Overexpression of IQGAP1 promotes the angiogenesis of esophageal squamous cell carcinoma through the AKT and ERK-mediated VEGF-VEGFR2 signaling pathway

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Abstract. Angiogenesis is crucial for the progression of esophageal squamous cell carcinoma (ESCC). Anti-angiogenesis by targeting important molecules has been considered as one of the most promising and efficient strategy for cancer therapy. Recent studies have demonstrated that the IQ-domain GTPase activating protein 1 (IQGAP1) plays critical roles in tumorigenesis and cancer progression. We previously reported that IQGAP1 is overexpressed in ESCC, and IQGAP1 knockdown can decrease cell proliferation and metastasis ability in vitro and in vivo. However, the effects of IQGAP1 on the angiogenesis of ESCC and its underlying mechanisms remain unknown. In the present study, we found that IQGAP1 overexpression promoted tumor angiogenesis confirmed by human umbilical vascular endothelial cell (HUVEC) tube formation in vitro and chicken embryo chorioallantoic membrane (CAM) assay in vivo. Moreover, IQGAP1 overexpression in ESCC cells increased expression of vascular endothelial growth factor (VEGF) and phosphorylation of vascular endothelial growth factor receptor 2 (VEGFR2). Meanwhile, we found that levels of AKT and ERK phosphorylation were upregulated in IQGAP1-overexpressing cells. Importantly, IQGAP1-knockdown cells showed the opposing results. Furthermore, AKT and ERK inhibitors not only significantly decreased VEGF expression and VEGFR2 phosphorylation in IQGAP1-overexpressing cells, but also abolished the pro-angiogenic effect of IQGAP1 overexpression on angiogenesis in the HUVEC tube formation and chicken embryo CAM assay. Taken together, this evidence confirms that IQGAP1 overexpression promotes tumor angiogenesis via the AKT and ERK-mediated VEGF-VEGFR2 signaling pathway in ESCC, and IQGAP1 may be an attractive therapeutic target for cancer anti-angiogenesis treatment.

Introduction

Esophageal cancer is one of the most aggressive and fatal gastrointestinal tract malignancies worldwide. Esophageal squamous cell carcinoma (ESCC) is the predominant histological type of esophageal cancer in China (1). Although clinical diagnostic and multidisciplinary therapeutic progress has been made, the overall prognosis of ESCC patients is still unfavorable due to rapid progression and metastasis. To date, the underlying mechanisms involved in the initiation and progression of ESCC are not fully understood. Therefore, a thorough understanding of the molecular mechanisms underlying the carcinogenesis and progression of ESCC is vital for discovering novel targets and innovative treatment strategies.

Angiogenesis, the process leading to the formation of new blood vessels from preexisting ones, is one of the major hallmarks of cancer, and is involved in the progression and growth of cancer (2,3). Understanding the molecular mechanisms responsible for tumor angiogenesis can benefit cancer diagnosis and treatment. Thus, suppression of tumor angiogenesis offers a promising strategy for targeted therapy of cancer.

IQ-domain GTPase activating protein 1 (IQGAP1) is a member of a family of scaffolding proteins, which regulate distinct cellular processes including cell adhesion, proliferation, migration and other cellular functions through interacting with diverse proteins (4-6). Thus, IQGAP1 is as a critical integrator of cellular signaling pathways. Several studies have shown that IQGAP1 expression is increased in various cancer tissues, including colorectal carcinoma (7,8), breast (9), ovarian (10), lung (11), pancreatic (12) and thyroid cancer (13). Furthermore, high expression of IQGAP1 promotes invasion...
and metastasis, and exhibits a significant correlation with poor patient prognosis (7-13). We reported that IQGAP1 is highly overexpressed in ESCC and the knockdown of IQGAP1 by small interfering RNA (siRNA) can decrease cell proliferation and metastasis ability in vitro and in vivo (14), indicating that IQGAP1 is a potential target for cancer treatment. However, the role of IQGAP1 in the angiogenesis of ESCC is not yet known.

In the present study, we investigated the role of IQGAP1 in regulating the angiogenesis of ESCC and explored its underlying molecular mechanisms. We report that IQGAP1 overexpression promotes angiogenesis of ESCC by the AKT and ERK-mediated vascular endothelial growth factor (VEGF)-vascular endothelial growth factor receptor 2 (VEGFR2) signaling pathway. These findings suggest an essential role of IQGAP1 in the angiogenesis of ESCC and provide novel insight into IQGAP1 as an attractive therapeutic target for cancer anti-angiogenesis treatment.

Materials and methods

Cell culture and stable transfections. Human ESCC cell lines EC9706 and KYSE150 were purchased from the Tumor Cell Bank of the Chinese Academy of Medical Sciences (Beijing, China). Human umbilical vascular endothelial cells (HUVECs) were purchased from the CHI Scientific Inc. (Jiangsu, China). The cells were maintained in Gibco Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories; GE Healthcare Life Sciences, Logan, UT, USA), penicillin and streptomycin at 5% CO₂ and 37°C in a humidified incubator. The GFP-IQGAP1 overexpression and control plasmids were purchased from GeneCopoeia, Inc. (Rockville, MD, USA) and transfected into EC9706 cells. Non-specific control and IQGAP1 shRNA (short hairpin RNA) plasmids were purchased from Shanghai GeneChem Co., Ltd., (Shanghai, China) and transfected into KYSE150 cells. Transfection of the plasmids was carried out using Invitrogen Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Inc., USA). Mouse anti-IQGAP1 antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Rabbit anti-AKT (1:1,000; cat. no. 9272), rabbit anti-p-ERK1/2 (1:1,000; cat. no. 9101) and total rabbit anti-ERK1/2 (1:1,000; cat. no. 4695) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Tube formation assay. A total of 50 µl of chilled Matrigel (BD Biosciences) was added to a 96-well plate and incubated at 37°C for 30 min. HUVECs (1x10⁴) in 100 µl of conditioned medium were seeded onto each well and incubated at 37°C in 5% CO₂ for 6 h. Images were captured by phase-contrast microscopy and the tubular structures were quantified by manual counting in three random fields per well to obtain the sum.

Chicken embryo CAM assay. Seven-day-old chicken embryos were windowed to expose the CAM and the conditioned medium was placed onto the CAM. The windows were sealed with cellophane tape and embryos were transferred back into the incubator. After 3 days, chicken embryos were fixed with stationary solution (methanol:acetone 1:1) for 15 min, CAMs were cut and harvested, and then photographed. The ability of angiogenesis in chicken embryo CAM was quantitated by measuring the number of vessels.

Statistical analysis. Statistical analysis was conducted using the SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA). All experiments were performed in triplicate. Data are presented as the mean ± SD. The differences between groups were assessed by one-way ANOVA and followed up using Dunnett's multiple comparison post hoc test. *P<0.05 was considered to indicate a statistically significant result.

Results

Generation of stable IQGAP1-overexpressing and silenced clones in the ESCC cell lines. In order to obtain insight into the effect of IQGAP1 on tumor angiogenesis, we over-expressed IQGAP1 in human ESCC EC9706 cells. Western blot analysis results showed that stable clones transfected with the expression vector carrying cDNA for human full-length IQGAP1 (named as IQ-1 and IQ-2) expressed fusion protein milk. The membranes were incubated overnight at 4°C with corresponding primary antibodies. To normalize protein loading, mouse anti-β-actin antibody (1:5,000; cat. no. A5441; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used. After incubation with HRP-conjugated secondary antibody for 2 h at room temperature, target proteins on the membrane were visualized using an enhanced chemiluminescence (ECL) detection system (Beijing ComWin Biotech Co., Ltd, Beijing, China). The band intensity was analyzed using Bio-Rad's Image Lab software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Mouse anti-IQGAP1 antibody was purchased from BD Biosciences (1:5,000; cat. no. 610612; Franklin Lakes, NJ, USA). Rabbit anti-GFP (1:1,000; cat. no. D110008) and rabbit anti-VEGFR2 (1:500; cat. no. D151118) antibodies were purchased from Sangon Biotech Company (Shanghai, China). Rabbit anti-VEGF (1:500; cat. no. A12303) and rabbit anti-p-VEGFR2 (1:500; cat. no. AP0382) antibodies were purchased from ABeconal Biotechnology Co., Ltd. (Wuhan, China). Rabbit anti-p-AKT (1:1,000; cat. no. 4060), total rabbit anti-AKT (1:1,000; cat. no. 9272), rabbit anti-p-ERK1/2 (1:1,000; cat. no. 9101) and total rabbit anti-ERK1/2 (1:1,000; cat. no. 4695) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Western blot analysis. The proteins in the cell lysates were quantified using the Bradford method. Proteins (70 µg) were processed by electrophoretic separation on 10% SDS-PAGE and transferred to a nitrocellulose membrane, which was then blocked with PBS/Tween-20 containing 5% non-fat milk. The membranes were incubated overnight at 4°C with corresponding primary antibodies. To normalize protein loading, mouse anti-β-actin antibody (1:5,000; cat. no. A5441; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used. After incubation with HRP-conjugated secondary antibody for 2 h at room temperature, target proteins on the membrane were visualized using an enhanced chemiluminescence (ECL) detection system (Beijing ComWin Biotech Co., Ltd, Beijing, China). The band intensity was analyzed using Bio-Rad's Image Lab software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Mouse anti-IQGAP1 antibody was purchased from BD Biosciences (1:5,000; cat. no. 610612; Franklin Lakes, NJ, USA). Rabbit anti-GFP (1:1,000; cat. no. D110008) and rabbit anti-VEGFR2 (1:500; cat. no. D151118) antibodies were purchased from Sangon Biotech Company (Shanghai, China). Rabbit anti-VEGF (1:500; cat. no. A12303) and rabbit anti-p-VEGFR2 (1:500; cat. no. AP0382) antibodies were purchased from ABeconal Biotechnology Co., Ltd. (Wuhan, China). Rabbit anti-p-AKT (1:1,000; cat. no. 4060), total rabbit anti-AKT (1:1,000; cat. no. 9272), rabbit anti-p-ERK1/2 (1:1,000; cat. no. 9101) and total rabbit anti-ERK1/2 (1:1,000; cat. no. 4695) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

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Figure 1. The establishment of stable IQGAP1-overexpressing and -knockdown ESCC cell lines. (A) EC9706 cells were stably transfected with the control (Con) or pEGFP-IQGAP1 expressing vectors (IQ-1 and IQ-2), and clones expressing a transfected GFP-IQGAP1 protein were analyzed by western blot analysis. The histogram represents quantitative densitometry of the proteins. Data are presented as mean ± SD, n=3, *P<0.01, compared with the control cells. (B) KYSE150 cells were stably transfected with the control (NC) or IQGAP1-shRNA vectors (shIQ-1 and shIQ-2), and IQGAP1 protein expression in the control and IQGAP1-knockdown stable cell lines was assessed by western blot analysis. The histogram represents quantitative densitometry of proteins. Data are presented as mean ± SD, n=3, *P<0.01, compared with the control cells. IQGAP1, IQ-domain GTPase activating protein 1.

Figure 2. Effect of IQGAP1 on the tube formation of human umbilical vascular endothelial cells (HUVECs). (A) Representative results of HUVEC tube formation cultured on Matrigel-coated plates with conditioned media from the IQGAP1-overexpressing (IQ-1 and IQ-2) and control (Con) cells. (B) The number of tubular structures was counted in each group. Data are presented as mean ± SD, n=3, *P<0.01, compared with the control cells. (C) Representative results of HUVEC tube formation cultured on Matrigel-coated plates with conditioned media from the IQGAP1 knockdown (shIQ-1 and shIQ-2) and control (NC) cells. (D) The number of tubular structures was counted in each group. Data are presented as mean ± SD, n=3, *P<0.01, compared with the control cell. IQGAP1, IQ-domain GTPase activating protein 1.
of GFP-IQGAP1, which was not found in the control vector (named as Con) (Fig. 1A). To further evaluate the roles of IQGAP1 in tumor angiogenesis and the potential of IQGAP1 downregulation for ESCC therapy, IQGAP1 stable knockdown was performed in the human ESCC KYSE150 cell line. As shown in Fig. 1B, two stable clones (named as shIQ-1 and shIQ-2) exhibited efficiently reduced expression levels of IQGAP1 protein compared with the control cells (named as NC).

**IQGAP1 overexpression promotes tube formation of HUVECs.** The tube formation assay can mimic certain stages of angiogenesis, which is a well-established in vitro angiogenesis test (15,16). To assess the functional role of IQGAP1 in angiogenesis in vitro, we investigated whether IQGAP1 is involved in capillary tube formation. As shown in Fig. 2A, the HUVECs spontaneously formed capillary-like tube structures after 6 h of incubation on Matrigel. The conditioned medium from IQGAP1 overexpressing cells increased the number of capillary-like structures. Quantification of the number of tubular structure showed that IQGAP1 overexpression resulted in a 5-to 6-fold increasing in tube formation by HUVECs (Fig. 2B). In contrast, IQGAP1 knockdown resulted in less elongated, broken and foreshortened tubes compared to the control shRNA-transfected cells (Fig. 2C). An approximate 6-to 7-fold decrease in tube formation was observed in the IQGAP1 shRNA-transfected cells (Fig. 2D). As a consequence, this finding confirmed that IQGAP1 functions as a promoter of tumor angiogenesis in vitro.

**IQGAP1 overexpression stimulates angiogenesis in the chicken embryo CAM assay.** Chicken embryo CAM assay is a well-known model of angiogenesis that can be widely used to investigate new vessel formation and inhibition in vivo (17). To further evaluate the potential effect of IQGAP1 on angiogenesis, chicken embryo CAM assay was employed. The results showed that IQGAP1 overexpression induced a stronger proangiogenic response in a chicken embryo CAM assay than the control (Fig. 3A). The number of branches of microvessels in the conditioned medium from the IQGAP1-overexpressing cells increased to 2.5-to 3-fold of the control (Fig. 3B). Conversely, IQGAP1 knockdown inhibited angiogenesis (Fig. 3C). Quantitative analysis revealed that conditioned medium from the IQGAP1-knockdown cells caused a 3.5-to 4-fold reduction in the number of blood vessels (Fig. 3D). These results further confirm that IQGAP1 overexpression induces tumor angiogenesis in vivo.
IQGAP1 overexpression enhances expression of VEGF and activation of VEGFR2. Considering that angiogenic factor VEGF is a prime regulator of angiogenesis (18), we first examined the expression of VEGF in IQGAP1-overexpressing and -silenced ESCC cells. As shown in Fig. 4A and B, IQGAP1 overexpression upregulated the expression levels of
VEGF, whereas VEGF expression was obviously decreased in the IQGAP1-silenced. VEGFR2 is the most biologically important receptor for VEGF (19). Thus, we subsequently examined VEGFR2 expression and activation in the IQGAP1-overexpressing and silenced ESCC cells. IQGAP1 overexpression significantly enhanced phosphorylation of VEGFR2, without obviously affecting overall VEGFR2 expression levels. Conversely, IQGAP1 knockdown inhibited the phosphorylation of VEGFR2, while the total levels of VEGFR2 had little change (Fig. 4A and B). These results clearly demonstrate that IQGAP1 overexpression can promote tumor angiogenesis by upregulating VEGF-VEGFR2 signaling.

**Figure 6.** IQGAP1 regulates expression of VEGF and phosphorylated (p)-VEGFR2 as well as angiogenesis through AKT and ERK signaling. (A) AKT inhibitor (LY294002) or ERK inhibitor (PD98059) (20 µM) was used to treat IQGAP1-overexpressing cells (IQ-1) for 48 h, and abrogated the effects of IQGAP1 overexpression-mediated upregulation of VEGF and p-VEGFR2. (B) The histogram represents quantitative densitometry of proteins. Data are presented as mean ± SD, n=3, *P<0.01, compared with the control cells (DMSO). (C) The promoting effect of IQGAP1 overexpression on HUVEC tube formation was attenuated by the AKT or ERK inhibitor. (D) The number of tubular structures was counted in each group. Data are presented as mean ± SD, n=3, *P<0.01, compared with the control cells (DMSO). (E) The pro-angiogenic function of IQGAP1 overexpression in chicken embryo CAM was abrogated when IQGAP1 overexpression cells were treated with AKT or ERK inhibitor. (F) The number of vessels in each group was counted. Data are presented as mean ± SD, n=3, *P<0.01, compared with the control cells (DMSO). IQGAP1, IQ-domain GTPase activating protein 1; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

IQGAP1 overexpression promotes tumor angiogenesis through AKT and ERK activation. To identify the potential molecular mechanisms of IQGAP1 in tumor angiogenesis, we analyzed the expression levels of various signaling proteins by western blot assay. The results showed that IQGAP1 overexpression markedly increased the levels of p-AKT and p-ERK, whereas the levels of total AKT and ERK were not altered (Fig. 5A). Silencing of IQGAP1 expression led to a significant decrease in the expression of p-AKT and p-ERK proteins, and had no effect on total AKT and ERK protein expression (Fig. 5B). To determine whether the IQGAP1-mediated increase in VEGF and p-VEGFR2 expression as well as tumor angiogenesis is mediated by regulating AKT or ERK signaling, we analyzed
the effect of specific AKT and ERK inhibitors (LY294002 or PD98059) on IQGAP1-overexpressing cells. As shown in Fig. 6A and B, the AKT and ERK inhibitor abolished the role of IQGAP1 overexpression on VEGF and p-VEGFR2 upregulation. Furthermore, we found that LY294002 or PD98059 could abrogate the effects of IQGAP1-mediated tumor angiogenesis by in vitro tube formation of HUVECs (Fig. 6C and D) and in vivo chicken embryo CAM assay (Fig. 6E and F). Taken together, these observations demonstrate that IQGAP1 overexpression promotes tumor angiogenesis by targeting the AKT and ERK-mediated VEGF-VEGFR2 signaling pathway.

Discussion

Esophageal squamous cell carcinoma (ESCC) is one of the leading causes of cancer-related death due to the high incidence of advanced disease, metastasis, and resistance to radiotherapy and chemotherapy (1). Thus, it is urgent to identify novel targets and new strategies to treat this disease. Angiogenesis plays a significant role in the continuous growth of tumors, invasion and metastasis as capillary formation in tumors can provide nutrients and oxygen to supply the growing tumor and also act as conduits for the metastasis of tumors (2,3,19). Consequently, more and more attention has been focused on tumor angiogenesis; and thus, anti-angiogenic therapy has become one of the most promising and efficient strategy for inhibiting tumor growth and progression.

The development of ESCC involves the accumulation of the abnormal expression of oncogenes involved in the initiation and progression of ESCC. IQGAP1 is a member of the IQGAP family of multidomain proteins (6,20). Cumulative evidence suggests that IQGAP1 is an oncogene and is overexpressed in several types of human cancers (4,21). Consistent with these findings, we reported that IQGAP1 is upregulated in ESCC tissues and is correlated with the invasive depth of ESCC tumors (14). However, it has not yet been elucidated whether IQGAP1 is involved in tumor angiogenesis in ESCC during which IQGAP1 is upregulated. In the present study, we found that IQGAP1 overexpression significantly increased the angiogenesis confirmed by HUVEC tube formation assay in vitro and chicken embryo CAM assay in vivo, whereas the angiogenesis ability was markedly suppressed when IQGAP1 expression was silenced. These results indicate that IQGAP1 is an attractive molecule for targeting tumor angiogenesis against cancer progression.

Angiogenesis is a complex multistep process which is regulated by several endogenous angiogenic activators and inhibitors (2,3,22). Of the numerous endogenous pro-angiogenic factors, VEGF is well known as a key regulator of the process of tumor angiogenesis by stimulating endothelial cell proliferation, migration and invasion. VEGF exerts its biological effects by binding to specific tyrosine kinase receptors on the cell surface, called VEGF receptors (VEGFRs), and VEGFR2 is the major mediator of VEGF-induced angiogenesis. The binding of VEGF to VEGFR2 leads to the intrinsic tyrosine kinase activation of the receptors followed by dimerization and autophosphorylation of VEGFR2, and then triggers a downstream signaling cascade (19,23,24). Therefore, we hypothesized that IQGAP1 regulates ESCC angiogenesis by regulating the VEGF-VEGFR2 pathway. In the present study, we observed that overexpression of IQGAP1 strongly increased VEGF expression and phosphorylation of VEGFR2, while knockdown of IQGAP1 obviously decreased VEGF and p-VEGFR2 expression. These findings showed that IQGAP1 could regulate tumor angiogenesis by controlling the activation of VEGF-VEGFR2 signaling. It has been reported that IQGAP1 can directly bind to VEGFR2 and also is necessary for VEGF to stimulate angiogenesis in MCF-7 and HUVECs (9,25-27), which is consistent with our findings. Considering that the VEGF-VEGFR signaling pathway is a significant factor underlying angiogenesis, numerous therapeutic strategies have been developed to target angiogenesis by blocking this pathway. Accordingly, this study further indicates that targeting IQGAP1 represents a promising therapeutic strategy for tumor angiogenesis.

AKT and ERK are serine/threonine kinases that are critical for many diverse processes, including cell proliferation, apoptosis, migration, angiogenesis and metastasis (28-31). IQGAP1, as a scaffold protein, contains multiple domains which mediate binding to a number of proteins. It has been reported that IQGAP1 can combine with AKT and ERK and regulate their activity (6,21,32-35). To explore the potential pro-angiogenic mechanisms of IQGAP1 in ESCC, we detected the expression of the AKT and ERK signaling pathway. The data showed that IQGAP1 overexpression could increase phosphorylation of AKT and ERK. Moreover, IQGAP1 knockdown could inhibit AKT and ERK activation. Furthermore, we observed that AKT and ERK inhibitors significantly decreased VEGF expression and VEGFR2 phosphorylation in IQGAP1-overexpressing cells. Moreover, the pro-angiogenic effect of IQGAP1 overexpression on angiogenesis in tube formation of HUVECs and a chick embryo CAM angiogenesis model was abrogated when IQGAP1-overexpressing cells were treated with the AKT and ERK inhibitor. These findings suggest that IQGAP1 promotes tumor angiogenesis mainly via AKT or ERK/VEGFR-VEGFR2 signaling pathway.

In summary, we demonstrated for the first time that IQGAP1 overexpression could promote angiogenesis in ESCC by targeting the AKT or ERK/VEGFR-VEGFR2 signaling pathway. Moreover, silencing of the expression of IQGAP1 inhibited tumor angiogenesis. Our studies not only demonstrated that IQGAP1 regulated the tumor angiogenesis of ESCC, but also revealed a therapeutic opportunity in targeting IQGAP1 for cancer treatment.

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Availability of data and material

All data generated or analyzed during this study are included in this published article.
Authors' contributions

CHL and XJS carried out the experiments and interpreted the data. SSN, CYY, YPH and JTK participated in the collection of the data. XXW and XZL designed the research, supervised the study, interpreted data and wrote the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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