ORIGINAL ARTICLE

Cervical Cancer Hela Cell Osteopontin Gene Suppression Model and Detection

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ABSTRACT

Objective: *To build an osteopontin (OPN) specific short interfering ribonucleic acid (siRNA) expression vector and transfect it into cervical cancer Hela cells, in order to obtain OPN stable transfection cell lines, thus providing support to the further study on the mechanism of OPN in cervical cancer Hela cells.*

Methods: *An OPN specific siRNA expression vector was bulit. Then the target fragment was connected to pcDNA3.1 (+) carrier and mediated with liposomes to transfect Hela cells, in order to obtain the RNA interference stable cell line. The OPN messenger RNA (mRNA) and protein relative expression in the OPN-siRNA group (transfected with the OPN specific siRNA), RANDOM group (transfected with non-interference plasmid) and pcDNA3.1 group (transfected with empty plasmid) were detected with reverse transcription polymerase chain reaction and Western blotting methods.*

Results: *The OPN mRNA and protein expressions were decreased significantly in the OPN-siRNA-1 group (*$p < 0.05$*).*

Conclusion: *The construction of OPN interference cervical cancer Hela cell line can provide a basis for the further study on the mechanism of the growth, metastasis and invasion of OPN-mediate cervical cancer cells.*

Keywords: Cervical cancer, osteopontin, ribonucleic acid interference

Detección y modelo de supresión del gen de la osteopontina de las células HeLa del cáncer cervical

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RESUMEN

Objetivo: *Construir un vector de expresión de ácido ribonucleico de interferencia pequeña (ARNip) específico de la osteopontina (OPN), y transfectarlo a las células HeLa del cáncer cervical, para obtener líneas celulares de transfección estable de OPN. De este modo, se brinda apoyo a la realización de estudios que profundicen sobre el mecanismo de la OPN en las células Hela del cáncer cervical.*

Métodos: *Se construyó un vector de expresión ARNip específico de la OPN. Luego el fragmento diana fue conectado al portador pcDNA3.1 (+) y mediado con liposomas para transfectar las células HeLa, a fin de obtener la línea celular estable de interferencia de ARN. El*

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DOI: 10.7727/wimj.2016.254

ARN mensajero (ARNm) de OPN y la expresión relativa de la proteína en el grupo de OPN-ARNip (transfectada con ARNip específico de OPN), el grupo RANDOM (transfectado con plásmido de no interferencia), y el grupo pcDNA3.1 (transfectado con plásmido vacío), fueron detectados mediante reacción en cadena de la polimerasa con transcripción inversa (RCP-TI) y métodos de electro-transferencia (Western blot).
Resultados: *Las expresiones de proteína y ARNm de OPN disminuyeron significativamente en el grupo de OPN-ARNip-1 (*$p < 0.05$*).*
Conclusión: *La construcción de la línea celular HeLa del cáncer cervical con interferencia de OPN, puede proporcionar una base para profundizar en el estudio del mecanismo de crecimiento, metástasis e invasión de las células del cáncer de cuello uterino mediadas por OPN.*

Palabras claves: Cáncer cervical, interferencia de ARN, osteopontina

West Indian Med J 2016; 65 (4): 609

INTRODUCTION

Cervical cancer is a common malignancy among women (1). In recent years, there is a tendency to onset at a younger age. With the development of research on cancer-related genes, more and more information shows that osteopontin (OPN) plays an important role in the process of tumour metastasis (2), and there are reports on the relationship between OPN and cervical cancer. Osteopontin is a secreted extracellular matrix protein. It has the effects of promoting cell chemotaxis, adhesion and migration which may promote cancer development and metastasis (3). The development of short interfering ribonucleic acid (siRNA) technology gives RNA interference (RNAi) a strong ability to inhibit gene expression and a high degree of sequence specificity (4), and it is more efficient compared with the previous tool to suppress gene expression. We designed an OPN specific short hairpin RNA (shRNA) with RNAi technology, and the OPN specific shRNA was expressed in cervical cancer Hela cells, and *via* plasmid vector mediation, the siRNA was produced, thus leading to OPN gene silencing (5). Then we can study the relationship between OPN and cervical cancer, as well as the ways and mechanism of OPN on cancer cells (6).

MATERIALS AND METHODS

The following are the materials: PMD19-T vector (TaKaRa, No. 6013), *Escherichia coli* (E *coli*) (TaKaRa, No. 9139), plasmid extraction kit (TaKaRa, No. 9761), DNA purification extraction kit (TaKaRa, No. 9762), pcDNA3.1 (+) (Invitrogen), Trizol reagents and enzymes used for reverse transcription polymerase chain reaction [RT-PCR] (TaKaRa, RR091A), synthesis and sequencing (Sangon Biotech Engineering (Shanghai) Co, Ltd), restriction endonuclease Hind III, EcoR I and T4 ligase (TaKaRa), Hela cells (Shanghai Bogoo Biological Technology Co, Ltd, No. BG217), 10% fetal bovine serum medium (Hangzhou Evergreen Company), 0.25% (W/V) trypsin containing 0.02% EDTA (Shanghai Jun-Swiss Biotechnology Company), rabbit anti-OPN (OPN FL-314 Santa Cruz Biotechnology, No. sc 20788), mouse anti-GAPDH (FL-335, Santa Cruz Biotechnology, No. sc-25778), liposomal transfection kit lipofectamine 2000 (Invitrogen Company), AMP (CAS: 71777-48-2, Shanghai Heng Yuan Biotechnology Co, Ltd), G418 (Cellgro), inverted microscope (Olympus IX73), centrifuge (Sigma 4–16), and quantitative real-time PCR instrument (Bio-Rad CFX96 Real-Time PCR Detection System).

Building of osteopontin specific siRNA expression vector

On the National Center for Biotechnology Information (NCBI) website, we found the OPN gene messenger ribonucleic acid (mRNA) complete sequence (GenBank: J04765.1). The Opti-RNAi tool was used to design OPN specific siRNA sequences. The designed sequence was used as a template to design according to the design complementary strand based on a complementary pairing. The Loop ring was added between two sequences. The designed shRNA primer sequences are shown in Table 1. The RANDOM group was the randomly designed meaningless shRNA and regarded as a control group. The designed primers found no homology with other genes in NCBI's Basic Local Alignment Search Tool (BLAST). Primers were synthesized by Sangon Biotech Engineering (Shanghai) Co, Ltd.

Table 1: Designed OPN shRNA sequences and randomized design sequence

OPN-shRNA notation	Targeted OPN mRNA sequence	Loop	Reverse complement sequence	Termination signal	Position in Gen
OPN 1	5'-CACAAGCAGTCCAGATTATATAA	TTCAAGAGA	TTATATAATCTGGTACTGCTTGTG-3	TTTTTTG	784
OPN 2	5'-GAGTCTCACCATTCTGATGAATC	TTCAAGAGA	GATTCATCAGAATGGTGAGACTC-3	TTTTTTG	330
RANDOM	5'-GTACAATCCTACACAGCTTTAAC	TTCAAGAGA	GTTAAAGCTGTGTAGGATTGTAC-3	TTTTTTG	

The primers of the two groups were diluted to 200 uM and placed in a water bath at 95°C for four minutes, annealed at room temperature in order to change the complementary strand into double-stranded DNA and then connected to the PMD19-T vector with T4 ligase, respectively. The vector was transferred into competent E *coli* and cultured in the AMP (100 ug/mg) containing lysogeny broth culture plate. Single colonies were picked, and the plasmids were extracted with plasmid extraction kit. Hind III and EcoR I double digestion was conducted, and 130 bp fragment was identified through electrophoresis. The plasmids were sent to Sangon Biotech Engineering (Shanghai) Co, Ltd for sequencing. A DNA purification kit was used to recycle the digestion products. The inserted carrier was confirmed to be mutated after sequencing. The expression vector pcDNA3.1 (+) (Invitrogen) was treated with Hind III and EcoR I double digestion, electrophoresis. Then the carriers were recycled with DNA purification kits. The vector and sequenced recovered fragments were connected with T4 ligase.

Culture and transfection of Hela cells

Hela cells were cultured in DMEM (high glucose) medium + 10% fetal bovine serum medium (Hangzhou Evergreen Company), and then cultured with 5% CO_2 at 37°C. Subcultured and passaged two to three times weekly with the passage ratio of 1:2. The cell growth was observed under an inverted microscope, and the Hela cells in growth period were digested by adding 0.25% (W/V) trypsin (containing 0.02% EDTA). Observation under an inverted microscope showed suitable conditions for digestion were increasing intercellular gaps and cytoplasmic retraction. Culture medium was added, the mixture centrifuged, the cells collected and seeded into the 24-well plates containing 50 µl antibiotic-free culture medium, 4×10^5 per hole.

The plasmids in the OPN1, OPN2, RANDOM and pc-DAN3.1 (+) groups were diluted with 50 µl Opti-ME I, then 2 µl Lipofectamine 2000 was diluted in 50 µl Opti-ME I medium, incubated at room temperature for five minutes and then transfected within 25 minutes. The diluted plasmids in four groups were mixed with Lipofectamine 2000 in four groups, placed at room temperature for 20 minutes, then 100 µl transfection solution were added to the cells in each hole.

Observation under an inverted microscope showed that the transfection efficiency was over 70%, cultured at 37°C and the culture medium can be replaced at five hours after transfection. After 48 hours, G418 was added for selection. The concentration was 700 mg/mL. After continuous culture for two weeks, the cells that did not transfect with plasmids were considered dead, while the survived cells that contained plasmids were diluted and cultured in 100 mm petri dishes. After cell colony formation, monoclonal cells were selected and cultured with 400 g/m G418 culture medium, and the monoclonal cells were divided into OPN-siRNA-1 group, OPN-siRNA-2 group, RANDOM group and pcDNA3.1 group.

RT-PCR primer design

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer sequences were chosen as internal reference (Table 2). The fluorescent labelling groups were synthesized at both ends in primer synthesis.

Table 2: RT-PCR synthesis primer

Notation	Primer sequence	Length (bp)
OPN	Forward,5'-GGAAAGCGAGGAGTTGA-3' Reverse,5'-GCTGTCCCAATCAGAAGG-3'	80
GAPDH	Forward,5'-ACTGCCACCCAGAAGA -3' Reverse, 5'-AGGTCAGGTCCACCACT -3'	196

Total RNA extraction and reverse transcription

Trizol reagent was used to extract the total RNA in each group according to manufacturers' instructions. DNA enzyme was used to remove DNA in the gene group. 1 µl total RNA and AMV were used to configure the reverse transcription reaction system so as to promote the formation of cDNA according otthe instrument of TaKaRa.

Real-time quantitative PCR

The reverse transcripted cDNA was used as a template. The synthesized fluorescent labelled primers (internal reference primers GAPDH and OPN primers) and DNA

polymerase TaKaRa Ex Taq HS were added on real-time PCR instrument. The PCR conditions were: 94°C denaturation for 10 minutes; 94°C denaturation for 30 seconds; 63°C denaturation for 30 seconds; 72°C denaturation for 40 seconds, and last for 40 cycles, then 72°C denaturation for six minutes. Then, the PCR instrument can be used to collect the fluorescence signals during extension, and the CT value of genes in each group through software analysis could be obtained.

Western blot detect the OPN expression

The Hela cells in logarithmic growth phase were collected from each group, and the total protein extract was used for cell disintegration, then centrifugation, and the total protein quantified by bicinchoninic acid method. Forty microgram protein was used in the 10% SDS-PAGE gel electrophoresis and transmembrane. Then, the transferred SDS-PAGE was blocked for three hours with 5% skim milk, diluted with OPN monoclonal antibody and GAPDH antibody (1:500), then incubated at room temperature for one hour. There was film exposure for colour rendering and fixing, and with ultra-violet product (UVP) software gathered, the image and data processing were carried out.

Statistical analysis

All data were expressed as mean ± standard deviation. SPPS 11.5 software was used for data acquisition and statistical analysis. The difference between groups was analysed with one-way analysis of variance, and $p < 0.05$ indicated statistical significance.

RESULTS

Osteopontin specific siRNA expression vectors

The positive clone was treated with restriction enzyme digestion, and sent to Sangon Biotech Engineering (Shanghai) Co, Ltd, for sequencing, and the results showed no mutation which was consistent with the designed sequence. The BLAST site of the NCBI website found there was no homology with other genes, thus an expression vector was constructed successfully.

Detection of the inhibition of OPN mRNA expression of each group

In order to verify the impact of designed OPN specifc siRNA on OPN gene expression, real-time PCR was used to test the relative expression of OPN mRNA (Fig. 1). The results showed that the OPN mRNA expression was decreased in cervical cancer Hela cells of OPN-siRNA-1 group and OPN-siRNA-2 group.

Compared with the pcDNA3.1 group, the expression of OPN mRNA of the OPN-siRNA-1 group decreased 73% significantly.

The relative expression of OPN mRNA of the OPN-siRNA-1 group was 8% less than that in OPN-siRNA-2 group ($p < 0.05$). The results indicated that the siRNA of OPN-siRNA-1 group had better effect in inhibiting the expression of the OPN gene. There was no significant difference in relative expression between the empty plasmid group and RANDOM group ($p > 0.05$).

Western blot showed that the OPN protein was decreased signficantly in the OPN inhibition group when compared with the non-gene inhibition group ($p < 0.05$).

DISCUSSION

Over-expression of OPN exists in many cell lines, and this over-expression is related to the metastatic potential of cancer (7). OPN-siRNA technology can down-regulate its expression and inhibit metastatic potential. Osteopontin can bind with various integrin receptors on the surface of cells through adhesion sequence RGD (8). On the one hand, OPN mediates the adhesion, migration and proliferation between cells and cells, and cells and extracellular matrix; on the other hand, it activates a range of cell signals, causes the synthesis of a variety of proteolytic enzymes, speeds up the degradation of fibronectin and laminin and other extracellular matrix, thus promoting tumour invasion and metastasis (9). Some reports have shown that OPN over-expression can increase the probability of malignant transformation of benign papillary epithelial cells (10).

It has been reported that over-expression of OPN was related to cervical cancer (11–13). In this study, the expression vector containing pcDNA3.1 (+) eukaryotic was selected, the expression of vector was observed after transfection into the cervical cancer cells, and the transfected recombinant cells were filtered out, and the pcDNA3.1-OPNsiRNA plasmids were constructed successfully. After liposome transfection and G418 selection, the positive cells OPN-1 and OPN-2 that stably transfected with pcDNA3.1-OPNsiRNA were obtained successfully. OPN-siRNA can effectively inhibit OPN gene, and reduce the OPN mRNA content, thus reducing the expression of OPN protein. The successful construction of cervical cancer Hela cell OPN gene inhibition model can provide a research model for further study on the mechanism of OPN gene inhibition in cervical cancer cells.

Since this experiment did not assess the growing states of cervical cancer Hela cell OPN gene after inhibition, as well as the ability of invasion and metastasis,

further experiments are needed for this. In addition, the detection of some cancer secreted related cytokines may be increased, such as MMP-2 and VEGF (14, 15), which might help reveal the mechanism of OPN gene in cancer cells.

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