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RNAi-mediated silencing of VEGF-C inhibits non-small cell lung cancer progression by simultaneously down-regulating the CXCR4, CCR7, VEGFR-2 and VEGFR-3-dependent axes-induced ERK, p38 and AKT signalling pathways

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ABSTRACT

Vascular endothelial growth factor C (VEGF-C) expression is associated with the malignant tumour phenotype making it an attractive therapeutic target. We investigated the biological roles of VEGF-C in tumour growth, migration, invasion and explored the possibility of VEGF-C as a potential therapeutic target for the treatment of non-small cell lung cancer (NSCLC). A lentivirus-mediated RNA interference (RNAi) technology was used to specifically knockdown the expression of VEGF-C in A549 cells. Quantitative reverse transcriptase-polymerase chain reaction, flow cytometry, Western blot, immunohistochemistry, cellular growth, migration, invasion and ELISA assays were used to characterise VEGF-C expression *in vitro*. A lung cancer xenograft model in nude mice was established to investigate whether knockdown of VEGF-C reduced tumour growth *in vivo*. Silencing of VEGF-C suppressed tumour cell growth, migration and invasion *in vitro*; suppressed tumour growth, angiogenesis and lymphangiogenesis by tail vein injection of lentivirus encoded shRNA against VEGF-C *in vivo*. More importantly, silencing of VEGF-C also trapped the VEGFR-2, VEGFR-3, CXCR4, CCR7-dependent axes, and down-regulated the AKT, ERK and p38 signalling pathways. These results suggest that VEGF-C has a multifaceted role in NSCLC growth, migration and invasion; that VEGF-C-mediated autocrine loops with their cognate receptors and chemokine receptors are significant factors affecting tumour progression; and that RNAi-mediated silencing of VEGF-C represents a powerful therapeutic approach for controlling NSCLC growth and metastasis.

1. Introduction

Lung carcinoma is the leading cause of cancer-related death worldwide and non-small cell lung cancer (NSCLC) accounts for 75% of all diagnosed lung cancers. Despite extensive research efforts in NSCLC screening, diagnostics and therapeutics, prognosis is poor and only 8–14% of patients survive >5 years from the time of diagnosis.[1] Clearly, current

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treatments for NSCLC are ineffective and different approaches are needed to improve the therapeutic ratio, including the accurate identification of optimal molecular targets for use in molecular targeted therapy.

Lymphangiogenesis is an important mediator of tumour cell dissemination, and many potential lymphangiogenic factors have been characterised including vascular endothelial growth factor (VEGF)-C.[2–4] VEGF-C plays a critical role in the control of lymphatic endothelial cell biology during embryogenesis, tumourigenesis and metastasis.[5–7] In some studies, the overexpression of VEGF-C significantly correlated with lymph node metastasis and lymphangiogenesis in primary tumours of thyroid, prostate, gastric, colorectal, ovarian, and breast cancers.[5,8] However, other reports could not confirm such correlations, or opposite relationships were found.[8] These results suggest that VEGF-C may be an important diagnostic and therapeutic target for treating malignant tumours, and indicate that the effects and interactions of the VEGF-C/VEGFR-3 system in cancer biology are complex and differ between malignancies. The functional role of VEGF-C in NSCLC remains unclear. Only a few clinical studies on VEGF-C expression in NSCLC have been reported and the prognostic significance of VEGF-C remains controversial[9–13]; the effect of VEGF-C knockdown on the metastatic phenotype in NSCLC is also unknown.

Tumour cells can acquire the ability to support autocrine signalling pathways by expressing growth factors and their cognate surface receptors. Tumour cell migration and metastasis share many similarities with leukocyte trafficking which is critically regulated by chemokines and their receptors. Recent reports suggested that the chemokine receptors CXCR4 and CCR7 were highly expressed in many kinds of tumours, and may be involved in metastatic processes[14–17] and the induction of tumour cell transport via the lymphatics in association with the secretion of VEGF-C.[18,19] Based on these data we hypothesised that a link between autocrine growth factors and chemokine receptors exists in tumour cells, and that a decrease in the expression of VEGF-C would result in down-regulation of the expression levels of CXCR4, CCR7, VEGFR-2, VEGFR-3 and their dependent axes and the suppression of tumour progression.

To assess the functional role of VEGF-C in the malignant phenotype of NSCLC cells, we developed a lentivector-mediated small interfering RNA (siRNA) approach to selectively knockdown the expression of VEGF-C in NSCLC A549 cells. We showed that RNA interference (RNAi) driven silencing of VEGF-C strongly inhibited NSCLC cell growth, migration and invasion and simultaneously down-regulated the CXCR4, CCR7, VEGFR-2 and VEGFR-3-dependent axes-induced ERK, AKT and p38 signalling pathways. Furthermore, an *in vivo* xenograft study showed that administration of VEGF-C siRNA significantly inhibited tumour growth, angiogenesis and lymphangiogenesis.

2. Materials and methods

2.1. Cell culture and selection of a high VEGF-C expression cell line

Five human NSCLC cells (A549, SPC-A1, SK-MES-1, Y90 and NCI-H460) were purchased from Institutes of Biochemistry and Cell Biology (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Shanghai, China) supplemented with 10% foetal bovine serum (FBS; Gibco) and 1% penicillin–streptomycin and maintained at 37 °C under 5% CO_2. The levels of VEGF-C expression were detected by Western blot analysis and quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR); the cell line with the highest level of VEGF-C expression was selected for the following assays.

2.2. Design and construction of lentiviral VEGF-C siRNA expression vectors

Potential RNAi oligonucleotides for silencing human VEGF-C (accession no. NM005429) were designed and synthesised by Invitrogen. The synthesised sequences were inserted into the BamHI and EcoRI enzyme sites of the pSIH1-H1-cop green fluorescent protein (GFP) (pSIH1) shRNA vector (System Biosciences) to construct human VEGF-C shRNA plasmids (Lv-VEGF-C-shRNA1, Lv-VEGF-C-shRNA2, Lv-VEGF-C-shRNA3 and Lv-siRNA-NS). The plasmids and the predicted secondary structure of the pSIH1 shRNA targeting VEGF-C are shown in Fig. 1A. Restriction endonuclease digestion analysis confirmed the structures of the recombinant vectors, and DNA sequencing was used to verify all inserted sequences.

2.3. Transduction of target cells by pPACKaged siRNA expression vector

Lentivirus production and subsequent target cell transduction were established using a lentiviral delivery system (System Biosciences) and performed according to the manufacturer's instructions. Briefly, expression vector pSIH1 carrying siRNA or vector control (pSIH1-NS) was mixed with Lentivirus Package Plasmid Mix and Lipofectamine™ 2000 (Invitrogen), and added to 293TN producer cells (System Biosciences). The supernatants containing lentiviruses were harvested at 48 h post-transfection and filtered through 0.45 μm polyvinylidene fluoride membranes (Millipore). The titre of virus was measured according to the expression level of GFP. Viral supernatant was used for transducing target cells. A549 cells transfected with lentivirus-encoded shRNA 1–3 against VEGF-C were named A549-s1, A549-s2 and A549-s3. A549 cells transfected with Lv-siRNA-NS or parental vector pSIH1 shRNA were controls and named A549-NS and A549-pS, respectively.

2.4. Tumour xenograft treatment model

Forty-eight, 6-week old BALB/c nude mice were obtained from Laboratory Animal Center (Shanghai, China). A suspension of A549 cells (1×10^7) was injected subcutaneously into the left flank of each mouse. When tumours formed on d 15 post-implantation, mice were randomly divided into four treatment groups: the Lv-VEGF-C-siRNA group treated with lentivirus encoded shRNA against VEGF-C; the Lv-siRNA negative group treated with negative control lentivirus; PBS treated controls; and untreated controls. Treatment groups received 250 μl lentivirus or PBS by intravenous (i.v.) injection into the tail vein every 24 h for 3 weeks. Upon termination, each mouse was weighted and tumours were harvested for