Overexpression of TRIB3 promotes angiogenesis in human gastric cancer

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Abstract. Tribbles homolog 3 (TRIB3) plays important roles in many types of malignancies. However, whether TRIB3 is involved in the development or progression of gastric cancer (GC) remains unclear. In this study, we analyzed TRIB3 expression in GC tissues from 191 GC patients categorized with stage I to IV disease, to examine the role of TRIB3 in GC, and examined the relationship between TRIB3 and tumor angiogenesis. We found that TRIB3 expression was significantly higher in GC tissues than in adjacent non-tumor tissues. TRIB3 expression was associated with VEGF-A and tumor microvessel density, as well as overall TNM stage, T stage, N stage, and distant metastasis in GC tissues. Furthermore, TRIB3 silencing downregulated VEGF-A expression in GC cells, which subsequently suppressed endothelial cell recruitment and vessel formation. In conclusion, overexpression of TRIB3 is associated with tumor angiogenesis and a poor prognosis in patients with GC. Our findings indicate that TRIB3 is a promising target for anti-angiogenic therapy in GC.

Introduction

Gastric cancer (GC) is one of the most common malignant tumors, and is responsible for many cancer-related deaths worldwide (1,2). Although several treatments for GC including excision surgery, radiotherapy and chemotherapy have been developed recently, the clinical outcome continues to be poor in patients with advanced GC (1). The mortality of GC is intrinsically related to metastasis, in which angiogenesis plays a crucial role (3). Angiogenesis is also indispensable to the continuous growth of the tumor. Therefore, one of the most promising yet challenging therapeutic approaches to cure GC is to develop safe, effective, and affordable anti-angiogenic therapies.

Tribbles homolog 3 (TRIB3, also named TRB3, NIPK, and SKIP3) belongs to the tribbles family of pseudokinases that were first identified in Drosophila to regulate cell division and migration (4-6). TRIB3 also participates in the activation of multiple signaling pathways, such as mitogen-activated protein kinase (MAPK) pathways (7-9). TRIB3 expression is upregulated by endoplasmic reticulum stress, hypoxia, and nutrient starvation (6,10,11). Recent studies suggest that TRIB3 is a potential oncogene, as evidenced by its elevated expression in colorectal cancer (12), breast cancer (13), liver cancer and other cancer tissues (14). Furthermore, TRIB3 is associated with an adverse prognosis in these cancers. However, whether TRIB3 is involved in the development and progression of GC has not been reported.

Previous studies have shown that TRIB3 expression is closely related to the progression of type 2 diabetes mellitus, and that TRIB3 mediated apoptosis in islet β cells, as well as insulin resistance. Together, these effects impair insulin-stimulated glucose uptake and maintain hyperglycemia in diabetes (15-17). Chronic hyperglycemia is a major initiator of diabetic angiopathy (18). Because impaired angiogenesis is a key pathological characteristic of diabetic microangiopathy (19), and it is well established that some inducers of TRIB3, such as endoplasmic reticulum stress, hypoxia, and glucose deprivation can also induce angiogenesis, we propose that the expression of TRIB3 is related to angiogenesis.

The present study analyzed TRIB3 expression in GC tissues from 191 GC patients categorized from stage I to IV, to examine the role of TRIB3 in GC. The study also examined the relationship between TRIB3 and tumor angiogenesis. We found that TRIB3 expression was elevated in GC tissues, and that TRIB3 overexpression is correlated to the severity and poor prognosis in GC. We also showed that TRIB3 suppression downregulated the expression of VEGF-A in GC cells, which subsequently suppressed the recruitment of endothelial cells.

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cells and formation of vessels. Thus, TRIB3 may be a promising target for anti-angiogenic therapy in GC.

Materials and methods

Patients and tumor tissue samples. Our study was approved by the Ethics Committee of Nanfang Hospital, the Southern Medical University. All tissues for diagnostic purposes were obtained with the consent of each patient diagnosed with primary GC and receiving resection surgery between 2004 and 2008. The clinical stages of the tumors were defined according to the National Comprehensive Cancer Network Guidelines (http://www.nccn.org/; version 1.2014, Gastric Cancer). No patient received any pre-operative chemotherapy or radiotherapy. A total of 191 tissue samples were used in this study.

Cells and cell culture. The human gastric epithelial cell lineGES-1, and GC cell lines BGC803, BGC823, MGC803, MKN28, MKN45, and SGC7901 were obtained from Foleibao Biotechnology Development Company (Shanghai, China). Human umbilical vein endothelial cells (HUVECs) were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). All cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) with 10% fetal bovine serum at 37°C and with 5% CO₂.

Immunohistochemistry. Immunohistochemical staining was used to evaluate the expression of TRIB3, VEGF-A, and CD31, as described previously (20). The primary antibodies used in this study were polyclonal rabbit antibodies for TRIB3 (Abcam, San Francisco, CA, USA), VEGF-A, and CD31 (AbClonal, Boston, MA, USA). The HRP-conjugated anti-rabbit secondary antibody was from CWBIO (Beijing, China). Specifically bound antibodies were detected with a 3,3’-diaminobenzidine staining kit (CWBIO). The percentage of positive cells was calculated and categorized as follows: 0 (0% of cells stained), 1 (1-25%), 2 (26-50%), 3 (51-75%), or 4 (76-100%). Staining intensity was visually scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). The final score (0-12) for the expression of TRIB3 and VEGF-A was calculated as the product of the percentage and the intensity scores for each case.

Tumor microvessel density (MVD) was determined by counting the number of endothelial cells positively stained for CD31, following the method described by Weidner et al (21). Slides were scanned initially at a low power (x100 magnification) in order to identify areas with higher vascular density (hot spots). Subsequently, counting of the stained microvessels was performed on four consecutive high power (x400 magnification) fields within the selected high density fields. Yellow-brown immunostained endothelial cells or an endothelial cell cluster that was clearly separated from adjacent microvessels were counted as vascular structures. The average number of microvessels counted in four 400x fields provided the MVD value for each case.

Gene silencing with siRNAs. Three siRNA sequences against TRIB3 were purchased from RiboBio (Guangzhou, China). Transfection was carried out using Lipofectamine® 2000 transfection reagent from Invitrogen (Carlsbad, CA, USA) following the manufacturer’s recommended protocol. A negative control siRNA was used to examine the effect of transfection alone.

Western blotting. Proteins from the tissues and cells were subjected to western blotting as described previously (20). Primary antibodies were polyclonal rabbit antibodies for TRIB3 (Abcam), VEGF-A (AbClonal), and α-actin (Proteintech, Wuhan, China). A secondary fluorescent goat anti-rabbit antibody (LI-COR, Lincoln, NE, USA) was used in this study. The Odyssey imaging system (LI-COR) was used to scan the blots.

Quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA of the cultured cells was extracted using a TRIzol kit (Takara Bio, Inc., Shiga, Japan) according to the manufacturer’s protocols. The First Strand cDNA synthesis kit (Takara Bio, Inc.) was used to synthesize cDNAs. qRT-PCR was performed using the SYBR-Green dye (Roche, Mannheim, Germany). The PCR primers used in this study were: 5’-ATTAGGCAGGGTGCTCCTGTA-3’ (TRIB3, sense), 5’-AGTATGGACCTGGGATTGTGG-3’ (antisense); 5’-CTTGCCTTGCTCCTAC-3’ (VEGF-A, sense), 5’-CACAGGAATGGGCTTGAAG-3’ (antisense); 5’-TTCATTGACCTC AACATACTG-3’ (GAPDH, sense), 5’-GTGGCAGTGATG GCATGGAC-3’ (antisense).

Enzyme-linked immunosorbent assay (ELISA). GC cells (3x10⁵/well) were seeded in 24-well plates and TRIB3 siRNA transfection was performed. The culture medium was collected 48 h after transfection and secretion of VEGF-A was determined using human VEGF-A ELISA kits (Baomanbio, Shanghai, China) according to the manufacturer’s instructions. The results are presented as mean ± standard deviation from three independent experiments.

HUVEC migration assay. GC cells (3x10⁴/well) were seeded in 24-well plates and TRIB3 siRNA transfection was performed. After culturing at 37°C for 48 h, a chamber with a porous (8.0 mm pore size) polycarbonate membrane filter (Millipore Corp., Bedford, MA, USA) containing 4x10⁴ HUVECs in 0.2 ml RPMI-1640 medium without fetal bovine serum, was inserted into each well. HUVECs were fixed with 4% paraformaldehyde after co-culture for 12 h at 37°C, and subsequently stained with 0.1% crystal violet for 30 min. The results were observed under an inverted microscope.

Scratch wound healing assay. GC cells (3x10⁴/well) were seeded in 6-well plates and TRIB3 siRNA transfection was performed. The culture medium was collected 48 h after transfection. Trypsinized HUVECs were seeded in 6-well plates at a density of 3x10⁵ cells per well. After reaching confluence, cultured cells were scratched with a sterile 200-µl pipette tip. The cells were then cultured in the GC cell-conditioned medium. Wound closure was observed at 0, 24, and 48 h under an inverted microscope.

Tubule formation by HUVECs. HUVECs mixed with 100 µl GC cell-conditioned medium were seeded at a density...
of 2x10^4 cells per well in 96-well plates containing 60 µl solidified Matrigel (BD Biosciences, New York, NY, USA) and cultured for 6-8 h at 37°C. Cultures were photographed under a microscope. The number of tubules was counted in three individual wells and presented as the mean ± standard deviation.
**Statistical analyses.** Statistical calculations were performed using SPSS 13.0 software (IBM, Chicago, IL, USA). Survival analysis was performed according to the Kaplan-Meier method. Differences of survival between groups were assessed with the log-rank test. The relationship between TRIB3 and MVD was evaluated by linear regression analysis. Student's t-test was performed to determine significant differences between two experimental groups. One-way ANOVA analysis was used to evaluate the statistical significance among multiple groups. *P*<0.05 was considered to indicate a statistically significant result.

**Results**

**TRIB3 expression is elevated in advanced GC and associated with a poor prognosis.** In order to detect the expression of TRIB3, immunohistochemical staining was performed in 27 normal gastric tissues and 191 GC tissues. Patients were divided into two groups, low (score 0-5) and high (score >5) expression levels, according to the TRIB3 staining score. TRIB3 was markedly upregulated in GC compared with normal gastric tissues. High expression levels of TRIB3 were observed in 61.8% (118/191) of the GC tissues. This was significantly >7.4% (2/27) seen in normal gastric tissues. High expression levels of TRIB3, immunohistochemical staining was performed in 27 normal gastric tissues and 191 GC tissues. Patients were divided into two groups, low (score 0-5) and high (score >5) expression levels, according to the TRIB3 staining score. TRIB3 was markedly upregulated in GC compared with normal gastric tissues. High expression levels of TRIB3 were observed in 61.8% (118/191) of the GC tissues. This was significantly >7.4% (2/27) seen in normal gastric tissues. Representative staining examples of normal gastric and GC tissues are shown in Fig. 1A.

The correlation analysis between pathological characteristics and expression levels of TRIB3 showed that high expression of TRIB3 was more frequent in patients with more advanced overall TNM stage (P=0.001, Fig. 1B), T stage (P=0.029, Fig. 1C), N stage (P=0.006, Fig. 1D), and distant metastasis (P=0.006, Fig. 1E). However, no statistical significance was found with age and gender (P=0.339 and 0.578, respectively, Table I).

Kaplan-Meier curves for overall survival duration of GC patients, according to TRIB3 expression levels using data from the TCGA database, showed that GC patients who had higher levels of TRIB3 expression exhibited a worse prognosis (log-rank =4.462, *P*=0.0347, Fig. 1F). Similar results were observed in the present study where high expression levels of TRIB3 were significantly correlated with short time to recurrence among patients in stages I-III (log-rank =20.20, *P*<0.0001, Fig. 1G), and short survival time among patients in stage IV (log-rank =6.205, *P*=0.0127, Fig. 1H).

**Overexpression of TRIB3 is associated with tumor angiogenesis in GC.** To clarify the potential relationship between TRIB3 and angiogenesis, a total of 91 GC tissues were immunostained for TRIB3 and angiogenesis-related markers. VEGF-A is an important hallmark of angiogenesis, while microvessels were identified by CD31 staining. Representative staining examples of low and high expression of TRIB3, VEGF-A, and CD31 in GC are shown in Fig. 2A. Tumors with high expression levels of TRIB3 had a significantly higher MVD than those with low expression levels (18.2±1.1 vs. 13.1±1.0, *t*=3.261, *P*=0.0016, Fig. 2B). Similar results were observed in the groups where both TRIB3 and VEGF-A expression were elevated (22.4±1.4 vs. 12.3±0.82, *t*=6.460, *P*<0.0001, Fig. 2B). Using data from the TCGA database, linear regression analysis revealed that TRIB3 and VEGF-A expressions were significantly correlated (r=0.416, *P*<0.01, Fig. 2C). Similarly, a significant positive correlation between the expression of TRIB3 and VEGF-A was found in the present study (r=0.468, *P*<0.01, Fig. 2D). TRIB3 positivity was also significantly correlated with MVD by linear regression analysis (r=0.346, *P*<0.01, Fig. 2E).

Using Kaplan-Meier analysis with the log-rank test, we found that high expression of VEGF-A was significantly correlated with short time to recurrence among GC patients in stages I-III (log-rank =5.776, *P*=0.0162, Fig. 2F). Similar results were observed in patients expressing high levels of VEGF-A combined with high levels of TRIB3 (log-rank =14.61, *P*<0.0001, Fig. 2G). In addition, high expression of VEGF-A was associated with short survival time among stage IV GC patients (log-rank =10.77, *P*=0.001, Fig. 2H). Similar results were observed in patients with high levels of both VEGF-A and TRIB3 (log-rank =5.025, *P*=0.025, Fig. 2I).

**Expression of TRIB3 and VEGF-A in GC surgical samples and GC cell lines.** To confirm the elevated expression of TRIB3 and VEGF-A in GC tissues, fresh surgically resected
specimens were analyzed by western blotting. Both TRIB3 and VEGF-A were expressed at higher levels in tumor tissues than in the adjacent non-tumorous gastric tissues (Fig. 3A). To better elucidate the expression profiles of TRIB3 and VEGF-A, we examined their mRNA and protein expression levels in a human gastric epithelial cell line (GES-1) and six GC
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cell lines (BGC803, BGC823, MGC803, MKN28, MKN45, and SGC7901). Except for MKN28 cells, the expression of TRIB3 mRNA was higher in GC (3- to 7-fold) than in GES-1 cells (Fig. 3B). Interestingly, a similar trend was observed in the expression of VEGF-A mRNA (Fig. 3B). The expression of TRIB3 and VEGF-A proteins were consistent with the mRNA results (Fig. 3C).

TRIB3 silencing downregulates the expression of VEGF-A in GC cells. The ability of three different TRIB3-siRNA
sequences to downregulate TRIB3 was determined by qRT-PCR. Only siTRIB3-#3 consistently suppressed the expression of TRIB3 mRNA in the BGC823 and MKN45 cells (Fig. 4A). Interestingly, the expression of VEGF-A mRNA was also downregulated by TRIB3-silencing siRNAs (P=0.0005 and P<0.0001, respectively, Fig. 4B). Both TRIB3 and VEGF-A protein levels were significantly reduced by TRIB3-#3-siRNA in the BGC823 and MKN45 cells (P=0.0002 and 0.0062, respectively, Fig. 4C) and MKN45 (P=0.0036 and 0.0006, respectively, Fig. 4C).

Figure 4. TRIB3 silencing downregulates the expression of VEGF-A in gastric cancer cell lines. (A) The ability of three different siRNA sequences against TRIB3 to downregulate this gene in the BGC823 and MKN45 cells was determined by qRT-PCR. (B) VEGF-A mRNA was downregulated in the BGC823 and MKN45 cells with TRIB3-silencing siRNAs. (C) VEGF-A protein expression was downregulated by TRIB3-silencing siRNAs in the BGC823 and MKN45 cells. (D) ELISA analyses of VEGF-A secreted protein levels using conditioned media derived from GC cells transfected with the negative control (NC) or TRIB3 siRNA. *P<0.05, **P<0.01 compared with the control group.
ELISA analyses showed that secreted VEGF-A protein was also decreased in the TRIB3-knockdown GC cells, when compared with control cells (P=0.0279 and 0.0329 in BGC823 and MKN45 cells, respectively, Fig. 4D). These data showed that TRIB3 silencing contributed to the downregulation of VEGF-A mRNA and protein expression in the GC cells.

TRIB3 silencing in GC cells compromises their ability to recruit endothelial cells via soluble factors. Angiogenesis
is dependent on the proliferation and migration of vascular endothelial cells. To examine whether TRIB3 silencing influenced angiogenesis, we evaluated the responses of HUVECs in terms of migration and tubule formation. The migration capacity of HUVECs was altered after co-culturing with GC cell-conditioned media as shown by both the migration and scratch wound healing assays (Fig. 5A and B). Silencing of TRIB3 in the GC cells significantly impaired their ability to recruit HUVECs. Significant differences in the tubule-like structure formation capacity of HUVECs were also observed between the control siRNA and TRIB3-3-siRNA groups (Fig. 5C).

Discussion

The purpose of the present study was to investigate the oncological significance of TRIB3 in GC. We evaluated the expression of TRIB3 in patients with GC in order to determine correlations with pathological variables, including TNM stage, survival time of patients, and incidence of cancer recurrence, and to examine the relationship with angiogenesis.

Tribbles, first identified in *Drosophila*, are members of the pseudokinase family of proteins with no associated kinase activity. Instead of direct phosphorylation of target proteins, tribbles act as adaptor or scaffold proteins, as well as decoy kinases, that impede the function of other kinases through obstructive binding (22,23). Currently, three mammalian homologs of the tribbles gene are known. One of these, TRIB3, plays an important role in multiple signaling pathways, coordinating crucial cellular processes such as apoptosis, glucose and lipid metabolism, adipocyte differentiation, and cell stress (15,24-27). TRIB3 is overexpressed in many cancers, but whether TRIB3 is upregulated in GC has not been reported previously.

To the best of our knowledge, this is the first study to evaluate the expression of TRIB3 in GC patients and GC cell lines, and examine the role of TRIB3 in tumor angiogenesis. Our results showed that TRIB3 was significantly upregulated in GC tissues compared with its level in adjacent non-tumor tissues, and that high expression levels of TRIB3 were more frequent in patients at a more advanced overall TNM stage, T stage, N stage, and with distant metastasis. High expression levels of TRIB3 predicted high cancer recurrence and mortality for GC patients. At the cellular level, TRIB3 was constitutively expressed at higher levels in all GC cell lines tested, except MKN28, when compared to a gastric epithelial cell line. These data show the potential utility of TRIB3 as a prognostic marker for GC.

In colorectal cancer, TRIB3 is a prognostic marker and has functional relevance to cell growth (12). In breast cancer, TRIB3 is involved in the ability of cancer cells to survive in hypoxic conditions and is associated with a poor prognosis (13). Similar relationships were noted in other types of cancer (14,28,29). Knockdown of TRIB3 in tumor cells significantly inhibited the invasive and metastatic ability of the cells by promoting mesenchymal-epithelial transition (30). The finding of elevated TRIB3 expression in different malignant tumors supports the clinical results of our study, implying that TRIB3 may be overexpressed ubiquitously in tumors and play an important role in promoting tumor progression.

The role of TRIB3 in multiple signaling pathways, such as the MAPK pathway, combined with its role in hypoxia, metabolism, and cell stress processes, originally spurred us to investigate its role in angiogenesis. Because angiogenesis is dependent on the proliferation and migration of vascular endothelial cells, where VEGF-A plays an extremely important role, specific studies were conducted to identify the role of TRIB3 in angiogenesis. Firstly, immunohistochemical staining of 91 GC tissues showed a significant positive correlation between the expression of TRIB3 and VEGF-A. In addition, tumors with high expression levels of TRIB3 had a higher MVD than those with low expression levels of TRIB3. At the cellular level, the expression of TRIB3 and VEGF-A were, with only one exception, consistent in a human gastric epithelial cell line and GC cell lines, suggesting that there is a correlation between the expression levels of the two genes. Both mRNA and protein expression levels of VEGF-A were significantly reduced by TRIB3-siRNA in GC cells. These data strongly suggested that TRIB3 silencing downregulated the expression of VEGF-A in GC cells. Further studies showed that TRIB3 silencing in GC cells significantly impaired their ability to recruit endothelial cells via soluble factors, implying that TRIB3 silencing may downregulate angiogenesis *in vitro*.

Overall, tumor angiogenesis is a complex process. Whether TRIB3 directly or indirectly modulates VEGF-A is still unclear and requires further investigation.

In summary, our study shows that: i) the expression of TRIB3 is significantly upregulated in GC; ii) high expression levels of TRIB3 are associated with a shorter survival time and a higher incidence of cancer recurrence and metastasis; and iii) TRIB3 silencing contributes to the downregulation of VEGF-A expression and angiogenesis in GC. Taken together, our data suggest that suppression of TRIB3 may be a novel anti-angiogenic approach for GC treatment.

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