IQGAP3 Promotes EGFR-ERK Signaling and the Growth and Metastasis of Lung Cancer Cells

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Abstract

Proteins of the IQGAP family display complicated and often contradictory activities in tumorigenesis. IQGAP1 has well documented oncogenic potential and IQGAP2 has putative tumor-suppressive function. IQGAP3 is the latest addition to this family and its role in cancer development remains to be defined. Here we demonstrate IQGAP3 expression is markedly increased in lung cancer tissues at both mRNA and protein levels. Overexpression of IQGAP3 promoted tumor cell growth, migration and invasion, whereas knockdown of IQGAP3 exhibited opposite effects. Moreover, suppression of IQGAP3 in a lung cancer cell line caused a reduction in the tumorigenicity of these cells in lung tissue after intravenous injection. Furthermore, we showed that IQGAP3 is able to interact with ERK1 and enhance its phosphorylation following treatment with EGF. These data suggest that IQGAP3 may contribute to the pathogenesis of lung cancer by modulating EGFR-ERK signaling.

Introduction

Lung cancer ranks first in cancer related mortality both in China and worldwide [1,2]. In China, there are approximately 300,000 new lung cancer cases and more than 250,000 deaths from this disease each year [3]. Histologically, as many as 85% of lung cancers are non-small cell type lung cancer (NSCLC), and the majority of these are either adenocarcinoma or squamous cell carcinoma [4-7]. As lung cancer may be occult, most patients are inoperable and have metastases to regional lymph nodes or to distant sites at the time of diagnosed. The NSCLC patients with distant metastases survive for a short time (from 9 to 12 months) [5,8]. There is therefore an urgent need to unravel the molecular mechanisms which lead to invasion and metastasis in lung cancer [9,10]. Such information will facilitate the development of novel therapies allowing improvement of the outcome in lung cancer patients [11,12]. Therapeutic approaches against EGFR or EGFR represent a promising direction for lung cancer therapy [13,14]. EGFR is expressed in normal cells of epidermal, mesenchymal and neurogenic origin, and its activation is strictly controlled in normal tissues [15,16]. However, binding of EGFR by its ligand results in receptor homo- or heterodimerization and activation of its intrinsic tyrosine kinase activity [17]. The downstream signaling cascade is thus initiated, ultimately leading to changes in such cell behaviors as proliferation, migration and differentiation [15,16]. Importantly, constitutive activation of EGFR or enhanced EGF signaling is frequently found in different types of cancers, especially in lung cancer, where it is associated with cancer initiation, tumor growth/progression, metastasis and poor prognosis [17-20].

The IQGAP family of proteins is well conserved in organisms from yeast to mammals [21]. It comprises three members, IQGAP1, IQGAP2 and IQGAP3 [22-24]. Among these, IQGAP1 is the best studied [25]. The name IQGAP is derived from the multiple functional domains these molecules harbor such as four IQ motifs and a RasGAP-related domain (GRD) [26,27]. IQGAP1 also contains putative coil-coil homodimerization domains, a tryptophan repeat motif (WW) of unknown function, a calponin-homology domain (CHD) that interacts with F-actin, and a RasGAP_C-terminus (RGCt) that interacts with numerous proteins including E-cadherin and b-catenin [27]. IQGAP1 has been suggested to function in regulation of the cytoskeleton and cell migration [28-30]. There is also evidence which indicates IQGAP1 plays a role in cancer progression [31,32]. In contrast, IQGAP2 seems to act as a tumor suppressor [33]. IQGAP3 is the latest addition to this family [24]. Data currently available suggest that it is involved in the proliferation of epithelial cells [34,35], however its role in tumorigenesis remains to be determined. In the current study, we provide the first evidence that IQGAP3 promotes lung cancer growth and metastasis by enhancing EGFR-mediated ERK signaling. IQGAP3 may therefore play a role similar to that of IQGAP1 in tumorigenesis.
Materials and Methods

Ethics Statement
This study was approved by the Ethics Committees of Peking University Health Science Center (Beijing, China) and the 306th Hospital of the People's Liberation Army of China (Beijing, China). For animal studies, all efforts were made to minimize suffering and when observed suffering was too great, humane euthanasia was used. Written consent was obtained from individual patients for use of tissue specimens.

Cell Lines and Patient Specimens
A549 and Hela cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA). HEK293T cells were cultured in DMEM medium supplemented with 10% fetal bovine serum. Cells were cultured at 37°C in a humidified 5% CO2 atmosphere.

For cell signaling assays, cells were serum deprived (0.5% fetal bovine serum) for 16 h prior to stimulation with 100 ng/ml EGF (Peprotech, Rockville, NJ, USA) for different lengths of time as indicated.

25 paired lung tumor tissue and adjacent normal tissue specimens were obtained from the 306th Hospital of the People’s Liberation Army of China.

Plasmids and Transfection
Myc-pCAGGS-IQGAP3 and control pCAGGS vectors were kindly provided by Dr. Kozo Kaibuchi. HA-tagged ERK1 or ERK2 expressing vectors were constructed by standard molecular techniques and verified by sequencing.

Cells were transfected with jetPRIME transfection reagent (Polyplus Transfection, Illkirch, France).

siRNA Transfection and Lentiviral Infection
Small interfering RNA oligonucleotides (oligos) against IQGAP3 or control siRNA oligonucleotides were obtained from GenePharma Co., Ltd (Shanghai, China). The targeting sequences of these siRNAs were as follows: si IQGAP3-1: 5'-GAGCCAAC-3'.

Figure 1. Increased expression of IQGAP3 in lung cancer tissues.
(A) EST expression analysis of the IQGAP3 gene in normal and cancerous tissues based on the ECgene database. (B) Quantitative real-time PCR for IQGAP3 expression in 25 pairs of lung cancer versus adjacent non-cancerous tissues. IQGAP3 expression was normalized against GAPDH. Relative levels were calculated for each sample, and a value of 1 was assigned for adjacent non-cancerous tissue of patient 13. Experiments were repeated in triplicate. Data from one representative experiment are presented as mean±SD. (C) Immunohistochemical analysis of IQGAP3 protein expression in lung cancer versus adjacent non-cancerous tissues. Representative images are shown for a squamous cell carcinoma and an adenocarcinoma. Ca, Cancer tissue; Adj, adjacent non-cancerous tissues. Magnification, ×200.

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CAGGACACUA-3'; si IQGAP3-2: 5'- GGCAGAAACUA-GAAGCAUA-3'; control siRNA: 5'- UUCUCCGAACGUGUCACGU-3'. siRNA oligos (50 nM) were transfected into A549 cells with jetPRIME transfection reagent.

Alternatively, the siRNA target sequence was cloned into the lentiviral vector pLL3.7. Upon sequence verification, the shRNA plasmid and packaging vectors pPSX2 and pLP/VSVG were transfected into the packaging cell line HEK293T using jetPRIME. The medium was changed 8 h post-transfection. 48 hours later, viral supernatant was harvested and incubated with the A549 cell line in the presence of 8 μg/ml polybrene (Sigma-Aldrich, Saint Louis, MO, USA). For stably transfected cell line selection, GFP fluorescence was used as a sorting marker. Cells with >75% infection efficiency were used for further analysis.

**Reverse Transcriptase-PCR and Real-time PCR**

TRIzol (Life Technologies) was used to isolate total RNA and then reverse transcribed into cDNA by the Reverse Transcription

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**Figure 2. Enhanced cell growth, migration and invasion with enforced expression of IQGAP3.** Myc-IQGAP3 or control vectors were transfected into Hela cells and then cells were harvested at 24 h after transfection for migration and invasion assay. Cells for proliferation assay was harvested at indicated time point. (A) IQGAP3 protein expression of transfectants was verified by Western blotting. Cell proliferation was assayed using the Cell Counting Kit-8. (B, C) Cell migration and invasion assays were performed using Chemotaxis chambers with or without coating with Matrigel. Each assay was repeated at least 3 times. Data from one representative experiment are presented as mean ± SD. *, P < 0.05; ***, P < 0.001. doi:10.1371/journal.pone.0097578.g002
System (Promega, Madison, WI, USA). Quantitative realtime PCR was carried out on a Bio-Rad Real-Time PCR system. The sequences of the realtime primers for IQGAP3 were as follows: forward, 5'-GTTCATCCATAGAGCCTGCCA-3'; reverse, 5'-GCGATGCTCTCACCAATAAGG-3'. The realtime primers for GAPDH have been described before [36]. Gene expression was quantified as the yield of IQGAP3 relative to that of GAPDH.

Immunoprecipitation and Western Blot Analysis

Myc-IQGAP3 and HA-ERK1 or HA-ERK2 constructs were transfected into HEK293T cells. Cells were harvested and lysed in lysis buffer with proteinase inhibitor cocktail (Roche, Basel, Switzerland) and phenylmethylsulfonyl fluoride. Then the cell lysates were incubated with mouse anti-HA mAb (Sigma-Aldrich), anti-Myc mAb (Sigma-Aldrich) or a control antibody (mIgG) (Sigma-Aldrich) and protein-A Sepharose (GE Healthcare, USA) and resolved by SDS-PAGE. For endogenous immunoprecipitation, cell lysate was prepared from A549 cells and immunoprecipitated with rabbit anti-ERK1 mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

For western blot analysis, equal amounts of protein from each sample was loaded and resolved with SDS-PAGE and transferred to blots. After blocking, blots were probed with indicated antibodies and evaluated with the Odyssey Imaging System (LICOR Bioscience, Lincoln, NE, USA). Antibodies used included anti-GAPDH (Bioworld Technology, Inc., St Louis Park, MN, USA), anti-IQGAP3 (Sigma-Aldrich), anti-ERK (Cell Signaling, Beverly, MA, USA), anti-phospho-ErkThr202/Tyr204 (Cell Signaling), anti-phospho-p38Thr180/Tyr182 (Cell Signaling), anti-phospho-AktSer473 (Cell Signaling), anti-Myc, and anti-HA.

Tissue Microarray and Immunohistochemistry

A lung cancer tissue microarray (TMA) was purchased from Chaoying Biotechnology Co. (Xi’an, China). Sections were incubated with anti-IQGAP3 antibody (1:50 dilution) overnight at 4°C. The primary antibody was identified using a horseradish peroxidase enzyme-labeled polymer conjugated to goat antimouse secondary antibody (Promega). The stained sections were reviewed and scored by a pathologist. The negative control was normal human lung tissue.

Figure 3. Reduced cell growth, migration and invasion upon inhibition of IQGAP3 expression. A549 cells were transfected with shRNA constructs to knockdown endogenous IQGAP3 expression. (A) IQGAP3 protein levels following infection with control (NC) or two different shIQGAP3 lentiviruses (KD1 and KD2). (B) Proliferation was assayed using the Cell Counting Kit-8 for lentivirus-infected A549 cells. (C, E) Migration of lentivirus-infected A549 cells in the chemotaxis chamber assay in the absence (C) or presence (E) of human EGF (100 ng/ml). (D, F) Invasion of lentivirus-infected A549 cells across Matrigel in the absence (D) or presence (F) of human EGF. Each assay was repeated at least 3 times. Data from one representative experiment are presented as mean±SD. *, P<0.05;**, P<0.01;***, P<0.001. doi:10.1371/journal.pone.0097578.g003
Cell Proliferation Assay
Cells were plated in 96-well plates at a density of $3 \times 10^3$ cells/well. Cell proliferation was evaluated every 24 h using the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Japan). Each experiment was performed in triplicate.

Cell Migration and Invasion Assay
Migration assays were performed in a 24-well Chemotaxis chamber (8-μm pore size, Corning Life Sciences, Corning, NY, USA) coated with 10 μg/ml fibronectin (Sigma-Aldrich). Cells ($3 \sim 5 \times 10^4$ cells/well) in 200 μl serum-free RPMI 1640 were seeded into the upper chamber. Then 600 μl RPMI 1640 with 10% fetal bovine serum or 100 ng/ml human EGF were added into the lower chamber. Cells in the upper chamber were removed using a cotton swab after 20 h of incubation at 37°C and 5% CO₂. Cells attached to the bottom of the membranes were fixed with methanol and stained by crystal violet. The degree of migration was expressed as the average number of cells in six 10× fields.

Cell invasion assays were carried out in essentially the same manner as the migration assay, except that the upper chamber was covered with 30 μl Matrigel (0.5 mg/ml; BD Biosciences). Experiments were performed in triplicate.

Luciferase Reporter Assay
The Elk-1 transcriptional activity were measured with the Dual-Luciferase Reporter Assay System (Promega). IQGAP3 siRNA or NC siRNA was co-transfected into A549 cells with the plasmids pFR-Luc (reporter plasmid), pFA2-Elk-1 (transactivator plasmid) or pRL-TK. Twenty-four hours later, cells were serum-starved for 16 h, then stimulated with or without 100 ng/ml EGF for 6 h. Cells were harvested and lysed. All reporter assays were repeated at least three times in triplicate. Firefly luciferase and Renilla luciferase activity were measured with a Centro LB960 luminom-
Firefly luciferase activity was calculated and normalized based on Renilla luciferase activity. Luciferase activity in control siRNA-transfected cells without EGF stimulation was set as 1.

Tumor Metastasis Analysis in vivo

NOD/SCID mice were purchased from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). Mice were raised in a pathogen-free facility at Peking University Health Science Center (Beijing, China).

A549 cells were infected with control shRNA or shIQGAP3 lentiviruses. Cells (1.5 × 10⁶) in 0.1 ml PBS were injected into the tail vein of 6-week-old NOD/SCID mice. Six weeks after infection, mice were sacrificed by cervical dislocation. Then lungs were removed, weighed, photographed, and fixed in 4% formalin. Tissue sections were also stained with hematoxylin-eosin (H&E) for histologic evaluation.

Statistical Analysis

Statistical analysis was carried out using SPSS, version 13.0 (SPSS, Inc.). The Student’s t-test was used to compare cell proliferation, migration, and invasion between two independent groups. The Chi-square test was performed to determine differences in patient’s age, gender, tumor stage and histology among groups with higher, equal or lower IQGAP3 expression in tumor versus adjacent non-cancerous tissues. P values <0.05 were considered statistically significant.

Results

IQGAP3 Expression is Upregulated in Lung Cancer Tissue

A potential role in tumorigenesis was first suggested for IQGAP3 by bioinformatic analyses of IQGAP3 expression in tumor tissue. ECgene (http://genome.ewha.ac.kr/ECgene/) showed elevated levels of IQGAP3 in a variety of tumor tissues or cells of origin in the ovary, lung, large intestine, stomach, bone marrow and breast (Figure 1A). RT-PCR was subsequently performed to verify these data. While IQGAP3 expression in normal tissues was restricted to the colon, small intestine and testis, high levels of IQGAP3 mRNA were observed in a number of tumors including lung cancer, hepatocellular carcinoma, renal cancer, gastric cancer, bladder cancer, colon cancer and leukemia (data not shown). Moreover, we saw production of autoantibodies against IQGAP3 in a significant fraction of patients with IQGAP3-positive lung cancer [37]. This prompted us to further inquire into the activity of this molecule in lung cancer development. IQGAP3 was upregulated at mRNA level in 20 lung cancer tissues as compared to adjacent non-cancerous tissues in 25 paired samples by real-time PCR analysis (Figure 1B). We next examined IQGAP3 protein expression in a tissue array containing 89 paired lung cancer tissues. Elevated levels of IQGAP3 protein were observed in 80 cancer tissues (Figure 1C and Table S1). Of note, statistical analysis showed that there was no significant correlation between IQGAP3 protein upregulation and parameters such as age, gender or histological grade (Table S1).

Alteration of IQGAP3 Expression Modulates the Growth, Migration and Invasion of Tumor Cells

To investigate the functional consequence of upregulated IQGAP3 expression in cancer, we monitored changes in cell behavior following alteration of its expression level in cancer cell lines. IQGAP3 was first overexpressed in Hela cells, which had a relatively low level of endogenous IQGAP3 expression. Overexpression of IQGAP3 promoted cell proliferation in these cells (Figure 2A). In addition, enforced expression of IQGAP3 resulted in enhanced cell migration and invasion (Figure 2B, C).

To further evaluate the effects of IQGAP3 on cancer cell growth and migration, IQGAP3 expression was knocked down in...
the lung cancer cell line A549, which had a relatively high level of expression of endogenous IQGAP3. Two shRNA constructs targeting different IQGAP3 sequences were used, both of which led to efficient downregulation of IQGAP3 in comparison to non-specific controls (Figure 3A). As anticipated, knockdown of IQGAP3 suppressed the proliferation of A549 cells (Figure 3B). Moreover, reduced IQGAP3 expression was also accompanied by decreased migration and invasion in A549 cells (Figure 3C, D). It is well-known that EGFR signaling is critically involved in lung cancer development. We therefore further tested whether IQGAP3 expression can modulate cell sensitivity to EGF stimulus. As shown in Figures 3E and F, knockdown of IQGAP3 caused inhibition of cell migration and invasion similar to that induced by EGF. Taken together, these data suggest IQGAP3 has oncogenic potential.

Knockdown of IQGAP3 Suppressed Lung Cancer Metastasis

In view of the potent impact of IQGAP3 expression on proliferation and migration of cancer cells cultured in vitro, we next sought to determine whether it also affected tumorigenesis in vivo using an established model of metastatic lung cancer [38,39]. A549 cells harboring a lentiviral vector expressing IQGAP3-specific shRNA were injected into the tail vein of NOD/SCID mice. Animals were sacrificed at day 42 and the lungs were removed and analyzed. Compared to the mock controls, IQGAP3 knockdown cells appeared to be less tumorigenic in vivo, as suggested by the greatly reduced bulk and weight of the affected lungs (Figure 4A, B). Immunohistochemical staining revealed that relatively normal lung structure was maintained in the IQGAP3 knockdown groups, while it was almost completely destroyed by the multiple tumor nodules in the mock control (Figure 4C). Taking the related in vitro data into consideration, the reduced tumorigenic potential of IQGAP3 knockdown cells in vivo may result from either impaired colonization or proliferation or both.

IQGAP3 Interacts with ERK1

As predicted by Scansite [http://scansite.mit.edu/], IQGAP3 contains multiple ERK D domains, which are known to mediate interaction with ERK family members. To explore this possibility, we transfected HEK293T cells with Myc-IQGAP3 and HA-ERK1 or HA-ERK2 constructs. Cell lysate was prepared and immunoprecipitated with anti-HA or anti-Myc antibodies. The resultant precipitate was reciprocally detected with anti-Myc or anti-HA. The result was of particular interest that immunoprecipitation of IQGAP3 was observed with ERK1, but not with ERK2.
Discussion

To date, three members of the IQGAP family have been described [22–24]. There is evidence that IQGAP1 primarily functions as an oncogene [27]. IQGAP2 displays anti-tumor activity, despite its structural similarity to IQGAP1 [33]. Here we demonstrated that IQGAP3 is highly expressed in a large proportion of lung cancer samples. While enforced expression of IQGAP3 causes accelerated proliferation and migration/invasion of cancer cells, suppression of its expression leads to a reduction in tumorigenic potential. At the molecular level, IQGAP3 interacts with ERK1 and promotes EGF-induced activation of ERK, which is associated with increased IQGAP3 expression. Although in our study, we demonstrate that upregulation of IQGAP3 is a common event in lung cancer, we should mention that there are a few lung cancer samples which show unchanged or even decreased expression of IQGAP3. This is not surprising. For example, Her2, the representing oncogene in breast cancer, is only upregulated in about 20% breast cancer [44,45]. Some genes, such as p16, which can be upregulated or downregulated in tumors and act as either a tumor suppressor or an oncogene depending on the context of the body [46]. Therefore, whether the different expression pattern of IQGAP3 suggests a more confound regulation and function of IQGAP3 in tumor development needs further studies.

The dysregulation of expression of IQGAP3 in cancer tissue suggests a potential role for this molecule in tumorigenesis. Consistent with this concept, overexpression of IQGAP3 enhanced tumor cell growth, migration and invasion, whereas knockdown of IQGAP3 expression displayed opposite effects. IQGAP3 is therefore functionally similar to IQGAP1, which is known to have oncogenic potential [31,32]. Like other members of the IQGAP family, IQGAP3 possesses structural motifs which may bind ERK. By reciprocal immunoprecipitation, we confirmed the interaction between IQGAP3 and ERK1. The functional relevance of this interaction is supported by the augmented phosphorylation of ERK and increased Elk1 transcriptional activity upon EGF stimulation in IQGAP3-transfected cells. More importantly, the proliferation-promoting effect was almost completely abolished by the ERK signaling inhibitor U0126, indicating that IQGAP3 exerts its function mainly by regulation of ERK signaling. It is interesting to note that despite their functional similarity, IQGAP3 and IQGAP1 display distinctly different specificities for binding partners. While IQGAP1 primarily binds to ERK2, IQGAP3 interacts exclusively with ERK1 [40]. The significance of such selectivity remains to be determined, however we speculate that IQGAP1 and IQGAP3 may cooperate in regulation of ERK signaling, thereby exerting a synergistic effect on tumor progression.

Intravenous inoculation of nude mice has been widely used to assess metastatic potential of cancer cells [38,39]. Using this model, knockdown of IQGAP3 in lung cancer cells was found to greatly impair its capacity to generate metastatic lesions in the lung. This result is consistent with our in vitro data which shows altered cell migration and invasion following manipulation of IQGAP3 expression. However, the specific details of these mechanisms remain unclear. Confocal microscopy revealed that IQGAP3 is enriched at the leading edge of migrating cells (Figure S1), suggesting its possible involvement in formation of leading edges. In addition, gene expression profiling showed an increased level of E-cadherin in IQGAP3 knockdown cells (Figure S2). It is well recognized that E-cadherin is very important for cell adhesion. Downregulation of it along with its associated catenin complex is concomitant with reduced intercellular adhesion and enhanced invasive capacity [47,48]. As such, IQGAP3 may facilitate cell migration and invasion partly through suppression of E-cadherin expression.

In summary, our studies reveal for the first time the involvement of IQGAP3 in lung cancer development and the potential molecular mechanisms underlying its tumor-promoting activity. These findings expand our knowledge of the complicated role of the IQGAP family in tumorigenesis. Further inquiry into the
potential of these molecules as targets for therapeutic invention is warranted.

Supporting Information

**Figure S1** IQGAP3 was enriched at the leading edge of migrating cells. A549 cells were placed on cover glass coated with 10 μg/ml fibronectin (Sigma–Aldrich). In order to leave a 6 mm-wide wet section with adhering cells, when the cells had adhered, the ends of the cover glass were wiped dry. After the cover glass was inverted and placed in the Zigmond chamber, serum-free RPMI 1640 containing 30μg/ml EGF was added to one side of the chamber and serum-free RPMI 1640 was added to the other side. The chamber was then incubated for 1 h at 37°C. Cells were fixed with 4% paraformaldehyde for 15 min at 37°C and were permeabilized for 5 min with 0.1% Triton X-100 (Sigma–Aldrich). Samples were blocked for 1 h with PBS containing 5% bovine serum albumin and probed with the anti-IQGAP3 antibody at 4°C overnight. After thorough washing, samples were incubated with secondary antibody and phalloidin (Sigma–Aldrich). Scale bar, 10 μm.

(TIF)

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