[69] A pharmaceutical composition contains an siRNA or vector of this invention with an organ targeting material and a pharmaceutically acceptable carrier for treating an infection with HBV. The dose of pharmaceutical composition can be determined, therapeutically, by a specific situation, such as the route of administration, the nature of the formulation, the phase of liver failure, the subject's size, weight, or distribution range, and the age and sex of patient.

[70]

[71] Practical and presently preferred embodiments of the present invention are illustrative as shown in the following Examples.

[72] However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

[73]

[74] **Example 1: constructing of a siRNA expression vector**

[75] In mammalian cells, previously siRNA vector has been designed to transcribe short

hairpin RNAs (shRNAs) from an RNA polymerase III promoter (such as U6, H1, or tRNA promoter) or a polymerase II promoter with a poly(A) signal sequence (Brummelkamp *et al.*, *Cancer Cell*, 2002, 2, 243; Tushcl, *Nat. Biotechnol.*, 2002, 20, 446; Xia *et al.*, *Nat. Biotechnol.*, 2002, 20, 1006). However, shRNA vectors show multiple drawbacks. Their non-natural secondary structure induces that it is hard to synthesize them in bacteria and to sequence, and DNA oligomers to generate them can be costly in the case of high through-put screening. Moreover, it is no facile to generate an siRNA expression cassette containing a promoter to a termination signal without additional tag-sequences for constructing diverse siRNA library. To circumvent these limitations of shRNA expression vectors, we constructed a vector for direct expression of siRNA, which is transcribed from convergent opposing promoters, and named it pRNAiDu (Kaykas and Moon, *BMC Cell Biology*, 2004, 5, 16; Zheng *et al.*, *Proc Natl. Acad. Sci. U S A*, 2004, 101, 135). See Fig. 3.

[76] Both the human U6 and H1 promoters were modified to contain polymerase III termination sequences of five thymidine nucleotides at the -5 to -1 position and a *Bam* H I site and a *Hin*d III site at each -12 to -6 position, respectively. As the U6 promoter prefers a purine nucleotide for transcription initiation, guanidine is inserted at the +1 position downstream of the U6 promoter. To minimize an artificial effect of induced this additional nucleotide and guarantee a consecutive hybridization between antisense siRNA and target RNA in the RNAi process, it was devised that the U6 promoter takes a charge of transcription for the antisense RNA, which directs RISC to cleave the homologous mRNA. To create the siRNA expression plasmids, pairs of 36-base oligonucleotides were annealed and ligated into pRNAiDu digested with *Bam*H I and *Hin*d III. Specially, in the pRNAiDu vector, the fusion gene of enhanced green fluorescent protein (EGFP) and firefly luciferase (FLuc), EGFP-FLuc, is contained under the SV40 promoter. Experimentally, this is useful to visualize and quantitatively monitor the transfection efficiency, and to standardize the RNAi activity via detection of fluorescence or luminescence.

[77]

[78] **Example 2: Inhibition of HBsAg expression by HBV siRNAs *in vitro***

[79] The Huh-7 cells were seeded at a subconfluent density of $4X10^5$ cells in 6 well culture plates. One day after, the cells were transfected with 0.5 μg of pcDNA-HBV1.3 and 1.5 μg of pRNAiDu, as a control vector, or a siRNA vector, using Lipofectamine 2000 (Invitrogen, USA) following the user guideline. At 1, 2 and 3 days after transfection, media were collected for quantitative detection of the level of HBsAg, and the cells were harvested for standardization of the transfection efficiency using firefly luciferase assay kit (Promega, USA). Experiments were performed in triplicate.

[80] The levels of HBsAg in 100 μl of the media of the transfected cells were measured

using HBsAg enzyme immunoassay kit (DiaSorin, Italy).

[81] To investigate the anti-viral activity of the HBV siRNAs, the levels of the secreted HBsAg in the culture media were quantified at 1, 2 and 3 days after transfection. See Fig. 4. The transfection efficiency in each experiment set was corrected by measuring the amount of FLuc protein in the cell lysates treated with the siRNA expression vectors. Compared with the control siRNA vector, HBsAg expression by HBV siRNA vectors was reduced by 80% in average in the cells at day 3 after transfection. Among all the siRNA expression vectors, pRNAiDuHBx-1 and pRNAiDuHBx-3 exhibited the most dramatic inhibition, as HBsAg were reduced 97% and 94% at day 3 post-transfection, respectively. It means that the strong siRNAs targeting HBx gene efficiently inhibit not only viral replication but also expression of other HBV genes by simultaneous degradation of all kinds of viral pregenomic and mRNAs containing homologous target X gene.

[82] To examine whether the siRNA expression cassette from the U6 promoter to the H1 promoter is enough to induce the siRNA-medicated RNA interference, the cassette was separated from the siRNA expression vector by digestion with restriction endonuclease. The linearized siRNA vectors were co-delivered with the HBV complete genome plasmid into Huh-7 cells. The results indicate that the linearized siRNA cassette, as well as the circular siRNA expression plasmid, is also able to induce the RNAi effect with decrease of the HBsAg level by about 90% in the media. See Table 1. This suggests that the siRNA expression cassette with two RNA polymerase III promoters, convergently opposing, is a useful tool to develop the PCR product-based anti-HBV gene therapeutics.

[83]

[84] Table 1

RNAi effect of the linearized siRNA expression cassette.

| | Relative amount of HBsAg (%) | |
|---|---|---|
| | control | HBx-1 |
| circular plasmid | 100 | 8±0.7 |
| linearized plasmid (*Eco*R I) | 100 | 6±0.5 |

[85]

[86] To confirm further the inhibitory effect of siRNA on HBV gene expression, we prepared synthetic siRNAs of control siRNA and HBx-1 siRNA. Then we conducted dose-response analysis by co-delivery with 0.5 μg of pcDNA-HBV1.3 and increasing amounts of synthetic siRNA into the Huh-7 cells and by monitoring the level of

HBsAg secreted into the media at day 1, 2 and 3 posttransfection. See Fig. 5. The results reveal that at least 10 nM of synthetic HBx-1 siRNA is sufficient for inducing strong inhibitory effect of HBV gene expression at day 1 (over 90%), comparing with control siRNA. Moreover, in the case of exposure of the HBV replication complete vector into the 40 nM synthetic HBx-1 siRNA, HBsAg protein was totally exhausted down to undetectable level. This definite inhibitory effect appears to last for 3 days. These results *in vitro* suggest that HBx-1 siRNA must be a specific and strong inhibitor and an ideal candidate for silencing of viral gene expression via RNA interference process.

[87]

[88] **Example 3: Reduction of viral transcripts by HBV siRNAs *in vitro***

[89] Total RNA was extracted from Huh-7 cells (about $10^6$) delivered with pcDNA-HBV1.3 and either control siRNA vector or HBV-specific siRNA vector, at day 2 posttransfection, using Trizol LS reagent (Invitrogen, USA) according to the manufacturer's instruction. The isolated total RNA was digested with RNase-free DNase (Promega, USA). Finally, absolute amount of RNA was determined by measuring UV-absorbance at 260 nm/280 nm using UV spectrophotometer.

[90] Antiviral activity was assessed by means of a quantitative real time RT-PCR (Sequence Detection System 5700, Applied Biosystems, USA). The real time RT-PCR was performed with 500 ng of total RNAs isolated from the transfectants in a reaction volume of 50 μl using the TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, USA). The primer and probe sequences, specific for HBV X gene, include 5'-TCCCCGTCTGTGCCTTCTC-3' (forward primer, SEQ. ID. NO: 6), 5'-GTGGTCTCCATGCGACGTG-3' (reverse primer, SEQ. ID. NO: 7) and 5'(fluorescein)-CCGGACCGTGTGCACTTCGCTT(TAMRA)-3' (probe, SEQ. ID. NO: 8). The total RNA amount was corrected, definitely, by carrying out real time RT-PCR targeting human β-Actin gene as an internal control, in parallel. The primer and probe sequences for β-Actin gene include 5'- GCGCGGCTACAGCTTCA-3' (forward primer, SEQ. ID. NO: 9), 5'- TCTCGTTAATGTCACGCACGAT-3' (reverse primer, SEQ. ID. NO: 10) and 5'-(fluorescein)CACCACGGCCGAGCGGGA(TAMRA)-3'(probe, SEQ. ID. NO: 11). All experiments were performed in triplicate.

[91] To determine whether HBV siRNA vector can reduce the viral RNA level *in vitro*, we monitored the RNAi activity induced by HBx-specific siRNA vectors using quantitative realtime RT-PCR. The relative amount of viral RNA transcripts was presented as percentages of the control siRNA vector. See Table 2. Compared with a control vector, pRNAiDu, significant reduction of the viral transcripts was detected when siRNA vector targeting specific HBx RNA were used. Specially, much more

dramatic reduction of viral RNA was detected by 70% and 60% in the total RNA prepared from cells transfected with pRNAiDuHBx-1 and pRNAiDuHBx-3, respectively, on day 2 posttransfection. These results demonstrate that RNAi can efficiently induce viral RNA degradation and inhibit HBV replication in cultured Huh-7 cells.

[92]

[93] Table 2

Quantitative measurements (by realtime RT-PCR) of HBV transcripts in the Huh-7 cells co-transfected with HBV DNA and siRNA expression vector.

| siRNA | Relative HBx RNA amount (%) |
|---|---|
| control | 100 |
| HBx-1 | 30±0.5 |
| HBx-2 | 46±3.0 |
| HBx-3 | 39±7.6 |
| HBx-4 | 53±9.0 |
| HBx-5 | 51±7.6 |

[94]

[95] **Example 4: Inhibition of HBsAg expression by synthetic HBx siRNA *in vivo***

[96] We performed *in vivo* experiments with female C57BL/6 mice weighing between 18 to 20 g (Orient, Korea). The complete HBV DNA, pcDNA-HBV1.3, and siRNAs were delivered into mice using the hydrodynamic injection method, by which 10 μg of pcDNA-HBV1.3 and 0.5 nmole siRNA dissolved in RNase-free 0.85% NaCl were injected into the mice tail vein (Zhan et *al., Hum. Gene Ther.*, 1999, 10, 1735; Lin *et al., Gene Ther.*, 1999, 6, 1258). In the dose-response assay, mice were injected with 10 μg of pcDNA-HBV1.3 together with increasing amounts of control siRNA or HBx-1 siRNA. The mouse serum was separated by eye-bleeding and assayed for HBsAg level at day 1, 2 and 3 after hydrodynamic injection.

[97] To visualize that synthetic RNA can reach the target organ, we prepared the synthetic double-stranded RNA with 21 nucleotides in length labeled with fluorescein at the 3' end of sense strand of RNA and injected 1 nmole RNA into the mice tail vein. At 20 h after injection, mice were sacrificed, and the livers were separated and dissected into pieces via cryosection.

[98] By exposure of the pieces of liver section on the fluorescence microscopy, spots with fluorescence were detected after 20 h postinjection. See Fig. 6. It shows that some portion of the synthetic RNA can be delivered to the target organ by escaping the

RNase attacks which abundantly distribute everywhere in the serum and the tissue of the mouse. It appears promising that the hydrodynamic injection methods must be a compatible tool to observe the synthetic siRNA-mediated RNAi efficacy in the mouse model.

[99] We selected an siRNA with the strongest *in vitro* inhibition effect on HBV gene expression for confirming its interference effect in the mouse model. By the hydrodynamic injection method, mice were received 10 μg of pcDNA-HBV1.3 plasmid separately, or together with 0.5 nmole of synthetic siRNA of control siRNA or HBx-1 siRNA. After 2 days, we separated serum samples and assessed theirs HBsAg level by performing ELISA assay. See Fig. 7. As expected, the negative control siRNA duplex did not cause reduction of the HBsAg level expressed from the HBV replication competent vector in the mouse. In accordance with the *in vitro* cell culture experiments, synthetic HBx-1 siRNA induced the prominent inhibition of HBsAg expression by 96% in the sera.

[100] To investigate the dose-dependant response of siRNA for inhibition of viral gene expression, we delivered 10 μg of pcDNA-HBV1.3 plasmid together with 0.05, 0.1, 0.5, 1 or 1.5 nmole of control or HBx-1 siRNA into mice and monitored the level of HBsAg in the serum at day 2 after the hydrodynamic tail vein injection. See Fig. 8. With as little amount of 0.05 nmole HBx-1 siRNA comparing with control siRNA, the HBsAg level was efficiently inhibited by 78%. Furthermore, dose of 0.1 nmole of the HBx specific siRNA was enough amounts for inducing the saturated inhibition effect for HBV gene expression.

[101] To investigate the kinetic inhibitory effect, the sera of mice injected with pcDNA-HBV1.3 and synthetic siRNA was harvested at different time intervals of day1, 2 and 3 after injection for measuring the HBsAg level. See Table 3. Results of a kinetic study displayed that the HBV gene expression in variable concentrations (0.05~1.5 nmole) of the synthetic RNA reached to undetectable range after day 2. The relative HBsAg levels induced by HBx-1 siRNA were presented as percentages of control siRNA. All experiments were performed in triplicate. In the ELISA assay, the saturated inhibition effect lasted for at least 3 days. This observation suggests that HBx-1 siRNA significantly and efficiently inhibits the viral replication via degradation of sequence specific viral RNAs and inhibition of the gene expression.

[102]

[103] Table 3

Kinetics of RNAi effect by the HBx-1 siRNA in mice.

| HBx-1 siRNA (nmole) | Relative levels of HBsAg (%) | | |
|---|---|---|---|
| | Day 1 | Day 2 | Day 3 |

| | | | |
|---|---|---|---|
| 0.05 | 21.6 ± 6.1 | 19.2 ± 13.2 | 27.3 ± 14.2 |
| 0.10 | 13.3 ± 4.0 | 4.7 ± 0.8 | 5.1 ± 1.3 |
| 0.50 | 9.5 ± 2.5 | 3.8 ± 0.4 | 5.6 ± 2.1 |
| 1.00 | 6.8 ± 0.6 | 1.8 ± 0.4 | 3.7 ± 0.7 |
| 1.50 | 4.4 ± 1.5 | 1.4 ± 0.2 | 3.2 ± 2.2 |

[104]

## Industrial Applicability

[105] The present invention relates to a siRNA specific for HBV X gene and a pharmaceutical use thereof. The siRNA of the present invention can be effectively used for treating diseases resulting from infection of hepatitis B virus, since the siRNA induces degradation of HBV pregenomic RNA and message RNAs, and finally inhibits the expression of viral proteins and the viral replication.

[106]

## Sequence Listing

[107] SEQ. ID. NOs: 1 ~ 5 are the nucleotide sequences of the siRNA molecules of the present invention.

[108] SEQ. ID. NO: 6 and SEQ. ID. NO: 7 are primers for real time RT-PCR to detect HBV X gene.

[109] SEQ. ID. NO: 8 is a probe for real time RT-PCR to detect HBV X gene.

[110] SEQ. ID. NO: 9 and SEQ. ID. NO: 10 are primers for real time RT-PCR to detect β-actin gene.

[111] SEQ. ID. NO: 11 is a probe for real time RT-PCR to detect β-actin gene.

# Claims

[1] An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group of SEQ ID NOs: 1 to 5, or a complement thereof, or a portion thereof.

[2] The isolated nucleic acid molecule according to claim 1, wherein the nucleotide sequence is SEQ ID NO: 1 or 3.

[3] The isolated nucleic acid molecule according to claims 1 or 2, wherein the nucleic acid molecule is a single stranded nucleic acid molecule.

[4] The isolated nucleic acid molecule according to any one of claims 1 to 3, further comprising a complementary strand thereof.

[5] The isolated nucleic acid molecule according to claim 4, wherein the nucleic acid molecule is a short interfering RNA (siRNA).

[6] The isolated nucleic acid molecule according to claims 5, wherein the complementary strands of the siRNA are covalently connected via a linker molecule.

[7] The isolated nucleic acid molecule according to claim 5, wherein the linker molecule is a polynucleotide linker or a non-nucleotide linker.

[8] The isolated nucleic acid molecule according to claims 1 or 2, wherein the isolated nucleic acid molecule binds to the HBV X gene.

[9] A method for treatment of an infectious disease related to HBV, comprising administrating to a subject a pharmaceutically effective amount of double-stranded siRNA molecules comprising the isolated nucleic acid molecule accroding to any one of claims 1 to 8.

[10] A DNA vector comprising a DNA seqeunce corresponding a nucleotide sequence selected from the group of SEQ ID NOs: 1 to 5, or a complement sequence thereof, or a portion thereof.

[11] The DNA vector according to claim 10, wherein the vector is suitable for siRNA expression.

[12] A pharmaceutical composition comprising the isolated nucleic acid molecule according to any one of claims 1 to 8 or the DNA vector according to claim 10 or claim 11 and pharmaceutically acceptable carriers or excipients, for treating, preventing or diagnosing Hepatitis B, liver cirrhosis or liver cancer.

# DRAWINGS

Figure 1

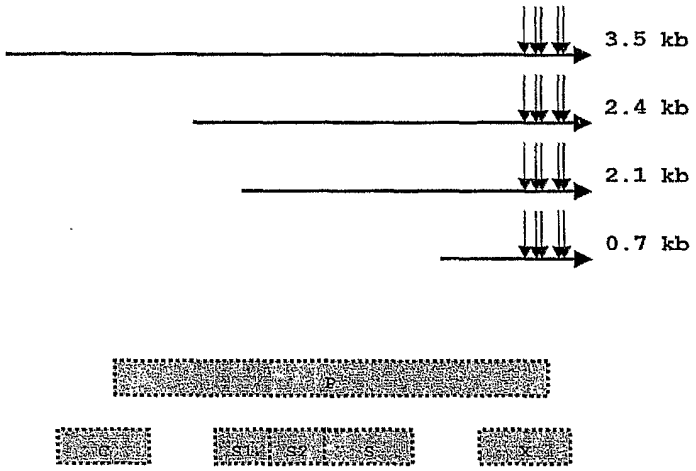



Figure 2

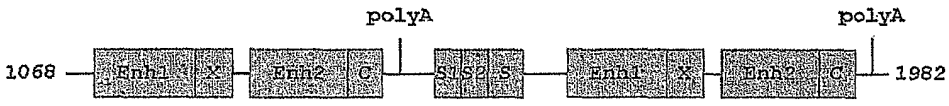



Figure 3

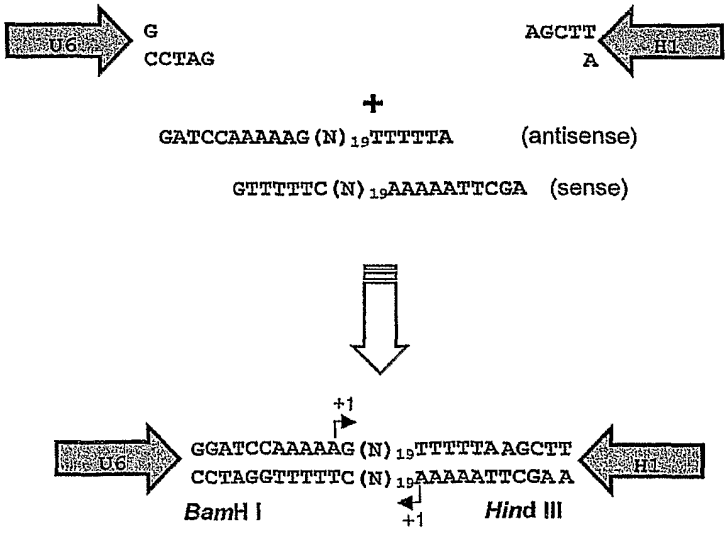


Figure 4

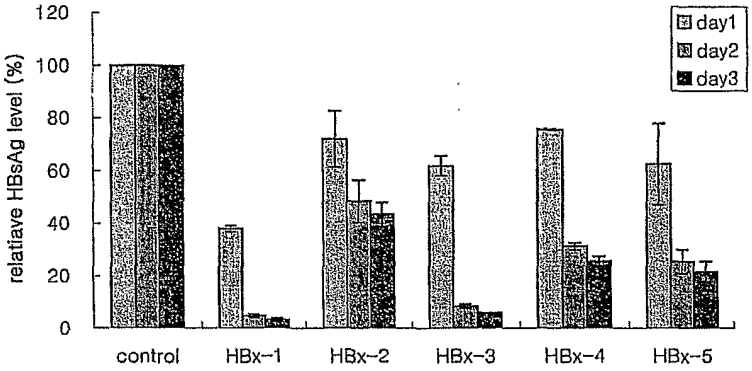



Figure 5

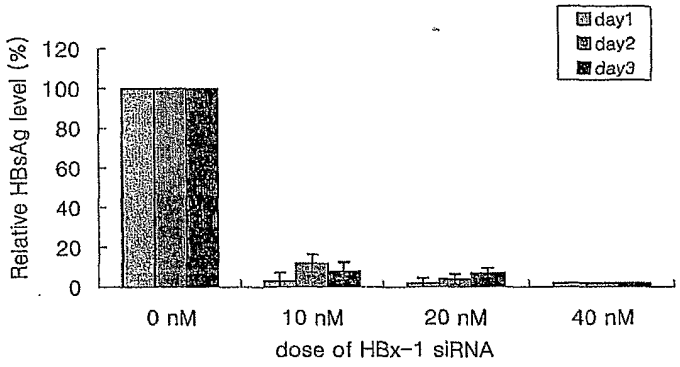



Figure 6

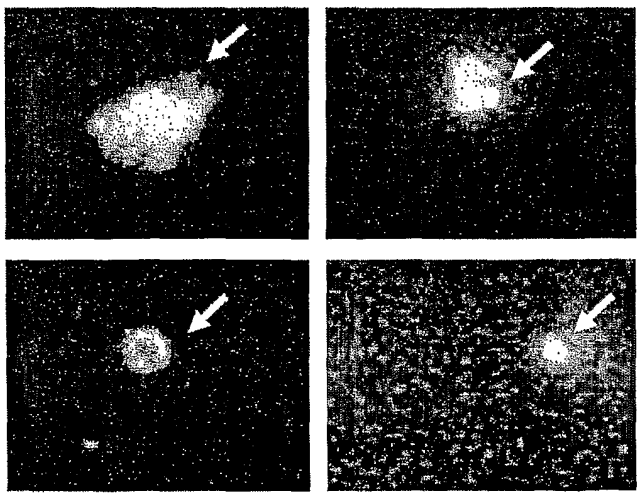


Figure 7

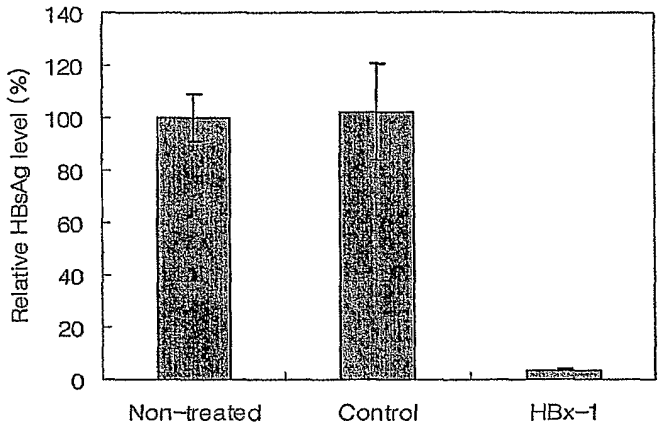
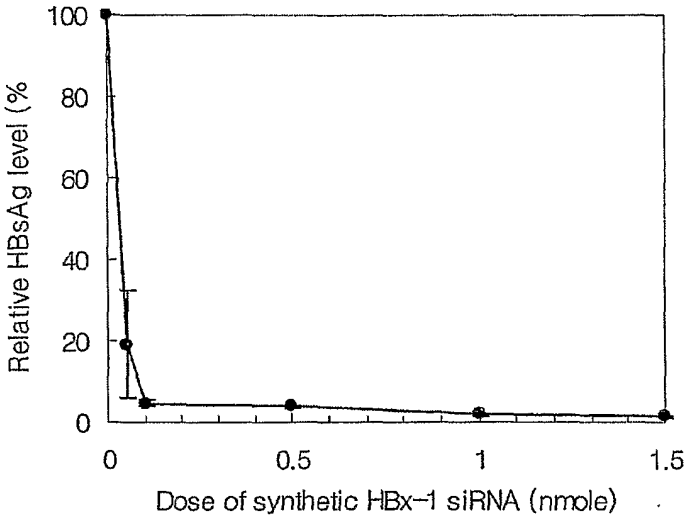



Figure 8



# INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/000837

## A. CLASSIFICATION OF SUBJECT MATTER

*C12N 15/11(2006.01)i, C12N 15/51(2006.01)i, C12N 15/09(2006.01)i*

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC8: C12N 15/11, C12N15/51

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)

Esp@snet, Pubmed, delphion

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | US 2003/0206887 A1 (MORRISSEY, D) 6 November 2003<br>see the whole document | 1-8, 10-12 |
| Y | WO 2004/078181 A1 (Capital Biochip Company, LTD) 16 September 2004<br>see the whole document | 1-8, 10-12 |
| P, Y | WO 2005/021751 (ARBUTHNOT, P) 10 March 2005<br>see the whole document | 1-8, 10-12 |

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 26 JUNE 2006 (26.06.2006) | **27 JUNE 2006 (27.06.2006)** |
| Name and mailing address of the ISA/KR<br>Korean Intellectual Property Office<br>920 Dunsan-dong, Seo-gu, Daejeon 302-701,<br>Republic of Korea<br>Facsimile No. 82-42-472-7140 | Authorized officer<br>KIN, Ji Yun<br>Telephone No. 82-42-481-8288 |

Form PCT/ISA/210 (second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/000837

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

   a. type of material
   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material
   - [ ] on paper
   - [x] in electronic form

   c. time of filing/furnishing
   - [x] contained in the international application as filed
   - [x] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/000837

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 9
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 9 directs to methods for treatment of the human or animal body, thus relates to a subject-matter which this International Searching Authority is not required to search under PCT Rule 39.1(iv).

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.
PCT/KR2006/000837

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| US 2003/0206887 A1 | 2003.11.06 | EP 01288296 A2 | 2003.03.05 |
| | | EP 01383782 A1 | 2004.01.28 |
| | | US 2003068301 A1 | 2003.04.10 |
| | | US 2003148985 A1 | 2003.08.07 |
| | | WO 02081494 A1 | 2002.10.17 |
| WO 2004/078181 A1 | 2004.09.16 | AU 2003258452 A1 | 2004.09.28 |
| WO 2005/021751 | 2005.03.10 | - None - | |