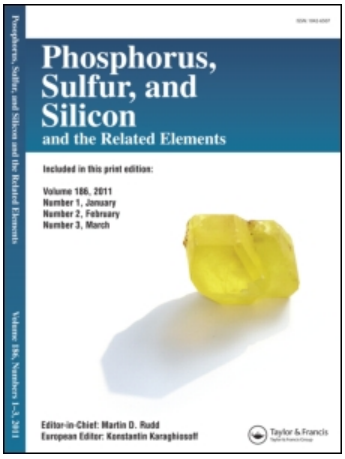

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Inhibition of Pokemon Gene Expression by Antisense Oligonucleotides

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Inhibition of Pokemon Gene Expression by Antisense Oligonucleotides

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Pokemon is an important transcription factor that regulates the important tumor suppressor alternative reading frame (ARF). Pokemon is overexpressed in multiple human cancers. Lack of Pokemon causes cellular senescence, apoptosis, and blockage of differentiation. We've designed and synthesized four 18-mer phosphorothioate modified antisense oligonucleotides targeting the coding regions of Pokemon mRNA, and investigated the down-regulation of Pokemon expression by measuring the fluorescence intensity of Pokemon-GFP fusion protein in COS-7 cells. Antisense oligonucleotide As4, targeting 1496–1513 sites of Pokemon mRNA, has been proven as the most potent compound and down-regulates the expression of Pokemon-GFP fusion protein to 22% at 40 nmol / L for 24 h.

Keywords Antisense oligonucleotides; EGFP; pokemon

INTRODUCTION

Pokemon belongs to the POK (POZ and Krüppel) protein family; it contains an NH_2-terminal POZ domain and a COOH-terminal DNA-binding domain. POK proteins are critical in embryonic development,[1,2]

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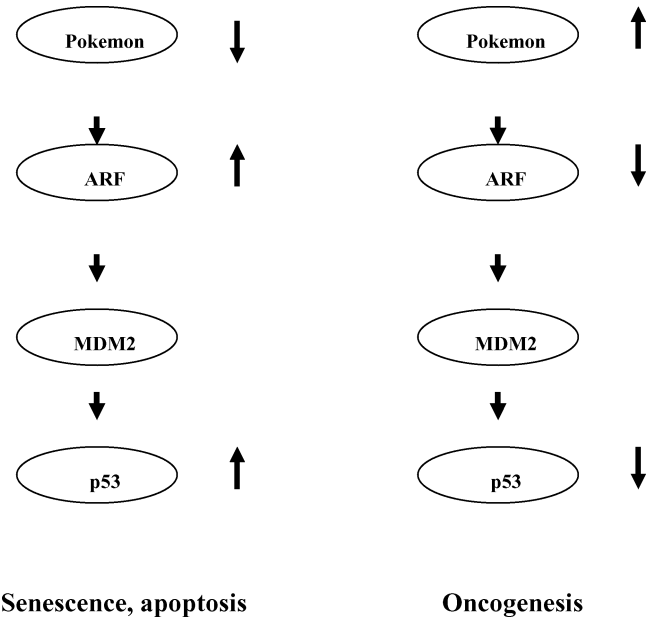

FIGURE 1 Proposed model of Pokemon pathway.

differentiation,[3,4] and oncogenesis.[5–7] Recently, Maeda et al.[7,8] found Pokemon to be a central regulator of the important tumor suppressor ARF, which binds the $p19^{Arf}$ promoter *in vivo* and thus represses its activity. They've also proposed the model of Pokemon-ARF-MDM2-p53 pathway (Figure 1). In view of its essential role in the oncogenic transformation, Pokemon is an attractive therapeutic target for human cancer therapy.

Antisense oligonucleotides can specifically hybridize and inhibit the expression of pathogenic genes, which is widely used in the gene therapy.[9–12] In our study, wedesigned a series of 18-mer phosphorothioate modified oligonucleotides targeting coding regions of Pokemon mRNA. Using the Pokemon-GFP fusion plasmid, we tested several antisense oligonucleotides inhibition effect *in vitro*.

MATERIALS AND METHODS

Preparation of Oligonucleotides

The secondary structure of the Pokemon mRNA (GenBank accession number NM_015898) was predicted on the Web (www.bioinfo.rpi.edu.cn/applications/mfold) to identify target sites likely to be accessible to antisense oligonucleotides. Based on the identified sites, we designed

TABLE I Oligonucleotides Targeted to the Pokemon Gene and Mismatched Control

Oligonucleotides	Target site	Sequence
As1	151–168	*CCAC*GCCGCCGGCC*ATCT*
As2	379–396	*CGCT*GACGAAGTCG*ATCT*
As3	805–822	*GGCCC*CGGCCCATAG*AAGT*
As4	1496–1513	*TTCA*GGTCGTAGTT*GTGG*
As5	Mismatch control	*ATGT*AGTCGTCTGC*CTAG*

Italic, phosphorothioate linkage.

four 18-mer phosphorothioates modified antisense oligonucleotides targeting coding region of Pokemon mRNA (Table I).

Plasmid Constructions

The coding domain sequence of Pokemon was cloned by RT-PCR and subcloned into the pEGFP-N_2 plasmid, which was digested by Hind and EcoRV. PCR was also performed using PrimeSTAR^{TM}HS DNA Polymerase (Invitrogen, USA), 5×Prime STAR^{TM} buffer (Mg^{2+} plus) and 10% dimethyl sulfoxide (DMSO) with cycling parameters of 98°C for 10 sec, 40 cycles at 98°C for 10 s, 70°C for 10 s, 72°C for 1 min, and finally 72°C for 4 min.

Cell Culture and Tansfection

COS-7 cells (American Type Culture Collection) were grown at 37°C in a 5% CO_2 atmosphere with in DMEM (Dulbecco's modied Eagle's medium), supplemented with 10% (V/V)fetal bovine serum, 100 mg/ml penicillin and 100 mg/ml streptomycin. At 24 h before transfection, cells were seeded in 24-wells plates at a density of 1×10^5 cells/well in a volume of 500 μL.

Cotransfection experiments were carried out with Lipofectamine 2000^{TM} (Invitrogen, USA). For each cotransfection, 1 μg DNA plasmid (pEGFP-N_2-Pokemon fusion plasmid) and the respective concentration of antisense oligonucleotides were mixed with 50 μL of Opti-MEM (Invitrogen, USA) medium without serum. In a separate tube, 2.5 μL of Lipofectamine-2000^{TM} per reaction was added to 50 ml of Opti-MEM medium and incubated for 5 min at room temperature. Two solutions were mixed gently, and incubated for an additional 20 min at room temperature. The mixed solution was then added to the cells in the 24-wells plates. At 6 h post-transfection, the medium was replaced by normal medium (containing 10% fetal bovine serum (FBS) and antibiotics) and cultured for 24 h.

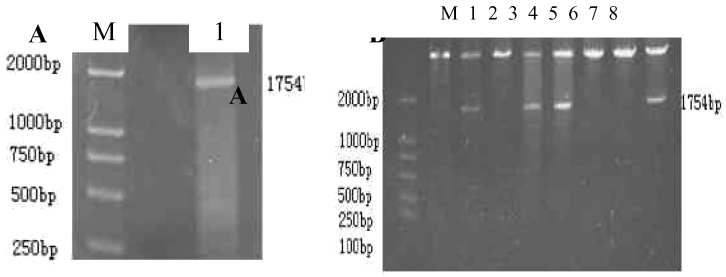

FIGURE 2 (A) Clone of Pokemon by RT-PCR and indentification of prokaryotic expression vector of pEGFP-N2-pokemon. M: DL 2000 DNA marker, 1: PCR product of pokemon using Hela cells cDNA as templater; (B) idendification of positive clones by digestion of Hind and EcoRV.

Fluorescence Microscopy

Antisense effects were analyzed by fluorescence microscopy. The medium was aspirated from the cells, 200 μl phosphate balanced solution (PBS) was added and the fluorescence images were taken directly from living cells using Olimpus fluorescence microscopy, and the fluorescence intensity was analyzed by Image-pro plus v. 5.02 software.

RESULTS AND DISCUSSION

Cloning of EGFP-Pokemon Fusion Constructs

The length of pokemon coding sequence (CDS) is completely in accordance with the mRNA of *Homo sapiens* in GenBank (Figure 2a). Meanwhile, pEGFP-N_2-pokemon fusion plasmid was digested by Hind III and EcoRV. The bands located at 1754 bp in lines 2, 4, 5, and 8 indicate the successful construction of EGFP-N_2-Pokemon fusion plasmid. (Figure 2b).

Screening Antisense Effects of Four Oligonucleotides *In Vitro*

Fusion proteins of the target protein with EGFP enable us to evaluate the potency of antisense oligonucleotides by fluorescence microscopy.[13] To identify antisense oligonucleotides that are potent and specific inhibitor of Pokemon expression, four phosphorothioates modified 18-mer antisense oligonucleotides were screened for their activity to inhibit EGFP-Pokemon fusion protein expression.

COS-7 cells were cotransfected with four different antisense oligonucleotides at a concentration of 40 nM and 1 μg of pEGFP-N_2-Pokemon fusion plasmid. As2 and As4 were found more efficient than As1 and As3 since they inhibited EGFP-Pokemon expression by 74% and 78%, respectively (Figure 3).

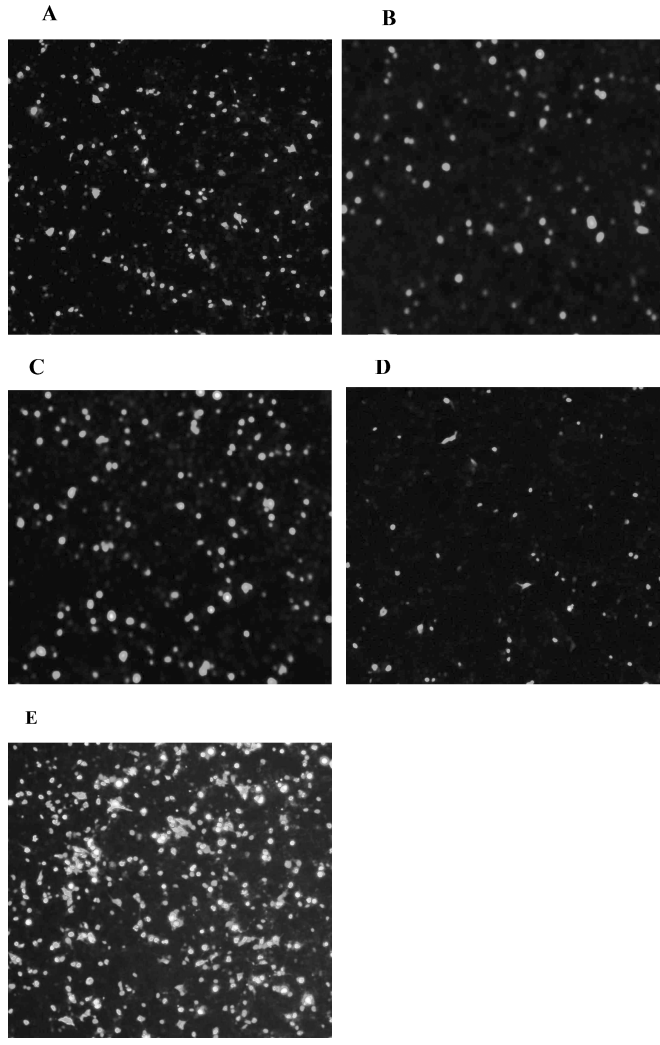

FIGURE 3 Inhibition of EGFP-Pokemon expression by different antisense oligonucleotides delivered with lipofectamine 2000™. Cells treated with the concentration of 40 n nmol/L for 24 h were analyzed by fluorescence microscopy. (A) AS1; (B) AS2; (C) AS3; (D) AS4; (E) AS5, mismatch control.

CONCLUSION

In the present study, we've constructed pEGFP-N_2-Pokemon fusion plasmid to identify the inhibition effects of antisense oligonucleotides targeting Pokemon coding sequence. Screening for the four antisense

oligonucleotides, we've found As2 and As4 were potent inhibitors of Pokemon. A further study of their effects as cancer therapeutic compounds is in process in our lab.

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