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Silencing Id-1 inhibits lymphangiogenesis through down-regulation of VEGF-C in oral squamous cell carcinoma

Zuoqing Dong [a], Fengcai Wei [a], Chengjun Zhou [b], Tomoki Sumida [c], Hiroyuki Hamakawa [c], Yingwei Hu [a], Shaohua Liu [a,*]

[a] Department of Oral and Maxillofacial Surgery, Qilu Hospital, and Stamatology Research Center, Shandong University, 107#, Wenhua Xi Road, Jinan 250012, PR China
[b] Department of Pathology, The Second Hospital of Shandong University, Jinan 250033, PR China
[c] Department of Oral and Maxillofacial Surgery, Ehime University Graduate School of Medicine, Ehime 791-0295, Japan

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SUMMARY

Our previous study demonstrated that overexpression of Id-1 (inhibitor of differentiation/DNA binding) was associated with lymphatic metastasis in human oral squamous cell carcinoma (OSCC). In this study, we further unveiled the association of Id-1 with vascular endothelial growth factor-C (VEGF-C) and peritumoral lymphatic vessel density (PLVD), and the effect of silencing Id-1 on inhibiting lymphangiogenesis in OSCC. We found that Id-1 was associated with VEGF-C ($r = 0.569$, $p < 0.001$) and PLVD ($r = 0.240$, $p < 0.001$) in OSCC. Lentivirus-mediated RNA interference targeting Id-1 in an OSCC cell line Tca8113 resulted in down-regulation of VEGF-C ($p = 0.003$, 0.007). Moreover, when Id-1 was suppressed by injecting Id-1-siRNA-lentivirus into the transplanted tumors in nude mice, VEGF-C was down-regulated ($p = 0.018$) and the PLVD decreased ($p = 0.001$). Our results suggest that Id-1 was correlated with lymphangiogenesis in OSCC. Silencing Id-1 could inhibit lymphangiogenesis through down-regulation of VEGF-C and it might be a promising treatment modality for the lymphatic metastasis of OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) is a common malignant tumor with poor clinical outcome and poor prognosis.[1] OSCC usually metastasizes via lymph vessels, which is the common cause of death. Lymphatic metastasis depends on lymphangiogenesis,[2,3] which is regulated by various lymphangiogenic factors. Vascular endothelial growth factor-C (VEGF-C) plays an important role in lymphangiogenesis. It has been confirmed that cancer cells could induce lymphangiogenesis and promote lymphatic metastasis by expressing VEGF-C.[4,5]

Id-1 (inhibitor of differentiation/DNA binding) is a transcription factor of the helix-loop-helix (HLH) family. It plays an important role in the development of cancer,[6] and is associated with poor tumor differentiation,[7] lymph node status[8] and poor clinical prognosis.[9] Our previous study found that Id-1 was overexpressed in OSCC and was associated with lymphatic metastasis.[10] Though the association between Id-1 and lymphatic metastasis has been well documented, the molecular mechanism is not clear, and whether there is some correlation between Id-1 and lymphangiogenesis needs to be investigated further.

To our knowledge, until now there was never any evidence that has shown a relationship between Id-1 and lymphangiogenesis in OSCC. In this study, using immunohistochemical methods, we investigated the relationship between Id-1 and VEGF-C and peritumoral lymphatic vessel density (PLVD), then small interfering RNA (siRNA) technique using lentivirus vector was applied to specifically inhibit the expression of Id-1 in an OSCC cell line Tca8113, and the expression of VEGF-C was detected. Finally *in vivo* model was used to evaluate the effect of silencing Id-1 on inhibiting lymphangiogenesis.

Materials and methods

Tumor samples and immunohistochemistry

Tumor samples were obtained from 128 patients with OSCC who were treated at Qilu Hospital of Shandong University from 2004 through 2008. All of the patients received local tumor resection and synchronal neck dissection. Informed consent was obtained from all patients.

Paraffin-embedded sample sections (4 μm thickness) were dewaxed and rehydrated. Id-1, VEGF-C and PLVD were detected using primary antibodies of Id-1 (rabbit polyclonal antibody, dilution 1:400, Santa Cruz, USA), VEGF-C (goat polyclonal antibody, dilution 1:50, R&D, USA) and LYVE-1 (rabbit polyclonal antibody,

* Corresponding author. Tel.: +86 13791122835; fax: +86 53182169274.
E-mail address: lshabccba@126.com (S. Liu).

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dilution 1:100, R&D, USA), respectively. The avidin–biotin technique was applied using DAB for visualization and hematoxylin for counterstaining. The immunoreactivity of Id-1 and VEGF-C was graded based on the intensity and the percentage of positive cells.[11,12] PLVD was determined by counting the number of LYVE-1 positive vessels per HPF in the tumor.[13]

Cell culture

Tca8113 cell line (derived from human tongue carcinoma) was grown in DMEM supplemented with 10% fetal calf serum. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

RNA interference

Tca8113 cells were cultured at a concentration of 2×10^5 cells per well in six-well plates. Growing to 30–40% confluence, the cells were transfected with Id-1-siRNA-lentivirus in the presence of 5 μg/ml polybrene at a multiplicity of transfection (MOI) of 50. As mock transfection, cells were treated with NC-siRNA-lentivirus. And the control group was used maternal cells without any treatment. Cells were used in assays after 96–120 h. The siRNA sequences (Shanhai GeneChem, China) were as followed:

Id-1-siRNA sequence: 5′-CATGAACGGCTGTTACTCA-3′.

NC-siRNA sequence: 5′-TTCTCCGAACGTGTCACGT-3′.

Real-time PCR analysis

Total RNA was prepared using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's protocol, and cDNA was synthesized using standard procedures. Real-time RT-PCR (Biored) was carried in the presence of SYBR green to measure the levels of Id-1 and VEGF-C mRNAs. The primers sequences used in this study were shown in Table 1. Real-time PCR was performed using the cycle profile: 1 cycle at 94 °C for 1 min, followed by 40 cycles of 94 °C for 20 s; 52 °C (for Id-1), 54 °C (for VEGF-C) or 50 °C (for β-actin) for 30 s, and 72 °C for 30 s; and then a final extension at 60 °C for 5 min. Melting curve analysis was performed to verify the specificity of the amplification reactions. The amplification curves were analyzed and C_t values were determined.

Western blotting analysis

Proteins were extracted from the cells with Protein Extraction Kit, and quantified using BCA Protein Assay Reagent Kit (Nanjing, China). Proteins were separated by 10% SDS–PAGE and transferred onto PVDF membranes (Nippon Genetics, Japan) at 90 V for 35 min. The membranes were blocked, washed, and incubated with the primary antibodies of Id-1 (dilution 1:1000), VEGF-C (dilution 1:1000) and β-actin (dilution 1:1000, Santa Cruz, USA) respectively, overnight at 4 °C. The next day, the membranes were washed and incubated with HRP-conjugated IgG (dilution 1:2000, Zhongshan Goldenbridge Biotechnology, China) at room temperature for 1 h. Signals were detected by enhanced chemiluminescence (ECL).

Secretory VEGF-C protein quantitation by ELISA

The cell culture supernate were removed and assayed to detect secretory VEGF-C proteins. VEGF-C proteins were analyzed by ELISA using Quantikine Immunoassay Kits (R&D, USA). ELISA was performed according to the manufacturer's instruction. After the colorimetric reaction, the optical density (OD) at 450 nm was quantified by an eight-channel spectrophotometer, and the OD readings were converted to picograms per milliliter (pg/ml) on the basis of the standard curves.

In vivo treatment

Tca8113 cell suspensions of 2×10^5 cells (0.1 ml) were submucosally injected into lingual central part in 30 BALB/c nu/nu male mice (Shanghai, China). The mice were 5-weeks old, with a body weight of 18–22 g. When the tumors were about 3.0–5.0 mm in diameter, 21 mice were divided into three groups. Tumors of each group were injected with DMEM, NC-siRNA-lentivirus (10^8 TU) and Id-1-siRNA-lentivirus (10^8 TU), respectively. Injection was performed intratumorally at several points, twice 1 week for 2 weeks. Three weeks later the mice were sacrificed and the tumor tissues were collected. Each tumor was divided into two halves, one was used to determine mRNA expression of Id-1 and VEGF-C, and the other was used to observe the expression of Id-1, VEGF-C proteins and PLVD by immunohistochemistry using primary antibodies of Id-1 (dilution 1:400), VEGF-C (dilution 1:50) and LYVE-1 (mouse polyclonal antibody, dilution 1:100, Santa Cruz, USA), respectively.

Statistical analysis

The results were expressed as mean ± SD. All statistical analyses were carried out using SPSS software (version 16.0, USA). Spearman's coefficient of correlation was used for bivariate correlation comparison. The Mann–Whitney test and Student's *t*-test were used to examine the association between Id-1 expression, VEGF-C expression and PLVD. *P*-value <0.05 was considered to be significant.

Table 2
Immunohistochemistry analysis of Id-1, VEGF-C and PLVD in orthotopic tumors of nude mice ($n = 7$, value indicated with mean ± SD).

	Control	NC-siRNA	Id-1-siRNA	*P*-value
Id-1	5.43 ± 0.98	5.52 ± 0.53	3.71 ± 0.49	0.003
VEGF-C	3.57 ± 0.53	3.43 ± 0.53	2.57 ± 0.53	0.018
PLVD	23.71 ± 4.61	22.29 ± 5.41	15.43 ± 1.62	0.001

Table 1
Primers sequences and size of PCR product.

Gene	Accession No.	Primer sequence	PCR protocol	Size (bp)
Id-1	NM002165	5′-TCTACGACATGAACGGCTG-3′ 5′-GGTCCCTGATGTAGTCGAT-3′	94 °C for 1 m, followed by 40 cycles of 94 °C for 20 s	117
VEGF-C	NM005429	5′-CACTTGCTGGGCTTCTTCT-3′ 5′-CACAGACCGTAACTGCTCCT-3′	52 °C (Id-1), 54 °C (VEGF-C), or 50 °C (β-actin) for 30 s	186
β-Actin	NM001101	5′-GCCAACACAGTGCTGTCT-3′ 5′-AGGAGCAATGATCTTGATCTT-3′	72 °C for 30 s, then 60 °C for 5 m	114