Inhibitory Effects of Chimeric Decoy Oligodeoxynucleotide in the Regulation of Transcription Factors NF-κB and Sp1 in an Animal Model of Liver Cirrhosis

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Liver fibrosis is a process of healing and scarring in response to chronic liver injury. Following injury, an acute inflammation response takes place resulting in moderate cell necrosis and extracellular matrix damage. To develop a novel therapeutic approach in hepatic fibrogenesis, we examined the simultaneous suppression of the transcription factors NF-κB and Sp1, which regulate acute inflammation and continuous deposition of extracellular matrix in liver fibrosis. We employed chimeric decoy oligodeoxynucleotide containing the consensus sequences of both NF-κB and Sp1 binding sites, to suppress these transcription factors simultaneously. Treatment of chimeric decoy oligodeoxynucleotide reduced the activity of hepatic stellate cells in vitro, and decreased the expression of fibrotic and proinflammatory gene responses in a mouse model of liver fibrosis. These results suggest that chimeric decoy oligodeoxynucleotide strategy can be a potential therapeutic application to prevent liver fibrosis.

Key words: Liver fibrosis, decoy, transcription factor, NF-κB, Sp1

Introduction

Liver fibrosis is the end result of chronic inflammatory reactions induced by a variety of stimuli including alcohol, ischemia, viral agents, medications or hepatotoxins [31]. The sustained hepatic inflammation in chronic liver injury can trigger an excessive accumulation of extracellular matrix (ECM) components, which leads to the formation of a permanent fibrotic scar. Cirrhosis is an advanced stage of fibrosis, characterized by the formation of regenerative nodules of liver parenchyma separated by fibrotic septa which result from cell death, aberrant ECM deposition and vascular reorganization. Preventing the progression to cirrhosis is a major clinical goal [23]. Thus, it is important to develop novel therapeutic approaches to regulate the progression of liver fibrosis.

Liver damage is accompanied by an activation and transdifferentiation of hepatic stellate cells (HSC) to myofibroblasts with increased proliferative capacity and contractility as well as other profound phenotypic alternations that include the loss of vitamin A and a-smooth muscle actin (SMA) expression [3,8]. During liver fibrogenesis, HSC are activated by inflammatory cytokines and growth factors in a paracrine and autocrine manner. Proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, are elevated during early hepatic inflammation and contributes to the activation of HSC [2,5,16,22]. In addition, transforming growth factor (TGF)-β1 is the most potent fibrogenic cytokine described for the activation of HSC and its expression affects excessive synthesis of ECM components, such as collagen and fibronectin [27].

Given the pleomorphic nature of stellate cell activation, attention has been directed toward transcription factors, such as NF-κB and Sp1. These transcription factors regulate various cell fate and activity, especially inflammation and fibrogenesis. Among them NF-κB is known to regulate inflammatory responses in many cell types [1]. NF-κB binding activity was found to increase in liver macrophages and hepatocytes of rats treated with CCL4 [21]. DNA binding activity of NF-κB is demonstrated in activated but not in quiescent HSC, and activation of HSC is associated with the nuclear translocation of NF-κB [10]. In contrast, transcription factor Sp1 plays important roles in regulating the transcription of matrix genes, such as TGF-β1 and type I collagen [28]. Sp1 mediates the stimulatory effect of TGF-β1 on COL1A2 transcription in activated HSC [7,11,12,15]. Therefore, NF-κB and Sp1 are believed to play majors role in HSC activation and liver fibrosis.

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To develop a new therapeutic approach, chimeric (Chi) decoy oligodeoxynucleotide (ODN) was used to inhibit both NF-κB and Sp1 transcription factors in an animal model of liver fibrosis. Chi decoy ODN, which contains both NF-κB and Sp1 binding sequences in a single decoy molecule, was designed to enhance the effective use of decoy ODN strategy. Chi decoy ODN could reduce the initial inflammatory response as well as fibrotic changes associated with liver fibrogenesis. The purpose of this study is to determine the inhibitory effects of Chi decoy ODN on HSC activation and liver fibrosis using an animal model of liver cirrhosis.

Materials and Methods

Synthesis of ring type decoy ODN and selection of target sequences

The following sequences of ODN were utilized (Consensus sequence is underlined):

Scrambled (Scr) decoy ODN: 5'-GAATTCAATTCAGGGTACGCAAAAAATTGCCTCGATACCTGATATT-3', NF-κB decoy ODN: 5'-GAAATTCAGGGAAAAAACCTCTCAAGAGGACCTCTCCTCCCT-3', Sp1 decoy ODN: 5'-GAATTCAATTCAGGGTACGCAAAAAATTGCCTCGATACCTGATATT-3', NF-κB decoy ODN: 5'-GAAATTCAGGGAAAAAACCTCTCAAGAGGACCTCTCCTCCCT-3', Sp1 decoy ODN: 5'-GAATTCAATTCAGGGTACGCAAAAAATTGCCTCGATACCTGATATT-3',

AGGGCGGTTCAAGAAACTTGAAAACCCCGCCCT-3', Chimeric NF-κB decoy ODN: 5'-GAATTCAATTCAGGGTACGCAAAAAATTGCCTCGATACCTGATATT-3', Chimeric Sp1 decoy ODN: 5'-GAATTCAATTCAGGGTACGCAAAAAATTGCCTCGATACCTGATATT-3'.

In considering the feasibility of a decoy ODN strategy, we designed ring type decoy ODN. These ODN were annealed 8 hr, while the temperature decreased from 80 to 25°C. The Scr, NF-κB, Sp1, Chi decoy ODN were predicted to form a stem-loop structure. Following the addition of T4 ligase (1U, Takara, Japan), the mixture was incubated for 18 hr at 16°C to generate a covalently ligated ring-type decoy molecule (Fig. 1A).

Cell culture with gene transfer

Primary cultured rat hepatic stellate cells (HSC) were obtained from Dr. Jeong (Kyungpook National University, Korea). HSC was cultivated at 37°C in an atmosphere of 5% CO₂ in minimum essential medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, NY, USA). Prior to the experiment, cells were transfected with 50 nM of decoy ODN combined

Fig. 1. Structure of Chi decoy ODN and effect of Chi decoy ODN in activated HSC. (A) Chi decoy ODN strategy (upper) and formation of ring type Chi decoy ODN (lower). Chi decoy ODN effectively suppressed TGF-β1, fibronectin and α-SMA (B) protein and (C) mRNA expression compared to Sp1 and NF-κB decoy ODN. NC, normal control; Scr, HSC treated with Scr decoy ODN; NF-κB, HSC treated with NF-κB decoy ODN; Sp1, HSC treated with Sp1 decoy ODN; Chi, HSC treated with Chi decoy ODN. *p<0.05 vs HSC treated with Scr decoy ODN. An arbitrary unit was defined as target gene/GAPDH band intensity.
with Trans IT-Oligo Transfection Reagent (Mirus, WI, USA) according to the manufacturer’s instructions. After incubation at 37°C for 8 hr, the cells were harvested.

**Animal model and gene transfer**
Male 6 week old (20-22 g) C57BL/6 mice (Orient Bio, Korea) were housed in a room at 22±2°C and a 12 hr light-dark cycle. The animal experiments were performed in accordance with the NIH guidelines for the care and use of laboratory animals. C57BL/6 mice were randomly divided into three groups: A normal group treated nothing (normal control, NC), a CCl4-liver damage treated with Scr decoy ODN (CCl4+Scr) and a CCl4-liver damage treated with Chi decoy ODN (CCl4+Chi). The liver damage received intraperitoneal injection of CCL4 (2 ml/kg) dissolved in corn oil (1:3 ratio) three times a week. The delivery of decoy ODN was transferred biweekly by injection of 10 μg Scr decoy ODN, or Chi decoy ODN through the mouse tail vein using in vivo Gene Delivery System (Mirus, WI, USA). Decoy ODN was introduced via tail vein 1 wk after the first CCl4 injection. Mice were sacrificed after 4 and 8 wk from first CCl4 injection.

**Liver function tests**
Blood of each mouse was collected and serum separated for analyzing the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by using automated analyzer.

**Enzyme-linked immunosorbent assay (ELISA)**
Serum TNF-α concentration was measured with a solid phase sandwich ELISA using a Quantikine mouse TNF-α kit (R&D Systems, MN, USA). Serum IL-1β concentration was also measured with a solid phase sandwich ELISA using a Quantikine mouse IL-1β kit (R&D Systems).

**Reverse-transcription polymerase chain reaction (RT-PCR)**
Total RNA was extracted from the cultured rat HSCs and frozen liver with TRI-zol Reagent described previously (Gibco, NY, USA). The purity and quantity of the RNA preparation were measured at optical densities of 260 nm and 280 nm. First strand cDNA was synthesized with oligo-d(T) primer and M-MLV reverse transcriptase (Promega). Aliquots of cDNA were used for PCR using primer sets specific to mouse TGF-β1, α-SMA, fibronectin, type I collagen and glyceraldehydes-3-phosphate-dehydrogenase (GAPDH) as a control. The primer sequences are: TGF-β1 forward primer, 5′-CCT GCT GTC TCC CTC AAC C-3′, TGF-β1 backward primer, 5′-CTG GCA CTG CTT CCC GAA TGT C-3′, fibronectin forward primer, 5′-TGT GAC AAC TGC CGT AGA CC-3′, fibronectin backward primer, 5′-GAC CAA CTG TCA CCA TTG AGG-3′, α-SMA forward primer, 5′-GAG AAC CCC ACC CAG TCG-3′, α-SMA backward primer, 5′-CTC TTT CTC TTG GCT TCA-3′, type I collagen forward primer, 5′-TGG TGC CAA GGG TCT CAC TGG C-3′, type I collagen backward primer, 5′-GGA CTT TCA ACA CCA CCT TCA CC-3′, GAPDH forward primer, 5′-GTG GAC ATT GTT GCC ATC AAC G-3′, GAPDH backward primer, 5′-GGG GGA GTT GTC ATA TTT CTC G-3′. PCR products were visualized by 1.2% agarose gel electrophoresis with EtBr staining.

**Western blot analysis**
Cells and tissues were lysed in buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 100 mM PMSF, 1 mM DTT, 10 mg/ml leupeptin and aprotinin; all from Sigma, St. Louis, USA) and centrifuged at 12,000 rpm for 30 min after storing 30 min on ice. Protein concentration was measured by the Bradford protein assay (BioRad). SDS-PAGE was performed on 8-12% polyacrylamide gels at 100 V for 3 h. The resolved proteins were transferred onto a PVDF membrane (Millipore, MA, USA) and probed with anti-TNF-α (Abcam, MA, USA), anti-IL-1β (Santa Cruz, CA, USA), anti-TGF-β1 (R&D Systems, MN, USA), anti-fibronectin (Abcam, MA, USA), anti-α-SMA (Sigma), anti-collagen I and anti-GAPDH (Santa Cruz, CA, USA) followed by secondary antibody conjugated to horseradish peroxidase (1:2,000) and detected with enhanced chemiluminescence reagents (Amersham Bioscience, NJ, UK). Signal intensity was quantified by image analyzer (Las3000, Fuji, Japan).

**Histopathology and immunohistochemistry**
Small pieces of liver from each lobe were kept in 10% formalin solution. Paraffin blocks were prepared. Cross-section taken from the blocks was stained with Masson’s trichrome. For immunohistochemistry, sections were incubated with anti-TNF-α (Abcam), anti-IL-1β (Santa Cruz), anti-TGF-β1 (R&D systems), anti-fibronectin (Abcam), anti-α-SMA (Sigma), anti-collagen I (Santa Cruz). After three serial washes with PBS, the sections were processed by an indirect immunoperoxidase technique using a commercial kit (LSAB
kit, DAKO, USA). Light microscopy was used to acquire the immunohistochemical image.

**Statistical analysis**
All values are expressed as means ± standard errors of the mean. Statistical differences in average between two groups and among three or more groups were assessed by unpaired t-test and ANOVA, respectively. All experiments were performed at least three times. p < 0.05 was considered significant.

**Results**

**Effect of Chi decoy ODN in activated HSC**
For this experiment, four kinds of ring type decoy ODN were designed. These included the Scr, Sp1, NF-κB and Chi decoy ODN, each containing their respective consensus binding sequence. Especially, Chi decoy ODN included two different Sp1 and NF-κB binding site, which expected potent regulatory control of these transcription factors (Fig. 1A).

To characterize the effect of Chi decoy ODN, activated HSC were transfected with the decoy ODN. The effect on their target genes were analyzed using RT-PCR and western blotting. Statistically significant interference of TGF-β1 expression was achieved with the Chi decoy ODN compared to those treated with Sp1 and NF-κB decoy ODN. The expression of fibronectin and α-SMA were increased in activated HSC, Chi decoy ODN treatment inhibited the expression of α-SMA compared to those treated with Sp1 and NF-κB decoy ODN (Fig. 1B and 1C). Although Sp1 and NF-κB decoy ODN also inhibited the expression of genes related to HSC activation, the effect of Chi decoy ODN was significantly greater than those of Sp1 and NF-κB decoy ODN alone. These findings suggest that Chi decoy ODN could effectively inhibit the expression of genes related to HSC activation both at transcriptional and translational levels.

**Chi decoy ODN treatment attenuates CCl4-induced liver fibrosis**

Based on the result of the Chi decoy ODN in HSC, we examined the effect of Chi decoy ODN on CCl4-induced liver fibrosis in mice. As shown in Fig. 2A and 2B, the level of serum AST and ALT was highly elevated in the liver treated with Scr decoy ODN. More than 10-fold increase of serum AST and ALT was observed after administration of CCl4 for 8 wk. In contrast, the level of serum AST and ALT were significantly reduced in mice treated with Chi decoy ODN.

Masson’s trichrome staining was done to determine whether in vivo transfection with Chi decoy ODN effectively blocked the accumulation of collagen fibers. Marked hepatic fibrosis with abundant matured collagen fibers were present 8 wk after CCl4 administration (Fig. 2D). Chi decoy ODN transfer showed only slight focal necrosis and deposition of blue collagen fibers at 8 wk after CCl4 administration (Fig. 2E). Together, it suggests that Chi decoy ODN transfer attenuated CCl4-induced liver fibrosis.

**Chi decoy ODN treatment suppresses the expression of proinflammatory cytokines CCl4-induced liver fibrosis**

Sustained hepatic inflammation provoked by long-term treatment with CCl4 is believed to induce liver fibrosis through the activation of transcription factor NF-κB, known to regulate inflammatory responses, such as TNF-α and IL-1β [16,22]. Next, we examined the effect of Chi decoy ODN in the production of TNF-α and IL-1β which are the key inflammatory cytokines in liver fibrosis. Serum concentration of TNF-α and IL-1β increased significantly in the control
mice, but decreased in the decoy ODN transfected mice (Fig. 3A and 3B). To investigate the effect of Chi decoy ODN on liver fibrosis, the expression of proinflammatory cytokines in the fibrotic liver was evaluated by immunohistochemistry. The cells positive for TNF-α and IL-1β were mainly distributed at the fibrous septa, areas of necrosis and hepatic lobule inflammatory cell infiltration sites in CCl4 exposed livers. When compared to Scr decoy ODN treatment, the expression of TNF-α and IL-1β in the Chi decoy ODN treatment was markedly decreased in the fibrotic liver (Fig. 3C and 3D). These results suggest that inhibition of NF-κB and Sp1 using Chi decoy ODN suppresses the proinflammatory cytokines responsible for the development of liver fibrosis.

Chi decoy ODN treatment inhibited fibrogenic gene responses in the fibrotic liver

The expression of TGF-β1 was progressively increased in CCl4 exposed liver. Chi decoy ODN treatment significantly reduced the elevated levels of TGF-β1 compared to Scr decoy ODN treatment. Also, treatment of Chi decoy ODN significantly inhibited the expression of α-SMA when compared with the Scr decoy ODN transfer. Liver fibrosis is associated with major alteration in both the quantity and composition of the ECM. This phenomenon is mainly due to upregulation of TGF-β1 expression [2,27]. Thus, we investigated the expression of ECM-related genes, such as fibronectin and type I collagen, in the decoy ODN treated fibrotic livers. The expressions of fibronectin and type I collagen were all upregulated in the fibrotic livers, consistent with the resulting increase of TGF-β1 expression. Chi decoy ODN treatment significantly abrogated this activation when compared to Scr decoy ODN treatment in the fibrotic liver (Fig. 4).

To investigate histological changes affected by Chi decoy ODN in liver fibrosis, immunohistochemical staining was performed. The cells positive for TGF-β1 was progressively increased in the bridging fibrous septa in CCl4 exposed livers treated with Scr decoy ODN. Chi decoy ODN treatments significantly attenuated the TGF-β1 expression in periportal area (Fig. 5A). The number of α-SMA, which is a marker of activated HSC in the fibrotic liver [4]. After 8 weeks of CCl4 injections, lots of α-SMA-positive cells were observed in regions of bridging necrosis in Scr decoy ODN treated mice, but they were hardly seen in Chi decoy ODN mice (Fig. 5B). These changes affect the expression of fibronectin and type I collagen, which are the main component of the ECM regulated by TGF-β1 [3]. The expression of fibronectin and type I collagen were also increased around the fibrotic septa in accordance with the reduction of TGF-β1 expression in CCl4 exposed livers treated with Scr decoy ODN. In particular, the cells positive for fibronectin and type I collagen was significantly reduced in livers treated with Chi decoy ODN compared to Scr decoy ODN (Fig. 5C and 5D). These observations suggest that Chi decoy ODN treatment inhibits

![Fig. 3. Effect of Chi decoy ODN by ELISA and immunohistochemistry of pro-inflammatory cytokines in fibrotic liver. Serum concentration of (A) TNF-α and (B) IL-1β was decreased by Chi decoy ODN treatment compared to Scr decoy ODN treatment. p<0.05 vs CCl4 treated with Scr decoy ODN. Chi decoy ODN prevents (C) TNF-α and (D) IL-1β expression in liver sections. Normal liver on 8 weeks (upper), Scr decoy ODN treated with CCl4-induced liver fibrosis on 8 weeks (middle), Chi decoy ODN treated with CCl4-induced liver fibrosis on 8 weeks (lower), respectively. Magnification: ×200.](image)

![Fig. 4. Effect of Chi decoy ODN the expression of fibrosis related genes in liver fibrosis in vivo. (A) Chi decoy ODN effectively suppressed TGF-β1, α-SMA, fibronectin and type I collagen protein expression compared Scr decoy ODN in liver fibrosis. (B) Chi decoy ODN effectively also suppressed TGF-β1, α-SMA, fibronectin and type I collagen mRNA expression compared to Scr decoy ODN in liver fibrosis.](image)
the fibrotic changes in CCl4-induced liver fibrosis.

Discussion

Recent advances in cellular and molecular research have provided new techniques to inhibit gene expression using DNA technology. One of the most successful DNA-based approaches is the decoy ODN technique which uses a synthetic double-stranded ODN containing an enhancer element [19,20,26]. Nucleic acid molecules with high affinity for a target transcription factor can be introduced into cells as decoy cis-elements that bind to these factors and alter gene expression. However, in vivo usage of decoy ODN is hampered by nuclease digestion. To overcome this limitation, we have developed ring type decoy ODN which has enzymatic ligation of two kinds of molecules with two hairpin-overhangs at both ends to prevent degradation by nucleases [13,14]. Especially, Chi decoy ODN included two different Sp1 and NF-κB binding site, which exerted potent regulatory control of these transcription factors. This novel decoy ODN possesses increased nuclease resistance and diverse effects in one decoy ODN molecule.

Recently several studies demonstrated that Chi decoy ODN strategy using NF-κB and Ets transcription factor was successful treatment of abdominal aortic aneurysm in a rabbit model [17,18]. Although some publications have described Chi decoy ODN in aortic aneurysm, there is little known about the effectiveness of Chi decoy ODN strategy in liver fibrosis. Based on previous studies for NF-κB and Sp1 in liver fibrosis [4,11,24], this study was designed using a Chi decoy ODN strategy targeting both Sp1 and NF-κB to inhibit both Sp1 and NF-κB simultaneously in liver fibrogenesis. To determine the feasibility of treating liver fibrosis using a Chi decoy ODN, this study was initially assessed the effects of the Chi decoy ODN on activated HSC. Chi decoy ODN significantly inhibited the activation of Sp1 and NF-κB as compared with transfection of Sp1 and NF-κB decoy ODN independently in activated HSC.

Based on our in vitro findings, this study examined the effects of Chi decoy ODN on CCl4-induced liver fibrosis. CCl4 is a hepatotoxin that causes acute liver injury and induces liver fibrosis when given repetitively at a low dose. CCl4-induced liver fibrosis is a well-established experimental model for liver fibrosis. This experimental fibrosis model resembles human fibrosis caused by alcohol consumption and serves as an attractive model to investigate the molecular events associated with chronic liver injury and also to screen antifibrotic agents [29,30]. Accordingly, this model is suitable to study the upregulation of several genes involved in liver fibrogenesis and also to knockdown the upregulated expression of these genes using decoy ODN based gene therapy.

One of the early pathogenic mechanisms of liver fibrosis involves increased proinflammatory gene expression due to the damage response from various liver pathogens. Sustained hepatic inflammation provoked by long-term treatment with CCl4 is believed to induce hepatic fibrosis through the activation of transcription factor NF-κB, known to regulate inflammatory responses [23]. Transcriptional activation of NF-κB was associated with inflammatory cytokines, such as TNF-α and IL-1β [16,22]. In CCl4-induced fibrosis model, Chi decoy ODN transferred in vivo ameliorated hepatic injury induced by CCl4 while suppressing production of TNF-α and IL-1β.

Many factors are involved in the progression of liver fibrogenesis and especially TGF-β1 is the most potent regulator in liver fibrosis [9]. It regulates ECM proteins by reducing matrix degradation and stimulating matrix accumulation in the development of liver fibrogenesis by the regulation of transcription factor Sp1 [6,25,28]. In this study, TGF-β1 expression was dramatically upregulated in CCl4-induced hepatic fibrosis. However, treatment with Chi decoy ODN significantly suppressed the expression of TGF-β1 compared to Scr decoy ODN treatment. The expression of α-SMA, fi-
bronectin and type I collagen were also suppressed by Chi decay ODN in accordance with the reduction of TGF-β1 expression when compared to Scr decay ODN treatment. The present study demonstrated that the Chi decay ODN reversed established liver fibrosis in mice. It also effectively attenuated the inflammatory, fibrogenic gene responses after CCl4 administration compared to each Scr decay ODN treatment.

In summary, this study demonstrated the feasibility of using Chi decay ODN against NF-κB and Sp1 to prevent liver fibrosis in a mouse model. Chi decay ODN attenuated the activation of HSC and prevented the fibrogenic and proinflammatory gene responses after CCl4 administration. Given the successful inhibition of liver fibrosis using Chi decay ODN in a murine model, gene therapy targeted to suppress NF-κB and Sp1 simultaneously might provide a new therapeutic strategy to prevent liver cirrhosis.

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References

초록: 간경화 동물모델에서 Chimeric decoy oligodeoxynucleotide로 억제되는 NF-κB와 Sp1

전사인자 발현 억제 효과에 대한 연구

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간상유화는 지속적인 간세포 손상에 대한 수복하장으로 일어나며, 급성 염증반응과 같은 손상이 주어진 후에는 간세포의 파사 및 세포원기기의 축적이 일어나게 된다. 간상유화에 대한 새로운 치료방법을 모색하기 위하여 본 연구에서는 간상유화 과정에서 염증 반응과 관련된 NF-κB와 세포원기기의 축적과 관련된 Sp1전사인자를 동시에 조절하여 간상유화 억제효과를 관찰하고자 하였다. 전사인자인 Sp1과 NF-κB를 동시에 억제하기 위하여 본 논문 내에 Sp1과 NF-κB의 전사인자와 결합하는 부위를 가지는 Chimeric (Chi) decoy oligodeoxynucleotide (ODN)을 제작하였다. Chi decoy ODN은 활성화된 간상세포에서 간상유화와 관련된 유전자 발현을 억제하였으며, 성형학 동물모델에서도 간 조직의 염증 반응 및 손상유와 관련 인자의 발현을 현저히 억제하였다. 따라서 Chi decoy ODN은 간상유화 및 활성화된 간상세포의 활성을 억제할 수 있는 유전자 치료제로 고려될 수 있을 것으로 사료된다.