Int J Clin Exp Med 2020;13(2):1185-1191
www.ijcem.com /ISSN:1940-5901/IJCEM0104089

Original Article

Gas6 protein promotes the progress of NSCLC cells through VEGFAKT pathway

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Received September 12, 2019; Accepted November 11, 2019; Epub February 15, 2020; Published February 28, 2020

Abstract: Purpose: To investigate the expression and mechanism of Gas6 in non-small cell lung cancer (NSCLC). Method: Western blot was performed to measure Gas6 expression in NSCLC tumor and adjacent tissues. Cultured NSCLC cell line A549 or normal epithelial cell HBE was treated with 100 or 200 ng/ml Gas6, and proliferation was measured by MTT assay. Caspase 3 activity was measured and cell invasion was described by Transwell assay. VEGF and AKT expression was detected by Western blot and the expression of interleukin-2 (IL-2) and IL-6 was assessed by ELISA. Results: Gas6 was significantly up-regulated in NSCLC tumor tissues compared to adjacent tissues ($P<0.05$). Treatment of A549 cells using Gas6 significantly facilitated tumor cell proliferation, decreased caspase 3 activity, facilitated tumor cell invasion, and enhanced VEGF protein level, pAKT, IL-2 and IL-6 expression ($P<0.05$) in a dose dependent manner. Conclusion: Gas6 is up-regulated in NSCLC tumor tissues and facilitates NSCLC cell proliferation or invasion, suppresses apoptosis to enhance NSCLC tumor progression probably via modulating VEGF/AKT signal.

Keywords: Gas6, non-small cell lung cancer, VEGF/AKT signal pathway, inflammatory factor, cell proliferation, tumor invasion

Introduction

Lung carcinoma is one of the most common and recurrent tumor worldwide, with a higher incidence and mortality. According to pathological types, lung cancer is grouped to small cell lung cancer (SCL) and non-small cell lung cancer (NSCLC), the latter of which is more frequent and consists of more than 80% of lung cancers [1, 2]. The incidence of NSCLC has been gradually increased worldwide. In China, the incidence and mortality of lung cancer is higher than other countries around the world [3, 4]. Currently the early identification, diagnosis and treatment of lung cancer are critical measures for improving survival rate and prognosis of lung cancer patients [5]. Although continuous improvement of medical techniques and treatment approaches has brought surgery, chemo-therapy and radio-therapy, plus intervention therapy, immune therapy and targeted treatment against lung cancer, the overall treatment efficiency is still unfavorable with higher recurrent and metastatic rate, resulting in poor prognosis and low survival rate, thus causing heavy mental and economic burdens, making it one major public health concern worldwide [6, 7]. Lung carcinoma has complicated pathogenic mechanism, which may be related to genetic, physical and chemical factors across multiple steps and tiers that have not been fully illustrated [8]. Therefore, identification of lung cancer pathogenesis related targets can provide evidences for the treatment and diagnosis of tumors.

Growth arrest specific gene 6 (Gas6) is one of newly discovered human growth factors and widely expressed in multiple cells [9]. As a type of vitamin K dependent protein, Gas6 receptor belongs to tyrosine kinase receptor family. Previous studies showed broad distribution of biological functions of Gas6 [10], as it can participate in various pathological and physiological processes including cell growth, apoptosis, viral infection, inflammation, autoimmune disease, and cardiovascular disease [11, 12]. Previous studies showed abnormal expression

of Gas6 in various tumor cells including gastric cancer, breast carcinoma and ovary cancer, and is closely related to tumor progression [11-13]. This study thus investigated the functional role and related mechanism of Gas6 in NSCLC.

Materials and methods

General information of patients

A total of 26 patients who were diagnosed as NSCLC by pathological examination in Qingdao Central Hospital (Qingdao, Shandong, China) between March 2016 and February 2017 were recruited in this study. All patients received surgical operation. Patients aged between 41 and 75 years, containing 16 males and 10 females. Tissue samples were collected during surgery for preparing frozen slices. Inclusive and exclusive criteria [14]: inclusive criteria: all patients were diagnosed as NSCLC for the first time and received surgery without chemo- or radio-therapy before surgery. All patients have signed informed consents. Exclusive criteria: patients having recurrent NSCLC or complicated with other subtypes of lung carcinoma were excluded. Patients having received surgery, radio- or chemo-therapy, or complicated with other disease such as infectious diseases, malignant tumor, severe diabetes, organ failure, systemic immune disorder or complication of malignant tumor were excluded. During surgery, both tumor and adjacent tissues were collected and preserved in liquid nitrogen. This study has been approved by the medical ethical committee of Qingdao Central Hospital (Qingdao, Shandong, China).

Major reagent and equipment

NSCLC cell line A549 (CCL-185™) and normal epithelial cell HBE were purchased from ATCC cell bank (US). Human recombinant Gas6 protein (rhGas6) was purchased from R&D (US). DMEM medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Hyclone (US). DMSO and MTT powder were from Gibco (US). Trypsin-EDTA digestion buffer were from Sigma (US). PVDF membrane was purchased from Pall Life Sciences (US). Western blot chemical reagents were purchased from Beytoime Biotech (China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-human VEGF monoclonal antibody and anti-phosphorylated AKT (pAKT) monoclonal antibody, rabbit anti-human AKT monoclonal antibody and HRP-conjugated goat anti-rabbit secondary antibody were from Cell Signaling (US). ELISA kits for interleukine-2 (IL-2) and IL-6 were purchased from R&D (US). Caspase-3 activity assay kit was purchased from R&D (US). Labsystem Version 1.3.1 microplate reader was from Bio-rad (US). Ultrapure workstation was from Sutai Purification (China). Transwell chamber was purchased from Corning (US). XO1000D ultrasonic rupture equipment was purchased from Xianou Corp (China). CO_2 incubator was purchased from Thermo (US).

A549 cell culture and grouping

A549 or HBE cells were resuscitated and passed, and were inoculated into culture dishes at a density of 1×10^7 cells per cm^2 and cultured in 90% high-glucose DMEM medium containing 100 U/ml penicillin and 100 ug/ml streptomycin at 3737-glucose DM_2. Cells were divided to control group, 100 ng/ml Gas6 group and 200 ng/ml Gas6 treatment group.

MTT assay for measuring the effect of Gas6 on A549 cell growth

A549 or HBE cells at log-growth phase were digested, enumerated, inoculated into 96-well plate (3000 cells/well) and grouped into control group and high/low dosage Gas6 treatment group, each of which was treated for 48 h in triplicated wells. 20 μ0 MTT solution (5 g/L) was added into each group of cells for 4 h continuous incubation in a CO_2 chamber. The supernatant was completely removed and 150 μc DMSO was added into each well for 10 min vortex. After complete resolve of crystal violet, and absorbance (A) value at 570 nm wavelength was measured by a microplate reader to obtain the cell proliferation rate.

Caspase 3 activity assay

The activity of caspase 3 among all groups of cells was measured following the instruction of test kit. In brief, trypsin was used to digest cells, which were then centrifuged at 600 g for 5 min under 4easured following the instruction of test kit. In br min, followed by 4 following the insat 20000 g for 5 min. 2 mM Ac-DEVD-pNA was added for measuring optical density (OD) values at 405 nm to calculate caspase 3 activity.

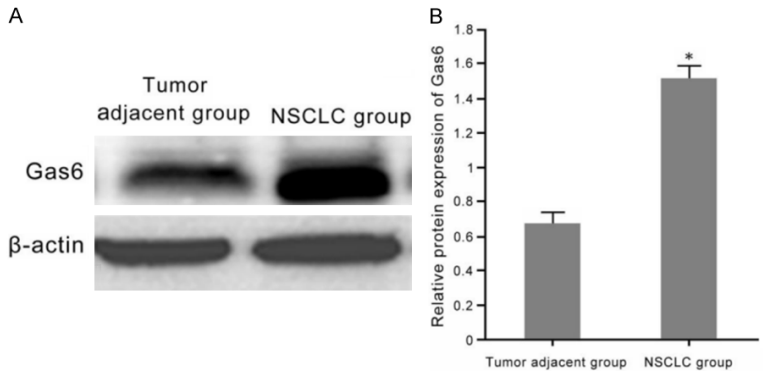

Figure 1. Gas6 expression in NSCLC tissues. A. Western blot for measuring Gas6 protein in NSCLC. B. Analysis for Gas6 protein expression in NSCLC tissues. **P*<0.05 comparing to tumor adjacent tissues.

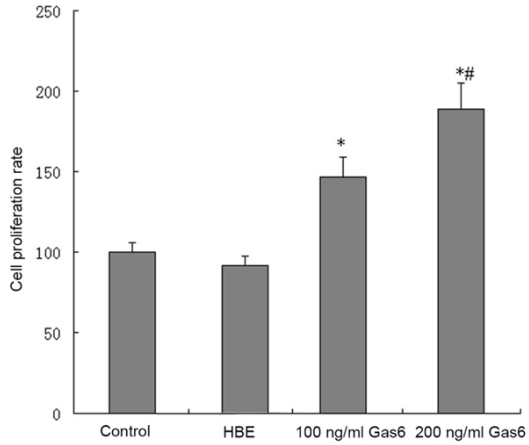

Figure 2. Effects of Gas6 on A549 cell proliferation. **P*<0.05 comparing to control group or HBE; #*P*<0.05 comparing to 100 ng/ml Gas6.

Transwell chamber assay for cell invasion

Serum-free culture medium was switched. The upper phase membrane and bottom side of Transwell chamber were pre-coated with 1:5 Matrigel dilution (50 mg/L), and were air-dried at 41:5 Mat μg FBS-containing DMEM medium and 100 μF tumor cell suspension prepared in serum-free culture medium were added into interior and exterior of the chamber, respectively. Each group was tested in triplicates. The chamber was placed into 24-well plate and control group utilized Matrigel-free Transwell chamber. After 48 h incubation, the chamber was rinsed with PBS to remove cells on the membrane. Cells were then fixed by cold ethanol. The number of cells at the lower phase of the membrane was enumerated and the experiment was repeated for three times.

Western blot for gas6, VEGF and AKT signal pathway change

Total proteins were extracted from both larynx carcinoma and tumor adjacent tissues, as well as Hep-2 cells. In brief, cells were lysed on ice for 15~30 min using lysis buffer. After rupture in ultrasound (5 s, 4 times), cell lysate was centrifuged under 10,000 g for 15 min at 4es) to obtain the protein which was quantified using Bradford approach. Proteins were separated by 10% SDS-PAGE, transferred to PVDF membrane, blocked with 5% defatted milk powder for 2 h and incubated with primary antibody against pAKT or AKT (1:1000 dilution) (Cell Signaling Technology), Gas6 monoclonal antibody (1:1000 dilution) (Cell Signaling Technology), or VEGF monoclonal antibody (1:2000 dilution) (Cell Signaling Technology) at 4ling Technolo Goat anti-rabbit secondary antibody (Cell Signaling Technology) was added for 30 min in dark followed by PBST washing and membrane development after addition of ECL reagent (Thermo Fisher Scientific) for 1 min. Data were processed by protein imaging processing software. All experiments were repeated for four times (n=4).

ELISA for IL-2 and IL-6 secretion in cell culture supernatant

Cell culture supernatant was collected from all groups of cells to measure the expressional change of inflammatory factors TNF-ouand IL-2, in accordance with the instruction of ELISA kit. Briefly, 50 μn serially diluted standard samples were added into 96-well plate for plotting the standard curve. 50 μs test samples were added into reaction well in triplicates. Sample concentration was calculated using the linear regression curve.

Statistical processing

SPSS 11.5 software was used for statistical analysis. Data were shown as mean were shown as as used fo and compared by ANOVA with Newman-Keuls post-hoc analysis, and comparison of means between groups was evaluated by student t-test. P<0.05 suggests a statistical significance.

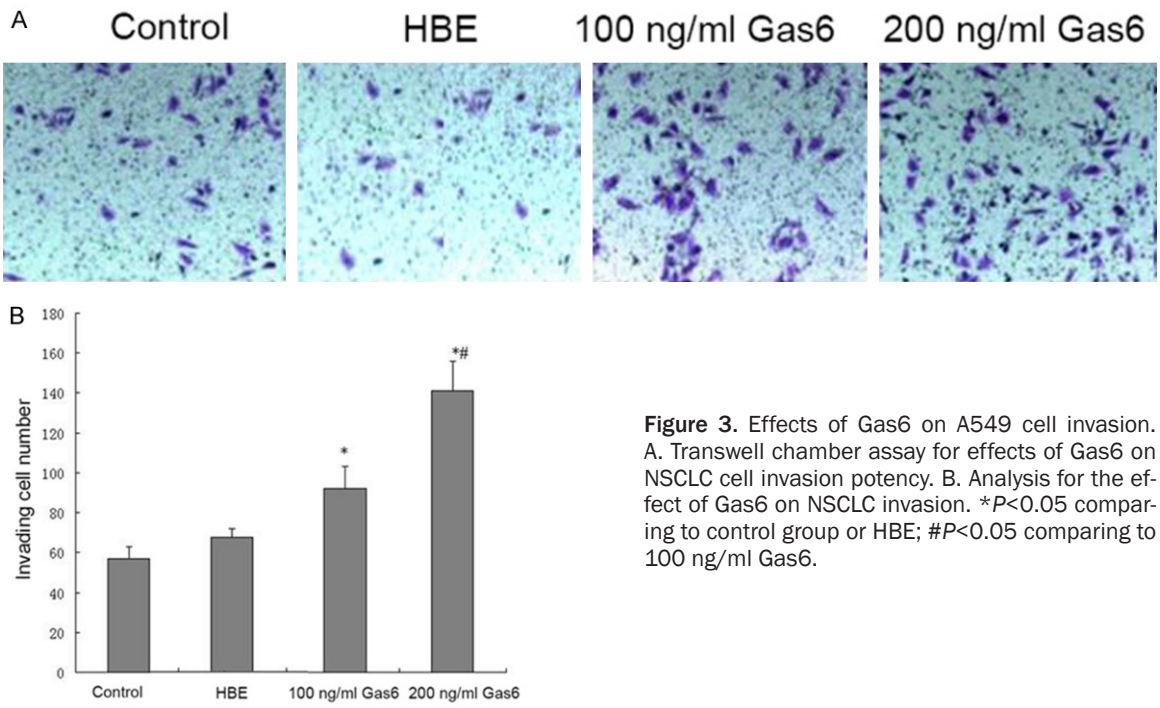

Figure 3. Effects of Gas6 on A549 cell invasion. A. Transwell chamber assay for effects of Gas6 on NSCLC cell invasion potency. B. Analysis for the effect of Gas6 on NSCLC invasion. *P<0.05 comparing to control group or HBE; #P<0.05 comparing to 100 ng/ml Gas6.

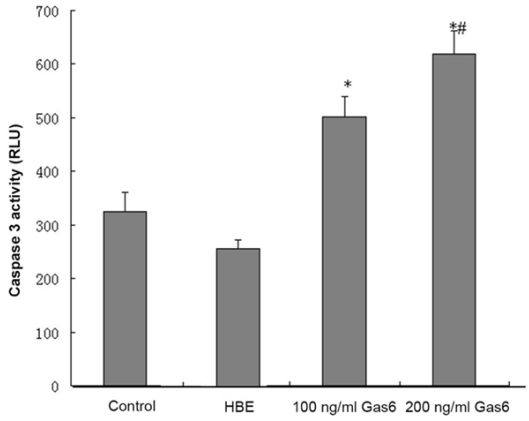

Figure 4. Effects of Gas6 on Caspase 3 activity of A549 cells. *P<0.05 comparing to control group or HBE; #P<0.05 comparing to 100 ng/ml Gas6.

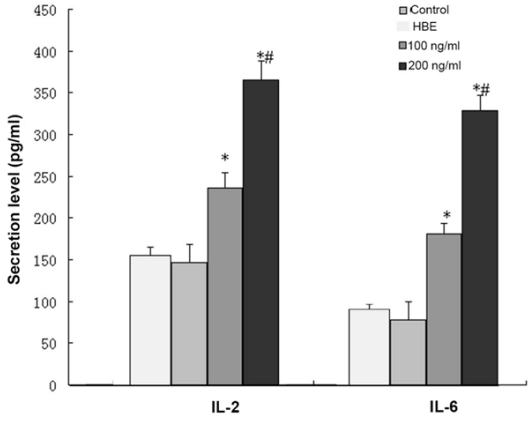

Figure 5. Effects of Gas6 on secretion of inflammatory factors from NSCLC cell A549. *P<0.05 comparing to control group or HBE; #P<0.05 comparing to 100 ng/ml Gas6.

Results

Gas6 expression in NSCLC tissue

Compared to adjacent tissues, Gas6 protein expression was significantly increased in NSCLC (P<0.05, **Figure 1**).

Effects of Gas6 on NSCLC cell proliferation

Addition of Gas6 into A549 cancer cell line significantly facilitated cell proliferation after 48 h incubation (P<0.05 compared with control group or HBE). With higher dosage, such facilitating role on tumor cell proliferation became more prominent (P<0.05, **Figure 2**).

Effects of Gas6 on NSCLC cell invasion potency

Addition of Gas6 into A549 cells significantly facilitated invasion of A549 cells (P<0.05 compared with control group or HBE). With more

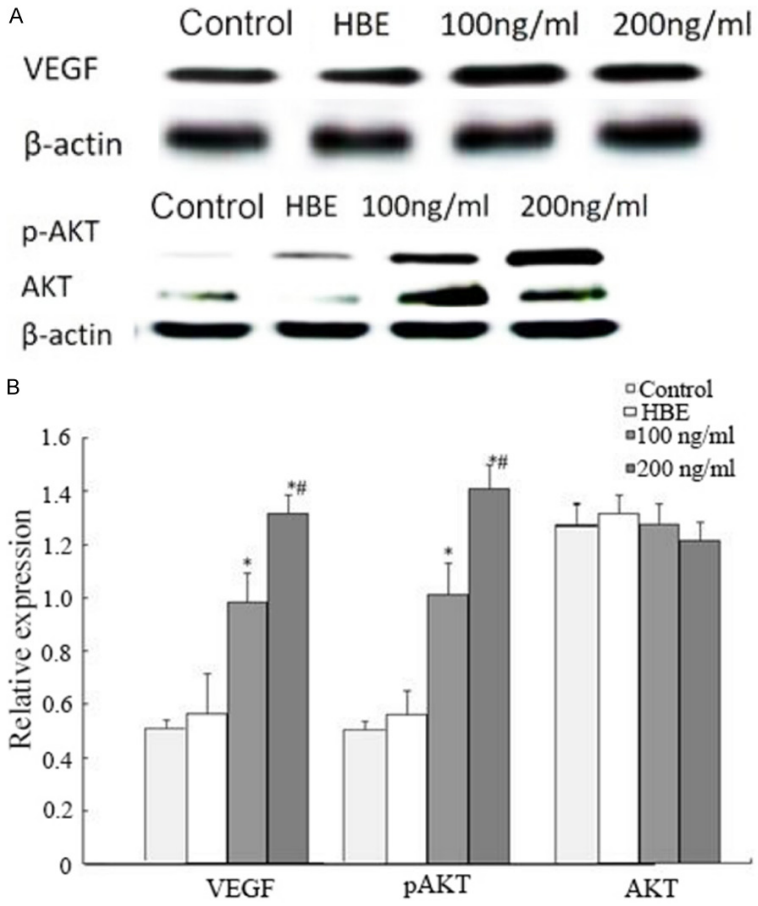

Figure 6. Effects of Gas6 on VEGF/AKT pathway in A549 cells. A. Western blot for measuring the effect of Gas6 protein on VEGF/AKT signaling pathway of A549 cells. B. Analysis for the effect of Gas6 on VEGF/AKT signal pathway of A549 cells. *P<0.05 comparing to control group or HBE; #P<0.05 comparing to 100 ng/ml Gas6.

dosage, such promoting effects on tumor cell invasion became more prominent (P<0.05, **Figure 3**). These data showed that Gas6 could affect invasion potency of tumor cells.

Effects of Gas6 on caspase 3 activity of NSCLC cells

We further measured the effect of Gas6 on Caspase 3 activity of NSCLC using test kit. Results showed that addition of Gas6 into A549 cells significantly inhibited caspase 3 activity (P<0.05). With higher dosage, such inhibitory effect on tumor cell caspase 3 activity became more prominent (P<0.05 **Figure 4**).

Effects of Gas6 on secretion of inflammatory cytokines from A549 cells

We further used ELISA to measure the effect of Gas6 on secretion of inflammatory cytokines in supernatant of cultured A549 cells. The addition of Gas6 into cultured NSCLC cells significantly potentiated the secretion of inflammatory factors IL-2 and IL-6 in supernatant of A549 cell culture (P<0.05). Such potentiation effect became more significant with higher dosage (P<0.05, **Figure 5**).

Effects of Gas6 on VEGF/AKT pathway of NSCLC cells

Addition of Gas6 into A549 cell culture system significantly up-regulated VEGF expression and facilitated AKT phosphorylation (pAKT) (P<0.05). The modulatory effect on VEGF/AKT pathway became more significant (P<0.05, **Figure 6**).

Discussion

NSCLC has rapidly increasing incidence nowadays, and patients present atypical symptoms at early phase, including cough, low fever, coughing blood, and chest pain. These symptoms frequently lead to misdiagnosis. In addition, NSCLC has relatively higher malignancy and is susceptible for metastasis. Therefore, many patients have already presented metastasis at the time of primary diagnosis, leading to unfavorable treatment efficiency [15]. Effective treatment against NSCLC can help to improve cancer patient survival and to benefit the prognosis [16].

As a novel cytokine, Gas6 can bind with its receptor to exert multiple effects including resistance against vascular endothelial cells, protecting smooth muscle cells from apoptosis, and facilitating cell growth [17]. More importantly, Gas6 showed up-regulation in various tumor cells [11-13]. Our study intends to analyze the expression and related roles of Gas6 in NSCLS. Results showed enhanced Gas6 expression in NSCLC tumor tissue, consistent with reports for Gas6 in other tumors [11-13]. Further studies focused on its role in NSCLS

and showed that Gas6 could facilitate tumor cell proliferation, decrease caspase 3 activity, and increase tumor cell invasion in a dose-dependent manner. These results illustrate that Gas6 can facilitate tumor invasion via potentiating tumor growth or proliferation or inhibiting apoptosis.

This study further explored related mechanisms and found that addition of Gas6 into A549 cell culture facilitated VEGF protein expression, up-regulated pAKT, and enhanced IL-2 and IL6 expression. As a trophic factor facilitating angiogenesis and vascular permeability, VEGF is an important angiogenesis cytokine for tumor growth and metastasis [18]. The expression of VEGF in vascular endothelial cells can facilitate proliferation, differentiation, migration and motility of VEGF to form vascular cavity like structure via elevating vascular permeability and degrading extracellular matrix [19]. During tumorigenesis, VEGF can facilitate angiogenesis [20], and protein kinase AKT participates in various biological behaviors of cells, making it an important signaling molecule in cell survival [21]. Gas protein itself can induce inflammation, and facilitate cellular interaction under inflammatory status [22, 23]. These results showed that Gas6 can induce inflammation via VEGF/AKT signaling pathway to further cause tumor progression. Further studies can be performed to analyze the functional role of Gas6 on NSCLC cells and related mechanisms, in order to consider it as an intervention target for clinical treatment.

In conclusion, Gas6 is up-regulated in NSCLC tumor tissues and induces inflammation probably via VEGF/AKT signaling pathway. It can also accelerate NSCLC proliferation and invasion, thus inhibiting apoptosis and facilitating NSCLC tumor progression. However, the therapeutic role of targeting Gas6 in patients with NSCLC was not investigated in our study. In the future, we plan to investigate the exact role of Gas6 in the development of NSCLC in patients as well as the therapeutic role in the treatment of NSCLC.

Disclosure of conflict of interest

None.

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