Transient Reduction of PTI-1 Expression by Short Interfering RNAs Inhibits the Growth of Human Prostate Cancer Cell Lines

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YU, L., WU, G., WANG, L., WANG, H. and ZHANG, G. Transient Reduction of PTI-1 Expression by Short Interfering RNAs Inhibits the Growth of Human Prostate Cancer Cell Lines. Tohoku J. Exp. Med., 2006, 209 (2), 141-148 —— The prostate tumor-inducing gene 1 (PTI-1) was originally identified by differential ribonucleic acid (RNA) display in human prostate carcinoma. PTI-1 is expressed in human prostate carcinoma but not in benign prostate hypertrophy or normal prostate tissue. PTI-1 may be a member of oncogenes that could affect protein translation and contribute to carcinoma development in human prostate. To investigate the role of PTI-1 in human prostate carcinoma, we constructed three different short interfering RNA (siRNA) vectors (pSilencer3.1-neo-Yu Lei [YL]1-2, -YL3-4 and -YL5-6), each of which was transfected into DU145 and PC3 human prostate cancer cell lines. Among these siRNAs, only pSilencer3.1-neo-YL1-2 could almost completely block the expression of PTI-1 in these two cell lines. The growth of the cell lines was then evaluated after transfection. The proliferation rate was retarded in DU145 and PC3 cells transfected with pSilencer3.1-neo-YL1-2, compared with the cells transfected with a control vector; namely, about 88.6% of DU145 and 80.2% of PC3 cancer cells were blocked at the G1 phase when transfected with pSilencer3.1-neo-YL1-2, compared to 62.0% in DU145 cells and 51.7% in PC3 cells, transfected with the control vector. Moreover, 68.3% of DU145 cells and 72.3% of PC3 cells were induced into apoptosis, while in control transfection, the population was 26.6% in DU145 cells and 28.4% in PC3 cells. These results indicate that blocking PTI-1 expression can inhibit the growth of certain prostate cancer cell lines. We suggest that PTI-1 may serve as a target for the gene-based therapy of human prostate carcinoma. ——— PTI-1; siRNA; prostate carcinoma; cell growth; gene therapy

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Adenocarcinoma of the prostate is now the most frequently diagnosed male malignancy with 1 in every 11 men developing the disease (Hayward and Cunha 2000). It is the second most common cause of cancer deaths in men. A variety of clinical, biochemical, pathological and imaging tools are currently involved in the diagnosis and staging of condition which all require fairly bulky
tumor regrowth before discovery (Salo et al. 1987). Finding a more sensitivity marker or oncogenic potential gene will provide us a useful tool to diagnose and cure prostate carcinoma.

Prostate tumor-inducing gene 1 (PTI-1) was originally identified by rapid expression, cloning and differential ribonucleic acid (RNA) display in human prostate carcinoma (Shen et al. 1995). The sensitivity of PTI-1 for detecting prostate cancer cells was ≥ 100-fold greater than the sensitivity of commonly used prostate cancer markers such as prostate specific antigen or prostate specific membrane antigen (Sun et al. 1997). The PTI-1 cDNA has been reported to consist of 2,123 bp containing a 630-bp 5’ untranslated region (UTR) with 85% identity to 23S ribosomal RNA from mycoplasma hypopneumoniae and a 1,494-bp region with 97.7% identity to human translation elongation factor 1α (EF-1α). The PTI-1 encodes a protein of 398 amino acids. Compared with EF-1α, at the fusion junction of N-terminal, PTI-1 has the deletion of 67 amino acids and introduction of 3 exogenous amino acids (Met-Gln-Ser). Further more, there presents six point mutations in specific amino acids, from positive charged to hydrophobic (Arg to Gly) and hydroxyl group-containing to nonhydroxyl group-containing amino acids (Ser to Gly). PTI-1 should encode a protein of 43.8 kDa, but it is translated to a ~46-kDa protein in vivo which may result from protein modification. The studies show that the function of PTI-1 is related with oncogenicity (Su et al. 1998). EF-1α is physically associated with and may be an important element in mitotic spindle formation (Marchesi and Ngo 1993), possibly playing a role in growth control, cell division and potentially enhanced survival. PTI-1 (mutant fragment of EF-1α) may through interrupting the function of EF-1α lead to the transformed phenotype of cancer cells. In summary, PTI-1 acts not only as a marker for the earlier diagnosis to the prostate cancer cells, but also as a target gene for the therapy.

To study the relationship between PTI-1 and proliferation of human prostate carcinoma, we constructed three RNAi vectors (pSilencer3.1-neo-YL1-2, YL 3-4, YL 5-6), which target the PTI-1 gene, to investigate whether silencing this oncogene can inhibit the growth of human prostate cell lines (DU145 and PC3) in vitro.

**Materials and Methods**

**Plasmic and material**

Using siRNA Design Tools on web site (http://www.Ambion.com/techlib/resources/RNAi/index.html) to design target sequences. A siRNA motif according to the AA-N19 rule was selected by applying find pattern program to human PTI-1 cDNA sequence (GenBank Table 1. Sequences of the siRNA targets.

<table>
<thead>
<tr>
<th>Code</th>
<th>Target sequence</th>
<th>Synthesized primer</th>
</tr>
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<tbody>
<tr>
<td>y1-1</td>
<td>aattaagctatgcagtcggaa</td>
<td>5′-GATCCGTTAAGCTATGCAAGCTGGAATTCAAAGGATTCCGACTGCAATTACGTTAAATTTTTTGGAAA-3′</td>
</tr>
<tr>
<td>y1-2</td>
<td>aattaagctatgcagtcggaa</td>
<td>5′-AGCTTTTCTAAAATTAAAGCTATGCAAGCTGGAATTACGTTAAATTTTTTGGAAA-3′</td>
</tr>
<tr>
<td>y1-3</td>
<td>aagctttgggtaagcttcc</td>
<td>5′-GATCCGCTTTGGGTAAGCTTCTCTCCAAGAGGAAAGAGCTTACCCCAAAGCTTTTTTGGAAA-3′</td>
</tr>
<tr>
<td>y1-4</td>
<td>aagctttgggtaagcttcc</td>
<td>5′-AGCTTTTCTAAAATTAAAGCTATGCAAGCTGGAATTACGTTAAATTTTTTGGAAA-3′</td>
</tr>
<tr>
<td>y1-5</td>
<td>aagctattgccctccttcct</td>
<td>5′-GATCCGCAGCTGGCCCTCCTCTCCAAGAGGAAAGAGCTTACCCCAAAGCTTTTTTGGAAA-3′</td>
</tr>
<tr>
<td>y1-6</td>
<td>aagctattgccctccttcct</td>
<td>5′-AGCTTTTCTAAAATTAAAGCTATGCAAGCTGGAATTACGTTAAATTTTTTGGAAA-3′</td>
</tr>
<tr>
<td>c1</td>
<td>aaagttcaacgaccagtagtc</td>
<td>5′-GATCCGTTCCAACGACCAGTATCTTGTTGACTCTGTGGACTTTTTTGAAA-3′</td>
</tr>
<tr>
<td>c2</td>
<td>(target no gene)</td>
<td>5′-AGCTTTTCTAAAATTAAAGCTATGCAAGCTGGAATTACGTTAAATTTTTTGGAAA-3′</td>
</tr>
</tbody>
</table>
accession no. L41498. Three target sequences were found (Table 1), and an insertion consequence (do not target any gene) was also synthesized. The four pairs of primers were annealed and cloned between BamH I and Hind III restriction site in pSilencer TM 3.1-H1/neo according to the manufacturer’s recommendations. The four constructs were named pSilencer3.1-neo-YL1-2, pSilencer3.1-neo-YL3-4, pSilencer3.1-neo-YL5-6 and pSilencer3.1-Control (pSilencer3.1-C). Mouse monoclonal anti-eEF-1α antibody was purchased from Upstate. Horseradish peroxidase (HRP)-conjugated secondary antibodies from BOSTER (Wuhan, China).

Cell culture and transfection
DU145 and PC3 human prostate cancer cell lines were purchased from the American Type Culture Collection (ATCC), cells grew in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a 95% air/5% CO2 humidified incubator. The cell lines used in this article were tested for mycoplasma contamination by using the Gen-Probe mycoplasma test kit (Gibco, Gaithersburg, MD, USA) and were found to be mycoplasma free. Twenty-four hours before transfection, cells were trypsinized and seeded in six-well culture plates at 1 × 105 cells per well. The cells were transiently transfected with siRNA expression vectors by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The medium was replaced with a fresh medium 5 hrs after transfection, and cells were harvested 60 hrs after transfection. Experiments were performed in triplicate.

Reverse transcription-polymerase chain reaction
The treated cells were trypsinized and then centrifuged for 2 min at 12,000 rpm at 4°C. Cell pellets were washed with phosphate buffer saline (PBS), collected, and lysed with Trizol reagent (Gibco BRL, Gaithersburg, MD, USA) and found to be mycoplasma free. Twenty-four hours before transfection, cells were trypsinized and seeded in six-well culture plates at 1 × 105 cells per well. The cells were transiently transfected with siRNA expression vectors by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The medium was replaced with a fresh medium 5 hrs after transfection, and cells were harvested 60 hrs after transfection. Experiments were performed in triplicate.

Western blot analysis
Cell culture monolayers in six-well culture plates were washed twice with ice-cold PBS and lysed with 150 μl of radioimmune precipitation assay (RIPA) lysis buffer (0.05 M Tris-HCl [pH 7.4], 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM ethylene diamine tetraacetic acid [EDTA]) containing protease inhibitors (1 mM phenylmethyl sulfon fluoride [PMSF], 1 μg/ml aprotinin and 1 μg/ml leupeptin). Proteins were measured (bicinchoninic acid [BCA] protein assay, Pierce), and samples were resolved by reducing SDS-PAGE, transferred to nitrocellulose, and incubated in blocking buffer (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.05% Tween 20, and 5% bovine serum albumin [BSA]), followed by incubation with the indicated antibodies at 4°C overnight. The membranes were then washed in blocking solution and incubated with HRP-conjugated secondary antibodies for 1h at room temperature. Antibody detection was performed by the Pierce chemiluminescence HRP substrate instruction. Expose the blot to x-ray for the appropriate time period.

Cell growth assay
Achorage-dependent cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis. Briefly, 2,000 cells/well were plated in 96-well plates and cultured for up to 7 days. Each day cell growth was determined by adding MTT solution (50 μg/well) for 4 hrs. Cellular MTT was solubilized with acidic isopropanol and optical density was measured at 570 nm with a plate reader. The result of the MTT assay correlated with results obtained by cell counting with a hemocytometer and by monitoring thymidine incorporation. The tripling time was then calculated for the exponential growth phase.

Soft agar assay
Cells (2 × 103/well) were suspended in 3 ml of 0.3%
Difco Noble agar supplemented with complete culture medium. This suspension was layered over 1.5 ml of 0.5% agar-medium base layer in 12-well plate. After two weeks, cells were stained with MTT (400 μg/well) overnight and colonies > 0.05 mm were counted.

**Flow cytometric analysis of apoptotic quantitation and cell cycle**

Measurements of apoptotic cells and cell cycle distribution were performed using a modification of the technique. Apoptotic cells were quantified by staining with FITC-conjugated Annexin V (Clontech, Palo Alto, CA, USA). Cells (1 × 10^6) were collected at 72 hrs for FCM and stained with FITC-conjugated annexin V and PI as instructed by the manufacturer, and then analyzed by flow cytometry using a FACS (BD Biosciences, San Jose, CA, USA) with an argon laser set to excite at 488 nm. In addition, cells were harvested at each time point and were fixed in 70% ethanol and stored at 4°C. Cell cycle distribution was performed at the indicated intervals following transfection. PI (40 μg/100 μl PBS) was added to 1 × 10^6 cells. Red fluorescence due to propidium-bound DNA was measured using a 630 nm-long bandpass filter. Data was analyzed as single-parameter frequency histograms in a SFIT model.

**RESULTS**

**Identify an efficient RNAi vector to silence the expression of PTI-1 without affecting the EF-1α**

The four constructs (pSilencer3.1-neo-YL1-2, YL3-4, YL5-6 and pSilencer3.1-C) were transiently transfected into DU145 and PC3 cell lines and their ability to downregulate expression levels of PTI-1 in mRNA and in protein was tested. Among the four constructs, only pSilencer3.1-neo-YL1-2 was shown to significantly reduce mRNA and protein levels of PTI-1 without affecting the EF-1α gene. RT-PCR showed that transfection of vector pSilencer3.1-neo-YL1-2 completely decreased level of PTI-1 without affecting the expression level of EF-1α. Whereas cells transfected with other vectors, pSilencer3.1-neo-YL3-4 and pSilencer3.1-neo-YL5-6, can also decreased the level (about 67% in DU145 and 37% in PC3 cells) of PTI-1, but they also decreased the expression of EF-1α (about 52% in DU145 and 21% in PC3 cells). pSilencer3.1-C did not affect the expression of both PTI-1 and EF-1α (Fig. 1). For the amino acids similarity of EF-1α and PTI-1, the antibody of EF-1α was used to detect the expression level of PTI-1. Western blot analysis of extracted proteins got the consistent result compared with RT-PCR. (Fig. 2). In protein level, by transient transfection the vector of pSilencer3.1-neo-YL1-2 can decrease level of PTI-1 (up to 95%) without affecting the EF-1α.
pSilencer3.1-neo-YL3-4 and pSilencer3.1-neo-YL5-6 can reduced both EF-1α and PTI-1. pSilencer3.1-neo-C did not affect both of them.

Transfection of pSilencer3.1-neo-YL1-2 inhibits anchorage-dependent and –independent cell growth in DU145 and PC3 cells

To assess the potential role of PTI-1 in prostate cancer cell growth, DU145 and PC3 cells were transfected with pSilencer3.1-neo-YL1-2, which can reduce the expression of PTI-1 without affecting the EF-1α. In order to investigate the anchorage-dependent growth rates, MTT assay was used. An equal amount of a control vector pSilencer3.1-neo-C was also introduced into DU145 and PC3 cells (untransfected cells as another control). After seven days cultured, we compared the three groups of cells proliferation rate. The significant difference was observed after five days culture. The cells transfected with pSilencer3.1-neo-YL1-2 obviously retarded the proliferation rate after five days culture. However, the cells transfected with pSilencer3.1-neo-C and untransfected cells showed the same proliferation rate (Figs. 3A and 3B). The MTT experiment showed the similar results in DU145 and PC3 cells. To determine anchorage-independent growth rate, soft agar assay was carried out. The cells (pSilencer3.1-neo-C) showed an average colony forming efficacy of 107.2 ± 16.7% (as compared to the control, wild type DU145 cells = 100%). In contrast, the positive clone showed a decreased (p < 0.05) colony forming ability of 60.7 ± 10.6% compared with DU145 cells (Fig. 3C). The PC3 cells (pSilencer3.1-neo-C) showed an average colony forming efficacy of 106.5 ± 15.8% and the positive clone showed a decreased (p < 0.05) colony forming ability of 67.0 ± 5.2% compared with PC3 cells (data not shown).

Transfection of pSilencer3.1-neo-YL1-2 induces apoptosis and cell cycle arrest of DU145 and PC3 cell lines

To explore the mechanism of growth suppression in PTI-1-silencing cells, cell cycle analysis was performed. The population of cells in Go/G1-phase was 88.6, 62.0 and 52.2% in the DU145 cells transfected with pSilencer 3.1-neo-YL1-2, those with pSilencer3.1-neo-C, and untransfected DU145 cells, respectively (Figs. 4A and 4B). Likewise, in PC3 cells, the population of cells in Go/G1-phase was 80.2, 51.7 and 48.1%, respectively (data not shown). The silencing PTI-1 caused a remarkable increase in subdiploid DNA content about 31.5% in DU145 and 28.9% in PC3 cells, suggesting increased cell death. Apoptosis was also assessed by annexin V-FITC staining to
detect phosphatidyl-serine on the cell surface in conjunction with propidium iodide staining. As shown in Fig. 4C, PTI-1 silencing cells showed an increase in annexin V-positive and PI-negative cells when compared with pSilencer3.1-neo-c and wild type control cells indicating increased cell death via apoptosis. Approximately 68.3% DU145 cells were induced into apoptosis (Fig. 4C) and the apoptosis PC3 cells were 72.3% (data not shown). The population of cells, which were induced into apoptosis, was 26.6% and 16.0% in the DU145 cells transfected with pSilencer3.1-neo-C and untransfected DU145 cells, respectively (Fig. 4C), while in PC3 cells, the population of apoptosis cells was 28.4% and 18.8%, respectively (data not shown). These results indicate that the anti-growth activity of silencing PTI-1 is associated at least in part with induction of cellular apoptosis.

**DISCUSSION**

The incidence and mortality of prostate cancer continue to rise and will continue to pose a major public health problem, especially for men. Despite progress in diagnosis and local therapy, fundamental questions remain with regard to the etiology, prevention, and treatment of prostate cancer. Although there have been the rapid development of molecular and cellular biology, our knowledge of prostate adenocarcinoma remains significantly less than of most other neoplasms (Ware 1994). Loss of the heterozygosity studies and comparative genomic hybridization techniques have suggested that chromosomes 6q, 8p, 9p, 10q, 13q, 16q and 18q are potential sites for genes associated with the initiation of prostate carcinoma (Bergerheim et al. 1991; Macoska and Trybus 1994). The most common abnormalities are found on 8p and 13q.

A rapid expression cloning strategy coupled with differential RNA display, screening of a human LNCaP cDNA expression library, and the rapid amplification of cDNA ends (RACE) approaches identified a putative prostate carcinoma tumor inducing oncogene, PTI-1 (Shen et al. 1995; Sun et al. 1997). Further studies show that:

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Fig. 3. Retardation of cell growth by transient transfection of pSilencer3.1-neo-YL1-2.
A and B: Anchorage-dependent cell growth was determined by MTT assay as described in Material and methods. Data showed that block PTI-1 by pSilencer3.1-neo-YL1-2 construct retards DU145 and PC3 cells proliferation. The x-axis indicates cultured time period. The y-axis represents the row OD value. Cell growth rate of cells transfected with control vector (pSilencer 3.1-neo-C) or wild type was comparable. C: Anchorage-independent cell growth was measured by the soft agar assay as described in Material and methods. Date are presented as means ± S.E.M.
1) PTI-1 is expressed in patient-derived prostate carcinoma tissues, but not in normal prostate or benign hypertrophic prostate tissues; 2) Using PTI-1 as marker to detect prostate cancer is more sensitive. Serial-dilution experiments indicate that PTI-1 can detect 1 prostate carcinoma cell in $10^8$ cells not expressing PTI-1; 3) PTI-1 is an oncogene and its expression is directly involved in controlling growth and maintaining the transformed phenotype. All of these characters not only make the PTI-1 gene as a perfect marker to early detect the prostate cancer, but also as a gene therapy target for the prostate cancer. Antisense, ribozyme and RNAi (Fire et al. 1988; Paul et al. 2002) are the mostly used methods to silence target gene. In this article, we chose RNAi technology to see if silencing the PTI-1 gene can convert the prostate carcinoma.

Fig. 4. Analysis of apoptosis and cell cycle after silencing the PTI-1 gene in DU145 cell line.
A: Cell cycle analysis. DU145 cells were transfected twice with pSilencer3.1-neo-YL1-2 by using Lipfection 2000 and were prepared for FACS analysis after 5 days. The pictures show one experiment, which was similar to 2 additional independent experiments. B: Cell cycle distribution. Shown is the percentage of cells with G0/G1, S and G2/M-phase DNA content. C: Apoptosis. Cells were stained with annexin V-FITC and PI to differentiate apoptosis and necrosis. Viable cells are FITC-negative/PI-negative; late apoptosis or cell death is FITC-positive/PI-positive; and early apoptotic cells are FITC-positive/PI-negative. Profiles show one experiment, which was similar to 2 additional independent experiments.
Finding a perfect target sequence to silence the PTI-1 gene without affecting EF-1α is the key step, but it is a very difficult job for the similarity between PTI-1 and EF-1α. The nucleotide sequence coding for PTI-1 and the truncated human EF-1α share 98.4% similarity and 97.7% identity. PTI-1 contains the same carboxyl terminus as human EF-1α, while the N-terminal differs due to a deletion of 67 amino acids. There is also an exogenous introduction of 3 amino acids (Met-Gln-Ser) derived from the fusion junction at the N-terminus of PTI-1. This includes the initiator Methionine and results in the creation of a continuous reading frame over the fusion sequence and six in-frame amino acid changes (Shen et al. 1995). By using the RNAi target design tool, we chose three target sequences, and constructed four RNAi constructions (including a control vector) as described in protocol. After transient transfection into the DU145 cell line for about 48 hrs, pSilencer3.1-neo-YL1-2 can completely silence the PTI-1 gene without affecting the EF-1α, which can induce cancer cells into apoptosis and block the cell cycle in G1 phage. To see if it is a common phenomenon, we also did the same experiment on PC3 cells that also expressed PTI-1 gene. The MTT experiment got the similar result. All these provide us a new early therapy of the prostate carcinoma. For the extensive expression in various human cancer cells including small-cell lung and breast (Shen et al. 1995), it may also be carried out to treat the PTI-1 positive cancer cells.

Acknowledgments

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References


