Polymerase synthesis and potential interference of a small-interfering RNA targeting hPim-2

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Abstract

AIM: To synthesize three small-interference RNAs (siRNAs) by T7 RNA polymerase-catalyzed reaction, and to investigate their efficacy on modulating the expression of serine/threonine kinase Pim-2 in human colon cancer cell line.

METHODS: siRNA I, II and III were synthesized by T7 RNA polymerase-directed in vitro transcription, then transfected into human colon cancer cells SW-480. After incubation for 6 h at 37 °C, 100 µL/L FBS in RPMI 1640 was substituted into each well. After the transfection was repeated twice to inhibit hPim-2 expression, the cell size and its role in the survival of cancer cells have been just determined recently[14,15]. It is believed to be a cancer-causing gene, or oncogene. Here, we sought to use siRNA-targeting hPim-2 to determine whether this technique could be used to specifically inhibit hPim-2 expression.

RESULTS: Compared to the control group, after transfected for 48 h with hPim-2 siRNA I, II and III, the relative inhibition rates of hPim-2 mRNA expression in colon cancer cells were 65.4% (P<0.05), 45.8% (P<0.05) and 56.1% (P<0.05), respectively. The protein level of hPim-2 was decreased at 72 h compared to the untransfected cells. The relative inhibition percentages of hPim-2 protein by siRNA I, II, III were 61.6% (P<0.05), 45.8% (P<0.05) and 55.6% (P<0.05), respectively.

CONCLUSION: The in vitro transcribed siRNAs can be useful for silencing oncogene hPim-2 expression specifically and efficiently. This may open a new path toward the use of siRNAs as a gene-specific therapeutic tool.

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INTRODUCTION

RNAi is an evolutionarily conserved mechanism known to control insects, plants, and mammalian cells[1-4]. In this process, introduced double-stranded RNAs (ds-RNAs) silence gene was expressed through specific degradation of their cognate mRNAs[5,6]. Importantly, RNAi can be achieved in mammalian cells following transfection of synthetic 21- and 22-nucleotide (nt) small interfering (si) RNAs, indicating that RNAi may serve as a powerful tool to block the expression of target genes specifically[7,11].

Pim-2 is a member of a family of serine/threonine protein kinases that consists of two other members, Pim-1 and Pim-3, and it exists at high concentrations in many tumor cells[12,13]. Though it was identified 20 years ago, its function that maintains the cell size and its role in the survival of cancer cells have been just determined recently[14,15]. It is believed to be a cancer-causing gene, or oncogene. Here, we sought to use siRNA-targeting hPim-2 to determine whether this technique could be used to specifically inhibit hPim-2 expression.
of the experiment. Transfection of the RNA oligonucleotides was performed using Lipofectamine 2000 (Invitrogen) as directed by the manufacturer to result in a final RNA concentration of 50 mmol/L. After transfection (incubation for 6 h at 37 °C), cells were washed with PBS and incubated in fresh culture medium until additional analyses.

**Analysis of hPim-2 mRNA by RT-PCR**

After transfection, total RNA was isolated using TRIZOL (Invitrogen) by a single-step phenol-extraction. Subsequent RT-PCR was performed (RT-PCR kit, Promega, Madison, WI.). Briefly, first strand cDNA was synthesized using an Oligo (dT)15 primer at 42 °C for 30 min. PCR for hPim-2 and β-actin was performed in a single reaction of 20 μL volume. The latter served as a control following 28 cycles of denaturing at 95 °C for 45 s, annealing at 58 °C for 40 s, and extending at 72 °C for 40 s. Under this PCR condition, the amplification showed linearity as determined experimentally (data not shown). PCR products were run on a 30 g/L agarose gel and visualized by ethidium bromide staining, and the intensities were then measured by scanning the gel with Gel Doc 1000 (Bio-Rad), and the inhibition percentage (%) was calculated according to the following formula: inhibition percentage = (1- A<sub>sample</sub>/ A<sub>control</sub>) ×100.

**Analysis of hPim-2 protein**

The expression levels of hPim-2 protein in cells transfected with siRNAs were measured by scanning the density of bands on Western blotting. The expression level of hPim-2 mRNA with siRNAs were measured by scanning the gel with Gel Doc 1000 (Bio-Rad), and the inhibition percentage (%) was calculated according to the following formula: inhibition percentage = (1- A<sub>sample</sub>/ A<sub>control</sub>) ×100.

**Statistics**

The data were expressed as mean±standard deviation (mean±SD). Statistical analysis was performed by Student's-t-test (two tailed). All data presented at least two independent experiments.

**RESULTS**

**Synthesis of siRNA by in vitro transcription**

To generate siRNAs by *in vitro* transcription, we designed the strategy presented in Figure 2. Target sequences for siRNA were identified by scanning the length of the hPim-2 gene with an appropriate genome database to eliminate any sequences with significant homology to other genes. Those sequences that appear to be specific are the potential siRNA target sites. Besides, it is noteworthy that T7 promoters are invariant and common to any target gene. A 40 mer DNA oligonucleotide template was synthesized according to the following strategy presented in Figure 2.
by a 21 mer oligonucleotide encompassing the T7 promoter with complementary sequence preceded by two additional nucleotides (reading the sequence 5'----3'). Following transcription reactions, sense and antisense transcriptions were annealed, ethanol precipitated and yielded what we refer to as T7siRNAs. The integrity of the transcriptions was checked on a 30 g/L agarose gel (Figure 3).

**Figure 3** Lane 1: T7 in vitro transcribed single-strand RNA. Lane 2: annealed double-strand DNA template. Lane 3: hybridized double-strand small interference RNA.

**Effect of siRNAs on hPim-2 expression**
The mRNA level of hPim-2 was determined by semi-quantitative RT-PCR. A 237-bp DNA fragment of hpim-2 gene and a 317-bp DNA fragment of β-actin gene were amplified by RT-PCR with specific primers, respectively. As shown in Figure 4A, mRNA expression level of hPim-2 was decreased when compared to the uninduced cells, while the mRNA level of β-actin as the control was almost unchanged. As shown in Figure 4B, after transfection with hPim-2 siRNA I, II and III and compared with the levels of β-actin, the relative inhibition rates of hPim-2 mRNA expression were 65.4% (P<0.05), 46.2% (P<0.05) and 56.1% (P<0.05) in colon cancer cells, respectively.

In order to verify the decrease in mRNA expression, which corresponded to the decreases at protein levels, Western blotting was performed. Figure 5A shows that the protein level of hPim-2 was decreased at 72 h compared to the uninduced cells. The relative inhibition percentages of hPim-2 protein by siRNA I, II and III were 61.6% (P<0.05), 45.8% (P<0.05) and 55.6% (P<0.05), respectively (Figure 5B).

**DISCUSSION**
Oncogene overexpression has been implicated in the development and progression of a variety of human cancers and, therefore, provides a potential target for cancer gene therapy[19-22]. For years, research has been focused on effective tools to specifically down-regulate oncogene overexpression such as antisense oligonucleotide strategy. However, there has been only limited success because of the lack of specificity and potency for this method. For example, screening of more than 20 oligomers is usually required before identifying one antisense that functions effectively, and the dose required for inhibiting gene expression is often not much different from the doses that lead to nonselective toxicity[23-25].

Recent progress of RNAi techniques has demonstrated the potential to overcome those limitations. The selection of targeting sequences of RNAi is less restricted, once the site is identified, sense and antisense oligonucleotides with 3’-UU overhangs can be designed, so the success rates of producing effective duplexes are higher. Just like in this experiment, siRNAs were designed complimentary to three different regions of the corresponding Pim-2 mRNA, and each of them has different level of inhibition efficacy, the suppression of hPim-2

![Image](image-url)
gene expression by these siRNAs directed at different sites varied from 45-65%. This indicates that screening potential target of RNAi is much more easy.

Besides, our results demonstrate that in vitro transcribed siRNA can effectively down-regulate oncogene expression with great efficiency. It has been suggested that siRNA may inhibit gene expression through diverse effects, inhibition of mRNA can occur through the formation of a nuclease complex called RISC (RNA-induced silencing complex) that targets and cleaves mRNA which is complementary to the siRNA. The damaged mRNA may deteriorate through the action of the RNA-dependent RNA polymerase (RdRNP), producing new siRNAs to target other mRNA. This incessant waterfall-like amplification can produce RNA interference effect at a very small dose, and inhibit the protein translation quickly and efficiently[28-30]. In our experiment, the dose required for inhibiting Pim-2 gene expression was 50 nmol/L, far below the dose required for the antisense oligonucleotide[31], indicating that siRNA synthesized by the in vitro transcription strategy can suppress the hPim-2 gene expression sensitively.

Here, we used the in vitro transcription method for the synthesis of siRNAs by T7 RNA polymerase and transferred them into cells. The main advantage of this technique is its simplicity. It provides a reproducible and highly efficient means to inhibit the target gene expression. Human Pim-2 gene, a regulated transcriptional apoptotic inhibitor, has a novel role in promoting cell autonomous survival. Over-expression of Pim-2 allows the tumour cells to ignore or become insensitive to boosters of the immune system[14]. Application of Pim-2-directed siRNA can significantly reduce Pim-2 mRNA and protein levels efficiently. Our next step is to try to manipulate the action of Pim-2 with siRNA, so that we can interfere with the survival of cancer cells.

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