

Original Article

GTPases enhance lung adenocarcinoma invasiveness and promotes cell motility

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Abstract: Objective: To understand the role of GBP-5 in lung adenocarcinoma invasiveness and cancer cell motility. Methods: In this study, we analyzed the possibility of GBP-5 being involved in the invasiveness of lung adenocarcinoma and its role in cell motility. The analysis was carried out by performing migration and wound healing assays using DMS-153 cells transfected with siRNAs. Results: The result from the RT-PCR analysis observed that the relative expression level of GBP-5 was much higher for GAPDH normalized for adenocarcinoma invasiveness compared to normal cells with a p -value < 0.05 . The study also revealed that transfected GBP5-siRNA cells have a low relative migration compared to the control cells. The result from the wound healing analysis also noted that the GBP5-siRNA transfected cells have a lower wound healing capacity. Conclusion: It is reported that GBP-5 promotes lung adenocarcinoma invasiveness and promotes the motility of cancer cells. Moreover, controlling the expression of GBP-5 may provide new avenues and a hope that may contribute to the development of a novel therapeutic strategy for treating lung adenocarcinoma.

Keywords: GTPases, GBP-5, lung adenocarcinoma, cell motility

Introduction

Lung adenocarcinoma (LAC) is one of the leading causes of cancer deaths, from which a large population of LAC is due to tobacco smoking [1]. There are reports which suggest that LAC is associated with environmental factors which include passive smoke inhalation, air pollution, household smoke, radon, etc among non-smokers [2]. LAC's are usually heterogeneous based on their genomic and morphological characteristics and mostly consist of two different histological subtypes [3]. On the other hand, GTPases belongs to the hydrolase family of enzymes and are involved in the binding and hydrolyzation of GTP (Guanosine triphosphate) to GDP (Guanosinediphosphate) which takes place in the G-domain conserved region [4]. GTPases also function as a molecular switch in various important cellular processes [5]. Among the GTPases family, the guanylate-binding protein (GBP) is one of these important proteins which is induced by IFN-gamma (Interferon-

inducible GTPase) and is a key protein in defense against microbial and viral pathogens [6]. The GBPs are a family of large cytokine-induced GTPases, whose activity is based on its structure and biochemical nature. There are seven types of GBPs found in human (hGBP1-7). Based on the structure the human GBPs are comprised of large globular N-terminal GTP-binding region and an elongated alpha-helical domain [7]. The GBP's family members such as GBP1, GBP2, and GBP5 play a crucial role in the activation of canonical and non-canonical inflammasome during a response to viral infection. GBP1, GBP2, and GBP5 are mostly induced in cells or tissues upon exposure to IFN-gamma. GBP1 is known to be involved in the inhibitory effects of inflammatory cytokines on the proliferation and migration of endothelial cells and epithelial tumor cells [8]. Whereas GBP2 and GBP5 are thought to be involved in a possible control factor in cancer cell proliferation and invasion [9]. Additionally, there are reports which suggest that the expres-

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sion of GBP1 in colorectal carcinoma is associated with the reduction of tumor cell proliferation and improved prognosis [10]. Whereas an increased expression of GBP2 and GBP5 is thought to be associated with improved prognosis in breast cancer. In spite of all these reports, the actual target and the biological mechanisms of GBP2 and GBP5's regulation on cancer metastasis is not properly understood [11]. Additionally, GBP2 is considered as a controlling factor for cancer cell proliferation and the spreading of tumor cells [12]. In better understanding the importance of GBP's in cancer, the present study aims to investigate the role of GBP-5 in lung adenocarcinoma.

Materials and methods

Ethics

This study was approved by the Ethics Committee of the Xiantao First People's Hospital Affiliated to Yangtze University, Xiantao, Hubei, China, 433000 and University Hospital of Wuhan University, Wuhan 430071, China. All participants and patients provided written, informed consent to participate in this study, and our Institutional Review Board approved the protocol vide Approval No. XFPH-472A-51C.

Cell culture and antibodies

Cell lines were obtained from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China and ATCC, Manassas, VA, USA. Cells were cultured in RPMI 1640 medium or DMEM medium supplemented with 10% fetal bovine serum (FBS) using a CO₂ incubator at 37°C. The antibodies were purchased from Genentech (CA, USA), Abnova (Taipei, Taiwan) and Sigma-Aldrich (MO, USA).

Tissue samples

Tissues samples of the tumors were collected from the patients confirmed with adenocarcinoma and the normal lung tissue samples were collected from both participating Hospitals following standard surgical procedures. The patients were not treated with any tumor-related therapy before the operation and excluded any therapy-based expression prior to surgical resection. Specimens from the patients with adenocarcinoma lung tissue (n = 6) and

normal lung tissue (n = 6) were collected and kept in a sampling tube which is frozen with liquid nitrogen.

RT-PCR analysis

Total RNA was harvested using TRIzol from the human samples and reverse transcribed using SuperScript II First-Strand kit (Invitrogen) following the manufacturer's protocol. The RT-PCR amplification parameters were set for 30 cycles at 96°C for 10 sec (denaturation), 30 s at 56°C (annealing), and 1 min at 70°C for 26 cycles (extension). GBP-5 was amplified using the primers 5'-ATCTATCGCACAGCATATT-3' (sense) and 5'-TTGACATGATGTCGTCCAA-3' (anti-sense) while E-cadherin using the primers 5'-CAATGGCTAAGAGCGCGATGA-3' (sense) and 5'-TCCGTTAGATAGTCACCGTA-3' (antisense). For amplification of GAPDH, the primers 5'-ACCA-CAGTCCATGCCATCAC-3' (sense) and 5'-TCCACCACCCTGTTGCTGTA-3' (antisense) were used. The PCR products were analyzed using 1% agarose gel electrophoresis and further stained with EtBr. The gel photographs were captured using a Bio-Rad Gel-Doc system and the signal intensities were quantified using ImageJ v.1.45 (NCBI, USA).

Western blot analysis

The lung adenocarcinoma cells were lysed using RIPA buffer which is composed of Triton X-100, 50 mM Tris (pH8.0), 150 mM NaCl, 0.5% sodium deoxycholate, protease inhibitor and 0.2% sodium dodecyl sulfate. The conditioned media was collected and centrifuged at 1500 rpm and the cell debris was discarded. The extracted proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and quantified using Lowry's method. The proteins were then transferred into a Hybond-P polyvinylidene difluoride membrane (GE Healthcare GmbH, Freiburg, Germany) and analyzed by western blot. The membrane was incubated with their respective antibodies at 34°C for 60 mins and the membrane was placed in a Western Blotting Detection Reagent (ECL™ Prime, GE Healthcare, USA) and visualized using an Rx film.

Transfection (gene knockdown)

All siRNA's including the control targeting the GBP-5 gene was obtained from Santa Cruz

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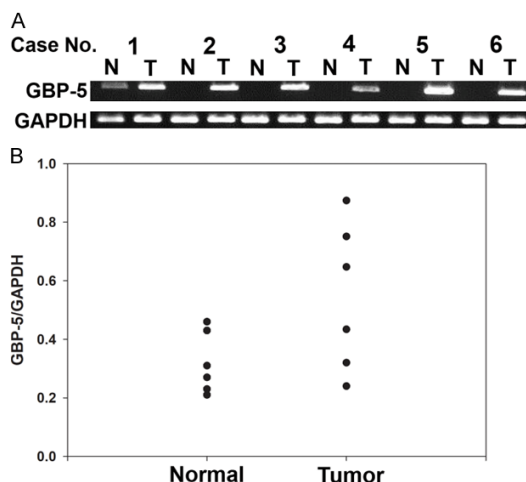


Figure 1. (A) Result from RT-RT-PCR analyses of from the surgical samples. "N" represent normal lung tissue and "T" represents lung adenocarcinoma tissue (B) Statistical analysis presenting the expression levels of GBP-5 using GAPDH as normalized factor.

Biotechnology, Inc. The small interfering RNAs were allowed to be transfected into DMS-153 cells using an RNAi-specific cationic lipid formulation such as Lipofectamine® RNAiMAX following the standard protocol and manufacturer's procedure.

Migration assay

Migration assay was carried out in a double chamber trans-well having a pore diameter of 8.0 mm using a 12-well plate. The upper membrane of the chamber was coated with fibronectin (1 mg/mL) in PBS solution for 3 hours at 34°C. The transfected DMS-153 cells containing the fibronectin after taking out the PBS were trypsinized and seeded in the upper chamber of the trans-well and fixed in a 6% PFA after passing the cells through the 8.0-mm membrane pores for 7-8 hours and stained using phalloidin, and the cells were counted using a confocal microscope.

Wound healing assay

DMS-153 cells were cultured on a gelatine-coated six-well plate (Nunc™) until confluence and transfected with siRNA. The cells on the plates were scratched after 48 hours of the formation of the confluent monolayer with the help of a microtip by drawing lines. The images of the monolayer wounds in the cells were taken immediately after scratching using

Axiovert 25 microscope (Zeiss) and the area of cell invasion was determined.

Immunohistochemical analysis

Formalin-fixed paraffin-embedded tumor sections of around 5 mm were treated with xylol and deparaffinized. It was further rehydrated in a descending ethanol concentration of 100%, 96% and 70% ethanol, respectively and autoclaved for 17 mins at 123°C in a citrate buffer. The slides were treated with hydrogen peroxide for 7 mins at 34°C to block endogenous peroxidases and further incubated with the anti-GBP-5 antibody for 45 mins at 34°C. The slides were then counterstained using Mayer's haemalum and kept for dehydration for 2 min in ethanol. They were then mounted in a DPX mounting medium and observed using light microscopy (Zeiss Axiovert 200M, Munich, Germany).

Statistical analysis

Statistical analysis was carried out using SPSS 17.0 Statistical Software (SPSS Inc, USA). Mann-Whitney U test was used to compare the relative expression level of GBP-5/GAPDH between normal and tumor samples. The student's t-test was used for identifying the significant differences from the clinico-pathological data. Fisher's exact test was used for analyzing the categorical data and variables. Results are expressed as means \pm standard deviation. Statistical significance ($P < 0.05$) was detected by one-way ANOVA using SPSS 17.0.

Results

RT-PCR and transfection analysis

The RT-PCR results from the normal lung and adenocarcinoma lung cancer cells revealed that the GBP-5 gene was not expressed in normal lung tissue. It is also observed that GBP-5 was abundantly expressed in lung adenocarcinoma tissue which is detected in 7 cases out of the total 12 cases (**Figure 1**). The relative expression of GBP-5 was 0.39 ± 0.21 compared to GAPDH after normalization for normal lung. Whereas for the lung adenocarcinoma, the relative expression of GBP-5 was 0.74 ± 0.24 normalized to GAPDH. Moreover, the relative expression level of GBP-5 was comparatively higher in the case of invasive lung adenocarcinoma compared to normal lung tissue

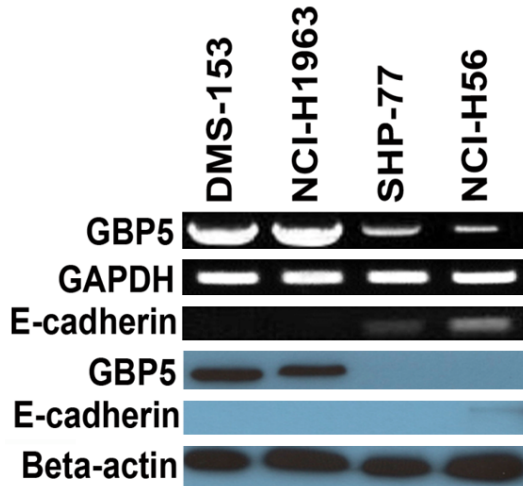


Figure 2. Western blotting and RT-PCR analysis of DMS-153, NCI-H1963, SHP-77 and NCI-H56 LAC cells.

with a *p*-value greater than 0.05. The western blot analysis also indicated that the protein expression level of adenocarcinoma cell lines DMS-153 and NCI-H1963 were higher in the mesenchymal-like cell lines compared to the epithelium-like cell lines viz. SHP-77 and NCI-H526 (**Figure 2**).

Analysis of migration assay

The migration assay was conducted in order to find out if GBP-5 is associated with the expansion of lung adenocarcinoma. This assay was carried out on DMS-153 cells with knockdown of GBP-5, which was further validated (**Figure 3**). The migration assay for DMS-153 cells observed that the scores were 38 ± 3.45 for control cells, 34 ± 2.34 for Ctrl-siRNA and 25 ± 2.1 for GBP5-siRNA respectively, which were counted after migration through a polycarbonate membrane and 5 hours of seeding. **Figure 3A** and **3B** represents the relative cell migration ability for DMS-153, Cntrl-siRNA, and GBP5-siRNA. The control cells were normalized to 0.87 ± 0.03 for Cntrl-siRNA and 0.58 ± 0.04 for GBP5-siRNA, respectively. In fact, the migration of cells with GBP-5 knockdown was comparatively lower than the control with a *p*-value of less than 0.05.

Analysis of wound healing assay

The wound-healing experiment was carried out using the transfected cells and their capacities

were measured after six-hours and twelve-hours. The healing capacities for DMS-153 were $48 \pm 1.8\%$ at 6 h and $59 \pm 1.7\%$ at 12 h. Whereas Cntrl-siRNA had a relative wound healing capacity of $38 \pm 2.1\%$ at 6 h and $58 \pm 2.5\%$ at 12 h. Lastly, the GBP5-siRNA had a relative wound healing capacity of $21 \pm 1.6\%$ at 6 h and $32 \pm 2.1\%$ at 12 h (**Figure 4**). In fact, the DMS-153 cells with GBP-5 downregulation revealed a drastic reduction in the wound healing capacity compared to the control cells at 6 h as well as in 12 h after scratching (*p*-value ≤ 0.05). On the other hand, **Figure 5** showed the proliferation of the tumor cells along the alveolar surface and its invasive components. From the figure, the GBP-5 immunoreactive signals of the invasive component in the cytoplasm were detected; however, the signals could not be detected in its lepidic growth of the component cells.

Discussion

In the present study, it is reported that GBP-5 is thought to play a crucial role in the lung adenocarcinoma cell invasion and promotion of cell migration. The clinico-pathological analysis also revealed the association of GBP-5 expression and lymphatic system invasion. In fact, GBP-5 acts as an indicator of malignant tumors and cancer associated with lung adenocarcinoma. The present study is one such report where GBP-5 induces the quick expansion of lung adenocarcinoma by stimulating cell motility. The GBP-5 proteins belong to the interferon- γ -inducible family which are large GTPases and are known for its high induction capacity by pro-inflammatory cytokines [13]. On the other hand, the GBP family of protein is categorized based on its ability to bind the immobilized guanine nucleotides which have similar affinities towards GTP, GDP and GMP [14]. Among these members of the family, GBP-1 is the best-characterized group that has been shown to portray a unique hydrolytic activity and play a crucial role in cancer cell proliferation, signal transduction and cell differentiation [15].

It is also reported that GBP mutations, which include the Ras superfamily of genes, results in the cell cycle degeneration and decreases the activity of the GTPase [16]. However, a recent study reported that GBPs homodimers are

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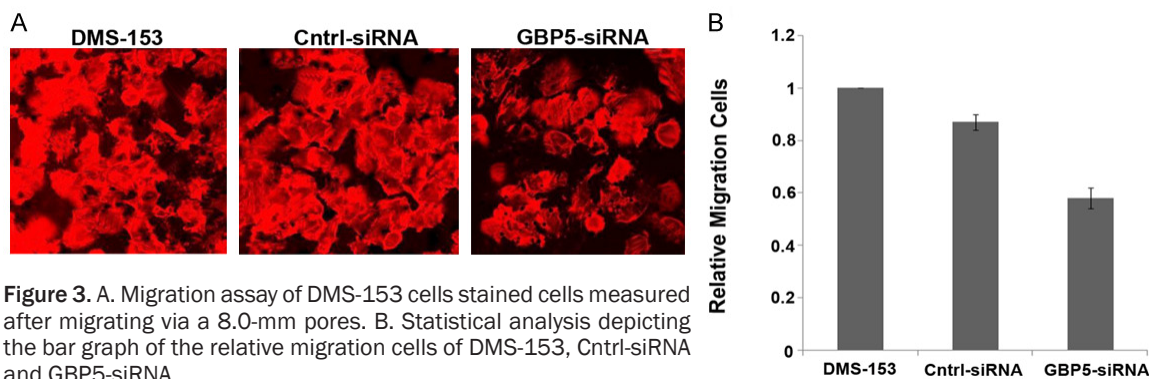


Figure 3. A. Migration assay of DMS-153 cells stained cells measured after migrating via a 8.0-mm pores. B. Statistical analysis depicting the bar graph of the relative migration cells of DMS-153, Cntrl-siRNA and GBP5-siRNA.

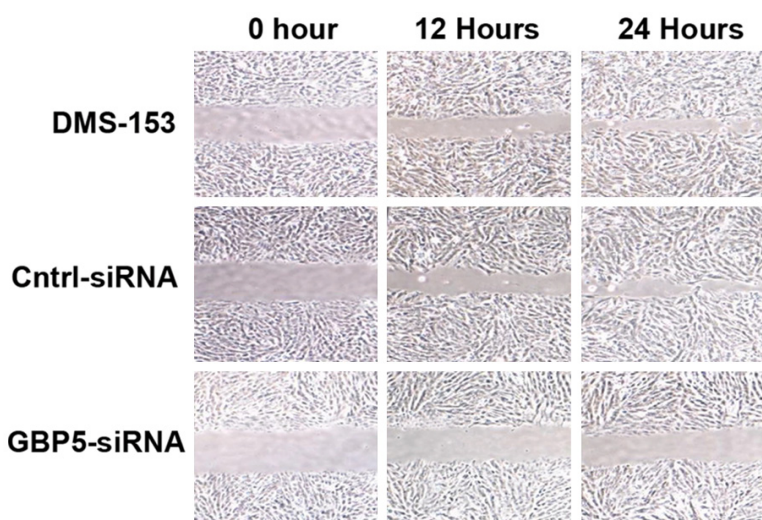


Figure 4. Micrographic images of the wound healing assay representing the scratching at 0 h, 6 h and 12 for the migrated cells of DMS-153, Cntrl-siRNA and GBP5-siRNA.

associated with the plasma membrane thereby localizing the actin cytoskeleton and promotes signal transduction and cytoskeleton organization [17]. In fact, the actin cytoskeleton organization is regarded as one of the prime factors for tumor cell invasion due to reduction in the adhesions of the cell-matrix [18]. This report is consistent with our findings that the GBP-5 promotes malignant lung adenocarcinoma by rapid cell invasion. Interestingly, the immunohistochemical analysis from the present study observed that the overexpression of GBP-5 in lung adenocarcinoma is relative to lepidic growth components, especially in the invasive components. About 15% of the tumors originated to be GBP-5 positive and the expression of GBP-5 was associated with the lymph vascular invasion. Moreover, more than 60% of the lepidic component of tumors was GBP-5-negative. In fact, the lympho-vascular invasion is considered as a good predictor for lung carci-

noma which leads to prognosis and risk factors for deterioration [19-21]. Additionally, it is reported that GBPs such as GBP 1, 2 & 5 are involved in increasing the metastasis of the lymph nodes in esophageal cancer, which put forward that the high expression of GBPs may cause grade III tumors or malignancy [22, 23]. Thus suggests that GBP-5 may act as a new biomarker of invasion in patients with LAC. In fact, GBP-5 is known to arbitrate interferon-inducible GTPase which is a key protein in defense against microbial and viral pathogens. GBP-5 also functions as an activator of NLRP3 inflam-

masome assembly and plays a major role in innate immunity and inflammation [24, 25]. The GBP-5 is also a member of the IFN-inducible subfamily of guanosine triphosphatases (GTPases) which is an important factor that plays a key role in cell-intrinsic immunity against various pathogens [26, 27]. These findings also imply that the function of GBP-5 is varied among various types of cancers and tumors [28]. From these results, it is implied that GBP-5 is observed to induce the malignancy and progression of LAC by promoting cell motility and invasion. Hence, the study suggests that GBP-5 may be a potent biomarker for lung adenocarcinoma invasiveness.

Conclusion

The study comes to the conclusion that GBP-5 promotes cell motility and is involved in the invasion of lung adenocarcinoma. The GBP-5

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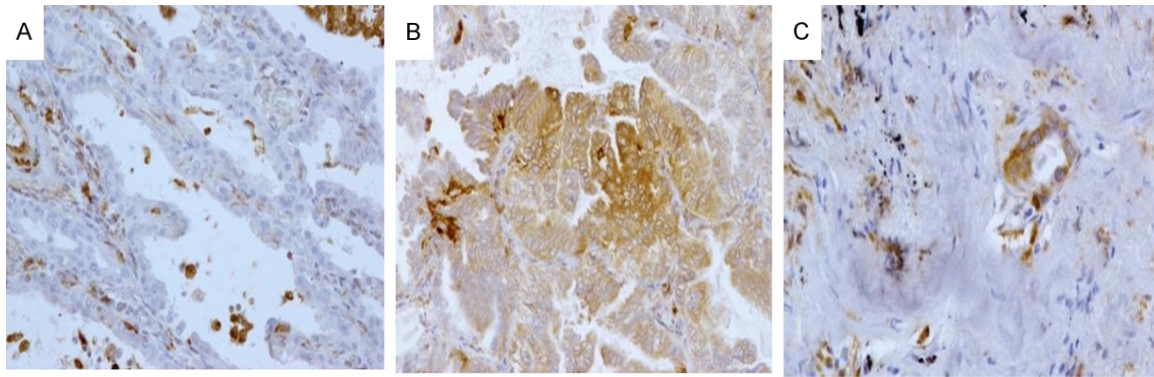


Figure 5. Immunohistochemical staining of GBP representing the (A) lepidic growth, (B) invasive component stained with hematoxylin and eosin and (C) immunostaining of GBP-5.

protein also promotes the activity of GTPase since it is a GTP-binding protein. The suppression of GTPase activity in GBP-5 may serve as a novel approach for controlling advanced stage malignant lung adenocarcinoma, especially in non-lepidic adenocarcinoma patients. Therefore this study concludes that GTPases enhance lung adenocarcinoma invasiveness and promote cell motility. However, additional investigation is required to understand the molecular mechanism of GBP-5 targeting the GTPase enzyme.

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Disclosure of conflict of interest

None.

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