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RNAi-mediated downregulation of urokinase plasminogen activator receptor inhibits proliferation, adhesion, migration and invasion in oral cancer cells

Xinhua Liang [a,b,*], Xiaoqin Yang [a], Yaling Tang [a], Hao Zhou [a], Xian Liu [a], Lin Xiao [a], Jiarang Gao [a], Zuyi Mao [a]

[a] *Department of Oral and Maxillofacial Surgery, West China College of Stomatology, Sichuan University, No. 14, Sec. 3, Renminnan Road, Chengdu Sichuan 610041, People's Republic of China*
[b] *State Laboratory of Oral Diseases, Sichuan University, No. 14, Sec. 3, Renminnan Road, Chengdu Sichuan 610041, People's Republic of China*

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Summary RNA interference (RNAi) has emerged as an effective method to target specific genes for silencing. Overexpression of urokinase-type plasminogen activator receptor (uPAR) has been implicated in progression and metastasis of oral cancer. In our study, RNAi was introduced to downregulate the expression of uPAR in the highly malignant oral squamous cell carcinoma (OSCC) cells. Our data demonstrated that siRNA targeting of uPAR leads to the efficient and specific inhibition of endogenous uPAR mRNA and protein expression as determined by quantitative real-time RT-PCR and Western blotting. Furthermore, simultaneous silencing of uPAR resulted in a dramatic reduction of tumor cell proliferation activity, adhesion, migration and invasion *in vitro* compared to the controls. These findings provide further evidence for the involvement of uPAR in a variety of cancer key cellular events as a versatile signaling orchestrator, and suggest that RNAi-directed targeting of uPAR can be used as a potent and specific therapeutic tool for the treatment of oral cancer, especially in inhibiting and/or preventing cancer cell invasion and metastasis.

Introduction

Despite the more widespread use of adjuvant radiation therapy or chemoradiation during the last 20 years, oral squamous cell carcinoma (OSCC) continues to portend a relatively unfavorable prognosis with a 5-year overall and

* Corresponding author. Address: Department of Oral and Maxillofacial Surgery, West China College of Stomatology, Sichuan University, No. 14, Sec. 3, Renminnan Road, Chengdu Sichuan 610041, People's Republic of China. Tel.: +86 28 81801173; fax: +86 28 85503479.
E-mail addresses: liang.xinhua@mayo.edu, lxh88866@yahoo.com.cn (X. Liang).

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disease-free survival estimated to be 56% and 58%, respectively.[1–3] New therapeutic measure are needed to improve the outcome for patients with OSCC.[4] The recent discovery of RNA interference (RNAi), a more powerful tool for the inhibition of gene expression, has provided new opportunities for cancer therapy.[5,6]

The predominant cause of death in patients with OSCC is the ability of cancer cells to invade surrounding tissues and form lymph and distant metastasis. Urokinase-type plasminogen activator receptor (uPAR), as the receptor of serine protease urokinase-type plasminogen activator (uPA), plays an essential role in the proteolytical degradation of extracellular matrix (ECM) and the basement membrane surrounding the primary tumour, which is a major determinant for the cancer invasion and metastasis. Recent studies indicate that uPAR not only functions as a proteinase receptor, but also affects migration, adhesion, angiogenesis, differentiation and proliferation through intracellular signaling pathways.[7–9] Therefore, it is widely accepted that uPAR has a crucial role in the invasion, metastases and progression of cancer including OSCC as a versatile signaling orchestrator.[8]

In previous studies, we and others have previously shown that the overexpression of uPAR was strongly related to the invasiveness, metastases and poor prognosis in OSCC.[10–13]

In the present study, we exploited siRNA targeting of uPAR to downregulate the expression of uPAR in OSCC cells, then investigated the inhibition of proliferation activity, adhesion, migration and invasion potential of OSCC *in vitro*.

Materials and methods

Cell line and reagents

The highly malignant oral squamous cell carcinoma (OSCC) cells (the highly malignant human tongue squamous cell carcinoma cell line—Tca8113 cells)[14] were obtained from Key Laboratory for Oral Biomedical Engineering Ministry of Education Sichuan University. The cell line was maintained as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS). Linearized RNAi-Ready pSIREN-RetroQ-ZsGreen Vector (pSIREN) was purchased from BD Biosciences Clontech (BD Biosciences Clontech, USA). The oligonucleotides were synthesized by BGI Life Tech Co. Ltd. (Peking, China). Lipofectamine™ 2000 Reagent was purchased from Invitrogen Corporation (Invitrogen, Rockville, MD, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Corporation (Sigma, USA).

Construction of a recombinant retroviral vector expressing siRNA for uPAR

RNAi-Ready pSIREN-RetroQ-ZsGreen Vector (pSIREN) was used for the construction of a vector expressing siRNA for uPAR (Scheme 1). Vectors were constructed in pSIREN containing the U6 RNA polymerase III promoter as per the manufacturer's instructions. In brief, the uPAR sequence from +889 to +907 (Genbank ENST00000221264) was used as the target sequence, and for convenience a self-complimentary oligonucleotide was used. The uPAR sequence 19 bases in length with a nine base loop region incorporated with BamH I and EcoR I sites at the ends (GATCCGggtgaagaagggcgtccaaTTCAAGAGAttggacgcccttcttcacCCTTTTTTACGCGTG) was used. The oligo was self-annealed in 6 × SSC using standard protocols and ligated on to the BamH I and EcoR I sites of a retroviral vector pSIREN. This vector was referred to as pU. A control vector (pUc) was constructed using a 19-nucleotide sequence (aacctgcgggaagaagtgg) with no significant homology to any mammalian gene sequence and, therefore, serves as a non-silencing control.

Cell culture and transfections

The highly malignant OSCC cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% glutamine, 100 mg/ml streptomycin, 100 U/ml penicillin and 10% fetal bovine serum (pH 7.2–7.4) in a humidified atmosphere containing 5% CO_2 at 37 °C. Transfections were performed with Lipofectaminet 2000 reagent using 1–2 mg of expression vector/ml serum-free medium as described by the manufacturer. After 5–6 h of transfection, the medium was replaced by serum-containing medium and incubated for a further 48 h.

Real-time RT-PCR

Total RNA was extracted from cells transfected with Mock (untreated/parental cells), empty vector (EV), pUc and the pU vector with the TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. To avoid DNA contamination, total RNA was treated with RNase-free DNase I (Takara, Kyoto, Japan) for 60 min at 37 °C, and extracted with the TRIzol reagent again. One microgram of total RNA was reverse-transcribed in a 20 μl reaction containing 5 units of AMV Reverse Transcriptase XL (Takara, Kyoto, Japan) and 50 pmol of a 9-mer random primer. One microliter of the cDNA solution was used for real-time PCR. The genes of *uPAR* were amplified in a 25 μl reaction containing PreDeveloped TaqMan Assay Reagents (Applied Biosystems, Foster City, CA, USA) using ABI PRISM 7700 (Applied Biosystems, Foster City, CA, USA). The conditions comprised an initial denaturation step at 94 °C for 1 min, then 45 cycles at 94 °C for 10 s, 55 °C for 30 s and 72 °C for 1 min and finally a extension step at 72 °C for 5 min. As an internal control of each sample, the glyceraldehyde 3-phosphatase dehydrogenase (GAPDH) gene was used for standardization, and the amplification was quantified in duplicate. Table 1 shows the PCR primer and probes sequences used in this study. Each PCR product was electrophoresed on an agarose gel to confirm that there was only one band with the expected size for the target gene. Quantitative analysis was carried out as follows: from the amplification curve for each gene, the PCR cycle number that is required for the production of a predetermined amount of the product, the threshold cycle (C_t) was noted. The ΔC_t value was calculated by subtracting the C_t value for GAPDH from the C_t value for uPAR gene, and it was adopted as the expression level of uPAR gene of cells transfected with Mock, EV, pUc and the pU vector. Furthermore, the ΔC_t value was calculated by subtracting the mean ΔC_t value in cells transfected with Mock and the expression change by RNAi was expressed as $2^{-\Delta\Delta C_t}$.[15] Student *t*-test was the