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Research Paper
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Inhibition of VEGF expression in tongue squamous cancer cells via RNA interference silencing of iNOS gene

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Abstract. The purpose of this study was to investigate the regulatory role of the inducible nitric oxide synthase (iNOS) gene on vascular endothelial growth factor (VEGF) expression in oral squamous cancer cells. The RNA interference (RNAi) technique was used to silence iNOS gene expression by transfecting an expression vector containing short hairpin RNA (shRNA) for iNOS into Tca8113 tongue squamous cancer cells using cationic liposomes. Reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting were used to monitor iNOS and VEGF mRNA, as well as protein expression. iNOS mRNA expression was significantly downregulated 24 and 36 h after transfection, and iNOS protein expression was significantly downregulated at 36 and 48 h ($P < 0.05$ versus control), showing that effective silencing was achieved. VEGF mRNA was significantly decreased 24 and 36 h post-transfection, and VEGF protein expression was significantly decreased at 36 and 48 h ($P < 0.05$). RNAi can decrease iNOS gene expression and achieve a gene silencing effect. iNOS gene silencing reduces VEGF expression levels in Tca8113 cells. Thus, there is a relationship between iNOS and VEGF expression in tongue squamous cancer cells.

Keywords: nitric oxide; inducible nitric oxide synthase (iNOS); vascular endothelial growth factor (VEGF); RNA interference.

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Vascular endothelial growth factor (VEGF) is an important mediator in the vascularization, growth, invasion and metastasis of a tumor, and its receptors have a major role in tumor angiogenesis. New therapeutic approaches targeting VEGF and its receptors is effective treatment for gastric cancer and oral squamous cell carcinoma (OSCC)[8,14]. Vascular growth is regulated by pro- and anti-angiogenesis factors. Tumor angiogenesis occurs when the equilibrium between the angiogenesis factors is disrupted. VEGF promotes tumor growth via multiple mechanisms and is the most important factor in tumor angiogenesis. The authors' recent research shows that inducible nitric oxide (iNOS) plays an important role in the growth, differentiation, metastatic capability and prognosis of tongue SCC[2,3,13]. The authors have studied how it interacts with VEGF in the complex transformation of oral epithelial dysplasia to invasive carcinoma and the role of angiogenesis in this process[1]. Inducible nitric oxide synthase generates high levels of nitric oxide (NO) in tissues. Increased iNOS

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expression has been demonstrated in a number of carcinomas, including head and neck SCC. Increased iNOS expression and the generation of high NO levels might have a role in oral SCC development[4]. Numerous studies have shown that iNOS and VEGF are involved in tumor angiogenesis. Overexpression of NOS and VEGF might contribute to tumor angiogenesis in OSCC. NO generation by NOS might be implicated in the VEGF-associated angiogenic process. Further investigation of the possible combined effect of NOS and VEGF on tumor angiogenesis is necessary[10].

RNA interference (RNAi) technology is one of the most effective tools in molecular biology for reverse genetic research. RNAi uses double-stranded RNA that is specific to a gene sequence and can specifically inhibit the expression of the gene. To investigate whether the iNOS gene exerts a regulatory role on VEGF expression in tumor cells, the authors transfected a plasmid vector containing iNOS-specific shRNA into tongue squamous cancer cells using liposome transfection. Reverse transcriptase polymerase chain reaction (RT-PCR) and Western blotting were used to detect changes in iNOS and VEGF gene expression.

Material and methods

Cell culture of Tca8113 tongue SCC strain

Frozen tongue SCC, the Tca8113 cell strain (obtained from the Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, China) were thawed and cultured in Dulbecco modified Eagle medium (DMEM) a low glucose culture media (Gibco USA) with 10% fetal bovine serum (FBS) (Hyclone, USA). Cells were cultured at 37 °C with 5% CO_2 in an incubator, and were moved into new media approximately every 3–4 days. The basic growth patterns of the cells were monitored.

Transfection of pGenesil-1 into tongue squamous cancer cells

pGenesil-1, the specific plasmid vector containing iNOS-specific shRNA, was designed by the Wuhan Jingsai Company. The specific iNOS target gene sequence selected prior to doing the experiment was AATGTGGAGAAAGCCCCCTG (sense). The target genetic sequence for the positive control (HK) was GACTTCATAAGGCGCATGC (sense). The day before transfection, cells were seeded onto T25 culture dishes with a density of 1×10^6 in DMEM containing low glucose and no antibiotics to achieve normal growth. On the day of transfection, Lipofectamine 2000 liposome suspension (Invitrogen, USA) was diluted with the plasmid in the ratio of 2.5:1 and mixed with the Tca8113 cells in OPTI-MEM I culture media without serum (Invitrogen, USA). Fluorescence microscopy was used to monitor the transfection of each cell, and flow cytometry was used to measure the transfection efficiency.

RT-PCR to monitor iNOS and VEGF mRNA expression in Tca8113 cells

PCR primers for iNOS, VEGF, and the internal control β-actin were designed using Olig5 software; the Shanghai Biosynthesis Company then synthesized them. The primer sequences were as follows:

iNOS gene, Forward primer:5'-GGA GCC AGC TCT GCA TTA TC-3'
Reverse primer:5'-TTT TGT CTC CAA GGG ACC AG-3'
VEGF gene, Forward primer:5'-GAT CCT GCC CTG TCT CTC TG-3'
Reverse primer:5'-GAC TCG CCC TCA TCC TCT T-3'
β-actin gene, Forward primer:5'-GTC CAC CTT CCA GCA GAT GT-3'
Reverse primer:5'-AAC CGA CTG CTG TCA CCT TC-3'

Cells in the experimental and control groups were transfected in culture dishes. Total cellular RNA was extracted 24 and 48 h after plasmid transfection using the Trizol Reagent (Invitrogen, USA). The RT (Fermentas, Czech) and PCR reagents (Takara, Japan) PCR condition for iNOS, VEGF, and the internal control β-actin were: 5 minutes at 95 °C, then 30 cycles of amplification at 94 °C for 30 s, 56 °C for 45 s, and 72 °C for 45 s, and an elongation step at 72 °C for 10 min.

Labwork 4.5 software (UVP) was used to analyze the data using the homogeneity of variance test. The SPSS 11.0 statistical software package (Chicago, IL, USA) was used to perform the LSD-t test and one-way ANOVA (test standard $\alpha = 0.05$).

Western blot examination of iNOS and VEGF protein expression of Tca8113

Tca8113 cells were divided into experimental and control groups and placed in T25 culture dishes. Plasmid transfection was done the next day. At 36 and 48 h post-transfection, cells were lyzed, and the protein was extracted. The bicinchoninic acid (BCA) method was used to measure total protein content, and Western blotting was used to examine iNOS and VEGF expression status. The primary antibody for iNOS was NOS2; the β-actin antibody was purchased from NeoMarker, USA; the VEGF antibody was purchased from Santa Cruz Co, USA; the secondary antibodies anti-rabbit-IgG-HRP and anti-mouse-IgG-HRP were obtained from CST, USA. AlphaImager 2200 software was used to semiquantitatively analyze the optical density values of the Western blot signals, and to perform integral processing where

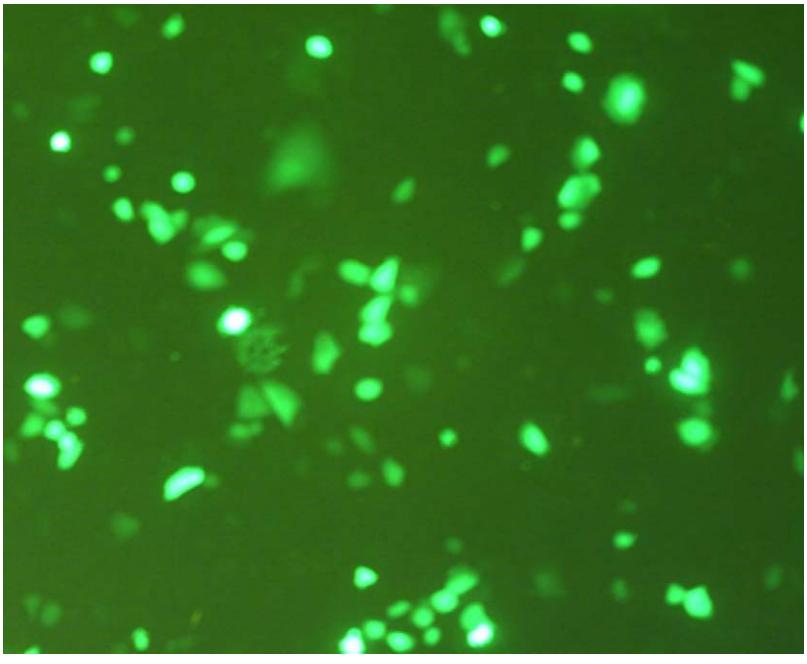

Figure 1. β-actin PCR electrophoresis chart.