Inhibitory Effects of Anti-sense PTTG on Malignant Phenotype of Human Ovarian Carcinoma Cell Line SK-OV-3*

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Summary: To construct eukaryotic expression vector expressing full length anti-sense pituitary tumor transforming gene (PTTG) mRNA and observe its blocking effect on the potential invasion of human ovarian carcinoma cell line SK-OV-3. PCR primers containing designed enzyme cut sites were used for cloning full-length PTTG gene fragment, and the resulting PCR product was inserted into the eukaryotic vector pcDNA3.1 in the antisense direction. The recombinant vector was then transfected into SK-OV-3 by Lipofectamine. The positive cell clone was screened by G418. PTTG and bFGF at protein level expression were detected by Western blot. The biological behavior change of transfection positive cells was observed by colony formation in soft agar assay. Our results showed that SK-OV-3 clones stably expressing full-length recombinant pcDNA3.1-PTTGas were obtained. The expressions of PTTG and bFGF protein in transfected cells were decreased by 61.5% and 52.3%, respectively as compared with non-transfected ones. The number of colony formation was reduced significantly in transfected cells as compared with empty vector transfected and non-transfected cells. It is concluded that the recombinant vector pcDNA3.1-PTTGas is a novel tool and provides an alternative anti-sense gene therapy targeted at PTTG in human carcinoma.
Key words: PTTG; ovarian carcinoma; anti-sense nuclei acid

Pituitary tumor transforming gene (PTTG) is a novel pro-oncogene that was demonstrated to be associated with tumorigenesis and metastasis[1]. PTTG protein was multi-functional in activating several tumor-associated genes and inhabiting chromatid separation during mitosis. Basic fibroblast growth factor (bFGF) promotes cancer cell proliferation, invasion and metastasis by inducing cancer angiogenesis. Studies have shown that PTTG could up-regulate the expression of bFGF and then deteriorate the phenotype of several tumors including ovarian carcinomas[2]. To study the mechanism of PTTG's promotional function in ovarian carcinogenesis, and to probe into the possibility of anti-sense gene therapy targeted at PTTG, we constructed recombinant eukaryotic vector pcDNA3.1-PTTGas, which expressed full length anti-sense of PTTG, and was then transfected into ovarian carcinoma cell line SK-OV-3 expressing high level of PTTG. On basis of these experiments, we observed differences in transforming ability between transfected and non-transfected cells.

1 MATERIALS AND METHODS

1.1 Construction of pcDNA3.1-PTTGas

Total RNA was isolated from SK-OV-3 cells using TRIZol™ Reagent (Gibco BRL, USA) according to the manufacturer's instructions. RNA (2 μg) was used for cDNA synthesis by reverse transcription. The RNA samples were incubated at 70 ℃ for 5 min with 0.5 μg oligo deoxythymidine primers in a final volume of 10 μl and then at 37 ℃ for 60 min in a 25 μl reaction volume containing 1.25 mmol/L deoxynucleotide triphosphate, 200 U Muloney murine leukemia virus reverse transcriptase and Muloney murine leukemia virus RT buffer (promega USA). The cDNAs obtained were amplified by using of cloning primers as follows: 5′ CCG GAA TTC CAC ACA AAC TCT GAA GCA CT 3′(sense) and 5′ CCG CTC GAG ATG AAT GCG GCT GTT AAG AC 3′ (antisense). The enzyme sites of *Xho* Ⅰ and *Eco*R Ⅰ were underlined respectively. A typical polymerase chain reaction (PCR) system consisted of 5 μl of cDNA or negative control sample (pure water), 0.2 mmol/L deoxynucleotide triphosphate, 1.25 mmol/L $MgCl_2$, 2.5 U Taq polymerase (MBI, Lithunia), 1× buffer, and 10 μM primers. The PCR profile was 95 ℃ for 1 min; 94 ℃ for 30 s, 56 ℃ for 1 min, and 72 ℃ for 1 min for 30 cycles, followed by extension for 7 min at 72 ℃. The amplified product was then recovered from the gel by Glassmilk gel recovery kit (New England Biotech, UK), ligated with pGEM-T vector (Promega, USA) by following the instruction manual. The ligation product was then transformed into DH5α competent cells. The recombinant vector was then screened by sequencing to confirm the correctness of insert sequences of PTTG. After being cut by *Eco*R Ⅰ and *Xho* Ⅰ (TaKaRa, Japan), full length PTTG was inserted into pcDNA3.1 which had also been cut by the

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same enzymes. After screening by enzyme digestion and sequencing, the recombinant pcDNA3. 1-PTTGas that expressed full length antisense of PTTG was constructed.

1.2 Cell Transfection and Screening

pcDNA3. 1-PTTGas and pcDNA3. 1 (as a blank control) were transfected into SK-OV-3 using Lipofectamine2000 (Gibco BRL, USA), G418 (Merck, USA) was added into the medium to screen positive cells. Positive cell clones were chosen and amplified, and then the *neo* gene expression was detected by RT-PCR using the primers 5′-AGAGGCTATTCTGCTATGAC-3 (sense) and 5′-GCTTCAGTGACAACGTCGAG-3′ (antisense).

1.3 Protein Extraction and Western Blot Analysis of PTTG and bFGF

Cells from each group (pcDNA3. 1-PTTGas transfection group, blank control group and non-transfected group) were washed three times with cold PBS and then subjected to lysis in lysis buffer. Cell lysate was centrifuged at 21 000 g for 10 min, and supernatants were analyzed. A total of 50 μg of protein from each cell-free extracts was denatured in SDS sample buffer, heated to 100 ℃ for 5 min, and loaded onto SDS-polyacrylamide gel. Electrophoresis was initially carried out at 100 V through the stacking gel and then at 150 V through the separation gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Amersham, USA). The membranes were blocked for 2 h with a blocking buffer containing 5 % non-fat dry milk and 0. 1 % (v/v) Tween 20 in Tris-buffered saline (TBST) at room temperature, incubated at 4 ℃ with the relevant primary antibodies (Goat anti hPTTG, Rabbit anti bFGF, Goat anti β-actin, Santa Cruz, USA) overnight, and washed three times (10 min each time) with TBST. Primary antibodies were detected using Alkaline Phosphatase-conjugated secondary antibody (Santa Cruz, USA) at a concentration of 1∶1 000, incubated for 2 h at room temperature and visualized with BCIP/NBT (SABC, Henan, China). Protein loading equivalence was assessed by the expression of β-actin.

1.4 Soft Agar Assay

Soft agar assay was carried out according to the method of the reference[3] with slight modification changes. Agarose (Gibco BRL, USA) was blended with PBS (Sigma, USA) at a concentration of 5 %, autoclaved, and thawed in boiled water before use. 2 ml agarose was blended well with 18 ml pre-warmed medium when it was cooled to 50 ℃, and then poured into 24-well plate (0. 8 ml per well) immediately. The gel was solidified at room temperature for about 30 min. Single cell suspend was made at a concentration of 1×10^3 cell per ml, diluted to 200 cell per 0. 7 ml. The cell suspension was blended well with 0. 1 volume of 50 ℃ 5 % agarose, and put into the above-mentioned plates (0. 8 ml per well made 200 cells each well). There were six repeat wells for each group. The cells were cultured for 2 weeks, and then clones containing above 40 cells were calculated and photographed[1].

1.5 Statistical Analysis

All experiments were repeated at least three times. Student's *t* test was used to evaluate the differences between experimental and control groups. All of the *P* values were considered significant when they are greater than 0. 05.

2 RESULTS

2.1 Construction, Transfection and Screening of the Recombinant Vector

Full length of PTTG sequences was confirmed by sequencing, and the electrophoretogram of enzyme digestion showed pcDNA3. 1-PTTGas was constructed successfully (fig. 1). *neo* expression detection by RT-PCR showed positive results in pcDNA3. 1-PTTGas transfection group and blank vector transfection group, and negative result in non-transfected group.

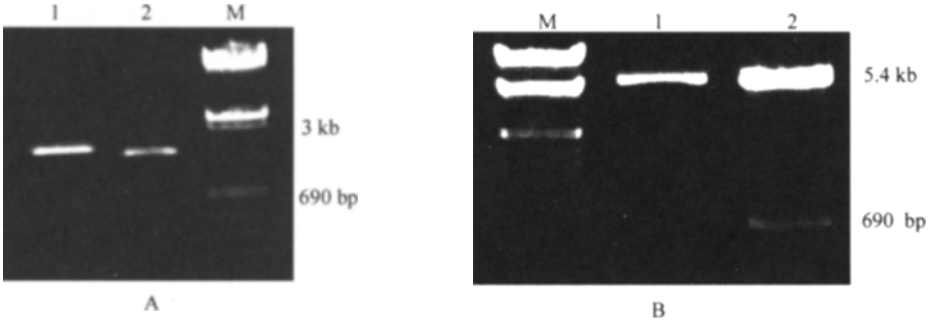

Fig. 1 Electrophoretogram of enzyme digestion
A: 1. T-vector-PTTG link product; 2. T-vector-PTTG link product after digestion of *Eco*R I and *Xho* I
B: 1. PCDNA3. 1-PTTGas; 2. PCDNA3. 1-PTTGas after digestion of *Eco*R I and *Xho* I;
M: λDNA/*Eco*R I + Hind Ⅲ Marker

2.2 PTTG and bFGF Protein Expression before and after Transfection

The expressions of PTTG and bFGF protein in transfected cells were decreased by 61.5 % and 52.3 %, respectively, as compared with non-transfected ones (fig. 2). The result indicated bFGF protein expression decreased after PTTG protein expression reduction.

Table 1 Numbers of cell clone formation of soft agar assay

Cell Group	Numbers of cell clone formation (per well)
Non-transfected SK-OV-3	23.3± 5.7
pcDNA3.1 transfected SK-OV-3	21.5± 7.9
pcDNA3.1-PTTGas transfected SK-OV-3	2.4± 0.8*

*$P<0.01$

2.3 Soft Agar Assay

The numbers of each clone were showed in table 1. The clone-formation ability decreased significantly in pcDNA3.1-PTTGas transfected cells when compared with blank vector transfection group and non-transfected group (fig. 3). The number of colony formation is reduced significantly in transfected cells (2.4±0.8) as compared with non-transfected and empty vector transfected cells (23.3±5.7 and 21.5±7.9, respectively, $P<0.01$).

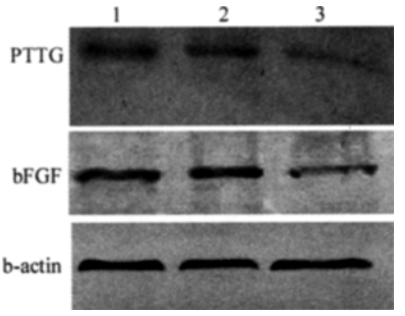

Fig. 2 Expression differences of PTTG and bFGF protein in different transfected SK-OV-3 cells (Western blot)
1: non-transfected SK-OV-3 cell group;
2: pcDNA3.1 transfected SK-OV-3 cell group;
3: pcDNA3.1-PTTGas transfected SK-OV-3 group

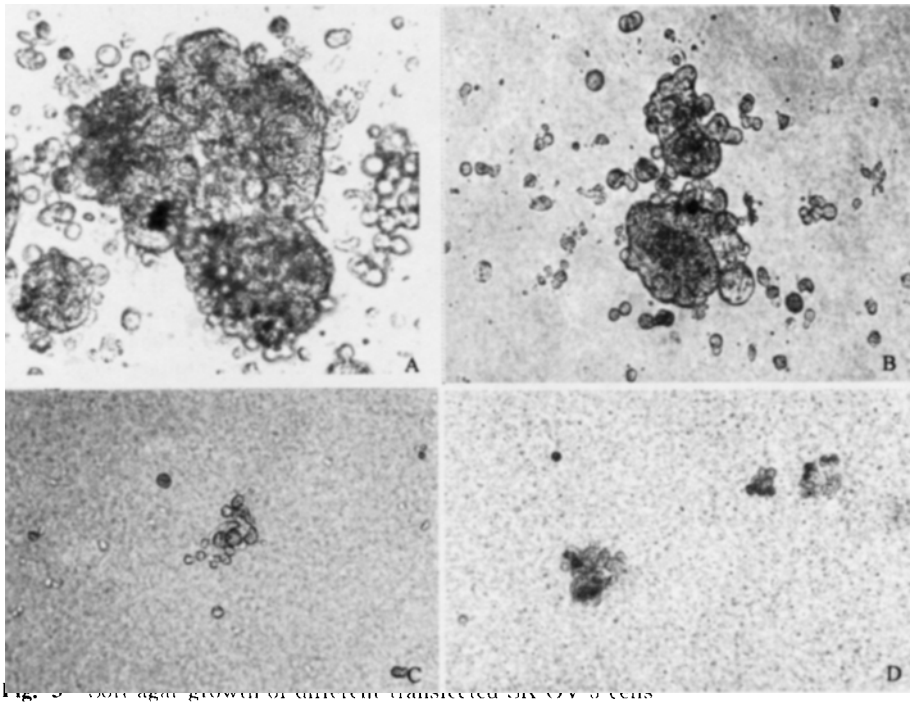

Fig. 3 Soft agar growth of different transfected SK-OV-3 cells
A: Non-transfected SK-OV-3 cell group;
B: pcDNA3.1 transfected SK-OV-3 cell group;
C and D: pcDNA3.1-PTTGas transfected SK-OV-3 group

3 DISCUSSION

PTTG is the first pituitary tumor transforming gene that isolated by differential display PCR (DD-PCR) using mRNA derived from rat pituitary tumor cell line GH4 and normal pituitary tissue. This gene encodes an unique protein which has been testified as human securin-like protein. Previous studies have shown that PTTG overexpressed in tumors tissues from lung, breast, colon, ovary and lymph as well as that from pituitary[2,4-6].

Current opinions about the mechanism of PTTG in carcinogenesis focus on three aspects. First,

as a securin, PTTG can inhibit sister-chromatid separation, cause aneuploidy of progeny cells, and then contributes to transformation and carcinogenesis[7]. Secondly, the Pro-X-X-Pro (PXXP) motif of PTTG protein could bind SH3 domain of several conjunction proteins, transform cells by recruiting certain signal molecules, transfer stimulus signals into nucleus, and then activate expression of relative tumor-associated genes. Thirdly, PTTG upregulates expression of bFGF, a major activating factor for mitogenesis and angiogenesis, then promotes carcinogenesis, tumor invasion and metastasis.

Recent studies showed that PTTG and bFGF expression were increased simultaneously in ovarian cancer tissues, suggesting that PTTG, as an upstream regulator, plays an important role in bFGF synthesis and secretion, and promote malignant phenotype of ovarian cancer[8]. On the other hand, overexpression of bFGF may stimulate PTTG expression, which formed an autocrine and paracrine loop[9]. So we hypothesized that if we could inhibit the expression of PTTG at mRNA or protein level, we might reverse carcinogenesis and metastasis phenotype of ovarian cancer at a higher level. If thus, PTTG may become a molecular therapeutic target for the biological intervention of ovarian cancer.

Antisense nucleic acid technology is an important tool in gene function and gene inhibition research. Because of its specificity, simplicity and stable effect, it has been extensively employed in tumor molecular biology.

To testify the correlation between PTTG and bFGF expression, we detected their expression at both mRNA and protein level by semi-quantitive RT-PCR and Western blot in A2780, SK-OV-3, COC1, OVCAR3 and SW626 ovarian cancer cell lines (data not shown). We observed the highest expression of both in SK-OV-3, which is of highest malignant nature. So we constructed pcDNA3.1-PTTGas, which expressed full length antisense PTTG, and transfected it into SK-OV-3 by using Lipofectamine2000. Stable transfected cells were obtained by G418 screening and confirmed by detection of *neo* expression. PTTG and bFGF protein expressions decreased significantly in pcDNA3.1-PTTGas transfected cells when compared with non-transfected cells. In the meantime, soft agar assay, a reliable method to judge the *in vivo* ability of carcinogenesis, showed that anchor-independent ability of pcDNA3.1-PTTGas transfected cells decreased drastically, indicating that the malignant phenotype of SK-OV-3 was inhibited after specific blocking of PTTG expression. Our study indicated that there was a distinct correlation between PTTG and bFGF expressions, and PTTG played a crucial role in tumor cell transforming. Considering the important effect of bFGF in angiogenesis[10], the regulation of PTTG was of great importance in the mechanism research and biological intervention of carcinogenesis. We could also draw a conclusion that there was an autocrine and paracrine loop in PTTG and bFGF interactive regulation, which provides a reliable experimental proof for potent gene therapy targeted at PTTG.

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