Name of Journal: World Journal of Gastroenterology

Manuscript NO: 90852

Manuscript Type: ORIGINAL ARTICLE

Basic Study

Kinesin 26B is related to immune infiltration in gastric cancer-spotlight on cancer-associated fibroblasts crosslinking with M2-polarized macrophage to regulate the immunosuppression and metastasis

Huang LM et al Kinesin 26B is related to immune infiltration in gastric cancer

Lianmeng Huang, Mingjin Zhang
Abstract

BACKGROUND
The regulatory effects of KIF26B on gastric cancer (GC) have been confirmed, but the specific mechanism still needs further explorations. Pan-cancer analysis shows that the KIF26B expression is highly related to immune infiltration of cancer associated fibroblasts (CAFs), and CAFs promote macrophage M2 polarization and affect cancers’ progression.

AIM
In this study, we hypothesized that KIF26B may regulate immune and metastasis of GC by influencing CAFs and related immune infiltration.

METHODS
We analyzed genes’ mRNA levels by qRT-PCR. Expression levels of target proteins were detected by immunohistochemistry, ELISA, and Western blotting. We injected AGS cells into nude mice for the establishment of a xenograft tumor model and observed the occurrence and metastasis of GC. The degree of inflammatory infiltration in pulmonary nodes was observed through hematoxylin-eosin (HE) staining. Additionally, transwell and wound healing assays were performed for the evaluation of cell invasion and migration ability. Tube formation assay was used for detecting angiogenesis. M2-polarized macrophages were estimated by immunofluorescence and flow cytometry.

RESULTS
KIF26B was significantly overexpressed in cells and tissues of GC, and the higher expression of KIF26B was related to GC metastasis and prognosis. According to in vivo experiments, KIF26B promoted tumor formation and metastasis of GC. In addition, KIF26B expression is positively associated with CAFs‘ degree of infiltration. Moreover,
CAFs could regulate M2 type polarization of macrophages, affecting GC cells’ migration, angiogenesis, invasion, and EMT process.

CONCLUSION
KIF26B regulated M2 polarization of macrophage through activating CAFs, thereby regulating the occurrence and metastasis of GC.

**Key Words:** KIF26B; Gastric cancer; M2 polarization macrophage; Tumor-associated fibroblasts

Huang L, Zhang M. Kinesin 26B is related to immune infiltration in gastric cancer-spotlight on cancer-associated fibroblasts crosslinking with M2-polarized macrophage to regulate the immunosuppression and metastasis. *World J Gastroenterol* 2024; In press

**Core Tip:** KIF26B could promote CAFs activation, thereby mediating macrophage M2 polarization and affecting the occurrence, lung metastasis, and abdominal metastasis of GC. This study provides useful insights for exploring new mechanisms of GC and suppressing its progression.

**INTRODUCTION**

The KIFs (kinesin superfamily proteins), known as molecular motor proteins, were dependent on microtubule or ATP and was used to transport membrane organelle [1-3]. KIFs have been identified as potential molecular targets in cancer treatment [4-6]. This study was based on the KIFs and conducts bioinformatics analysis to screen out the most likely potential gene (KIF26B) involved in gastric cancer (GC) progression and prognosis.

KIFs have been confirmed to participate into the regulation of multiple cancers. For example, silencing of KIF15 reduce cell proliferation and enhance cell apoptosis to retard the progression of osteosarcoma [7]. Moreover, KIF11 aggravates the progression
of breast cancer and results into poor prognosis [8]. KIF20B strengthens tumorigenesis in
tongue cancer [9]. Besides, suppression of KIF15 weakens cell proliferation to inhibit the
progression of triple-negative breast cancer [10]. In existing studies, it has confirmed the
role of the KIFs in GC, such as KIF15 promotes GC progression by promoting
proliferation and inhibiting apoptosis [11], and KIF2A downregulation inhibits GC cell
invasion by inhibiting MT1-MMP [12]. However, little research has focused on the
regulation of GC progression by KIF26B, and currently only one study from
2017 suggests that KIF26B promotes GC cell proliferation and metastasis through VEGF
pathway activation [13].

In recent years, many literatures have verified that TME (tumor microenvironment)
promotes tumor occurrence, progression, and metastasis [14–16]. One of the main
functions of TME is to stimulate the immunosuppressive environment around tumors
through various mechanisms [17, 18]. CAFs, cancer associated fibroblasts, are the key cells
in TME [18, 19]. According to pan-cancer analysis, KIF26B expression is highly associated
with CAFs immune infiltration [20].

Macrophages interact with tumor cells in the TME and cause tumor progression [21].
M1 polarization of macrophage is characterized by the increased pro-inflammatory
activity, the enhanced antigen presentation, and the inhibition of tumor growth [22, 23].
Macrophages’ M2 polarization contributes to malignant angiogenesis, tumor cell
proliferation, and growth [24, 25]. Furthermore, cancer cells regulate macrophages to
aggravate tumor metastasis [26]. Studies have illustrated the relationship axis between
CAFs/macrophages/cancer cells, clearly indicating that CAFs promote macrophage M2
polarization by secreting CXCR12, thereby affecting cancer cell behavior and worsening
cancer prognosis [27].

We hypothesized that KIF26B may regulate immune suppression and metastasis of
GC through influencing CAFs immune infiltration in this study.

MATERIALS AND METHODS

Clinical samples
We obtained tumor and adjacent normal tissues in pair \((n = 50)\) from GC patients from 2019.07 to 2023.03. This study was approved by the Medical Ethics Committee of 901th Hospital of PLA (202311006). The written consents from each participant have acquired.

**Survival prognosis analysis**

To verify GC patients in the high-KIF26B or low-KIF26B expression subgroups, the Kaplan-Meier analysis with the “survminer” R package was performed. Next, the log-rank test was utilized to determine the significance of differences.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

We extracted total RNAs from frozen tissues by the Trizol reagent (Invitrogen, Carlsbad, USA). Complementary DNA from RNAs was synthesized through the reverse transcription kit (Invitrogen, Carlsbad, USA). Then, qRT-PCR was made through a SYBR® Green qRT-PCR Kit (Promega, USA). Finally, we calculated relative mRNA expression levels by means of the \(2^{-\Delta\Delta C_t} \) method (GAPDH as the internal reference).

Primer sequence: KIF26B, forward: 5’-CCACCUCUUU GAGAAGGATT-3’, reverse: 5’-UUCCUUCUAAGAGGUGGT-3’; α-SMA, forward: 5’-CGCCCTCGCCACCAGATCTG-3’, reverse: 5’-TAGCCTTTCATAGATG GGAC-3’; ACTA2, forward: 5’-GAGGGAAGGTCTAACAGCC-3’, reverse: 5’-GCTTCACAGGATTCCGTCT-3’; CXCL12, forward, 5’-CGCGCCTTGCCT GAGCGACCGGAAG-3’, reverse, 5’-CTTGTITAAAGCTTCTCCAGGTACT-3’; FAP, forward: 5’-TGGGTCTCCAGT-GAAGGAGTATG-3’, reverse: 5’-TGATATT CTTTTGCTGTCCG-3’; ITGB1, forward: 5’-CCTTCAGCTCCAGCGGTG-3’, reverse: 5’-TGCTCTGCTCCTGACTCACTCC-3’; PDPN, forward: 5’-CGAAGATG ATGTGGTGAAC-3’, reverse: 5’-CGATGGAATGGCTGTAC-3’; THY1, forward: 5’-GAAGGTCTCTCTACCT ATCCGCC-3’, reverse: 5’-TGATGCCCTACA CTGACCAG-
3'; GAPDH, forward: 5'-GGTGAAGGTCGGAGTCACAACG-3', reverse: 5'-CAAAAGTTGTCATGGATGACC-3'.

**Immunohistochemistry (IHC) staining**

We collected paraffin sections of GC tumor tissues and normal adjacent tissues (n = 3) from GC patients as well as tumor tissues from mice. Tumor tissues were fixed, embedded (paraffin), and then sliced into sections (4-μm-thick). Afterwards, we deparaffinized paraffin sections and then rehydrated, and restoring the antigen was carried out by means of citrate buffer at a high temperature and pH 6.0. We incubated these sections mixed with primary antibody anti-KIF26B (ab121952, 1/200, Abcam), anti-Ki67 (ab15580, 0.1 μg/mL, Abcam), anti-α-SMA (ab5694, 1/200, Abcam) and anti-CD163 (ab182422, 1/500, Abcam) at 4°C overnight. Then, these sections were washed by an incubation with secondary antibodies Goat F(ab')2 Anti-Rabbit IgG F(ab')2 (HRP) (ab6112, 1: 500, Abcam) for 30 min. The sections were stained with hematoxylin and diaminobenzidine, and observed under a microscope. The IHC images were analyzed through Image J software.

**Cell transfection**

We purchased HGC-27/AGS and GES-1 cells from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium with 10% FBS and 1% penicillin, 37°C, 5% CO₂.

The plasmids (RiboBio, Beijing, China) include sh-KIF26B, pcDNA-KIF26B, and negative controls (sh-NC, pcDNA-NC) were utilized. We transfected above mentioned plasmids into HGC-27 and AGS cells with Lipofectamine 3000 (Invitrogen) and cultured for 24 h. The transfection efficiency was detected utilizing western blotting.

**Nude mice tumorigenesis assay**

We bought mice (5-week-old; BALB/c nude mice; males) from Vital River Laboratories (Beijing, China). AGS cells (5×10⁶) transfected with sh-KIF26B/sh-NC were
injected into the mice’s inguinal skin. After four weeks, we killed nude mice with an overdose of pentobarbital. All animal experiments were approved by the Animal Ethics Committee of Beijing Viewsolid Biotechnology Co. LTD (VS2126A00168). The authors have read the ARRIVE Guidelines, and the manuscript was prepared and revised according to the ARRIVE Guidelines.

*H&E (hematoxylin and eosin) staining*

We fixed the lung lymph node tissue of mice in neutral formalin (10%) and then embedded in paraffin. We cut the tissues into sections 4 μm, and then stained them with H&E staining. The images were observed under a microscope.

*Western blotting*

We dissolved the HGC-27 cells by RIPA buffer (Beyotime, Shanghai, China), and the total protein was purified and quantified through bicinechoninc acid (BCA) protein kits (ThermoFisher, USA). After that, we detached proteins by 10% SDS-PAGE and moved them to PVDF membranes. After blocking with skimmed milk (5%), we incubated proteins with the primary antibodies anti-KIF26B (1:1000, FNa04559, FineTest, Wuhan, China), anti-E-cadherin (1:2000, ab40772, Abcam), anti-MMP-2 (1:5000, ab92536, Abcam), anti-N-cadherin (1:2000, ab76011, Abcam), anti-MMP-9 (1:5000, ab76003, Abcam) and anti-β-actin (1:2000, ab8227, Abcam) overnight at 4°C. Proteins were continued to incubate with the anti-rabbit secondary antibody (1:5,000; SA00001-2, SanYing, China) for one hour. We examined protein blots by ECL chemiluminescent system. Image J was applied for the quantification of protein blots.

*Enzyme-linked immunosorbent assay (ELISA)*

We resuspended the HGC-27 cells (1×10⁶) with 300 μL of complete medium and plated them in 96-well plates, and collected the supernatant 3 days later. ELISA was performed according to ELISA kits’ instructions (Invitrogen, USA). We detected and recorded the absorbance value at 450 nm.
Transwell assay

In order to evaluate the ability of cell invasion, the transwell chamber was used. The supernatant (500 μL) that collected from HGC-27 or CAFs was added into the upper chamber. Into the bottom chamber, we appended the serum-free medium (about 200 μL). Then, the cell plate was cultured for 2 d at 37°C with 5% CO₂. Next, we removed the bottom chamber’s cells by cotton swabs, and the upper chamber’s cells were exposed to crystal violet (0.2%) to stain for 5 min. We used the inverted microscope for counting the number of invasion cells.

Flow cytometry

We cultured THP-1 monocytes in 6-well plates (10^6 cells/well) in RPMI medium together with 5% FBS and the supernatant of CAFs that transfected with sh-KIF26B, pcDNA-KIF26B, and negative controls for 72 h. Then, the macrophages were separated and incubated in complete medium with 0.02% NaN₃ for 30 min on ice. There followed the incubation of macrophages with blocking antibodies anti-CD14/anti-CD206 and anti-F4/80/anti-CD206 (BD Biosciences Pharmingen, USA) for 20 min in Brilliant stain buffer on ice. We fixed the cells with 1% paraformaldehyde and performed the flow cytometric acquisition by BD LSR Fortessa (flow cytometry, BD Biosciences Pharmingen, USA). The data analysis was made through FlowJoTM 10.8.1 software.

Immunofluorescence assay

We used an immunofluorescence assay for the detection of CD206 expression, that CD206 is the marker of M2 macrophages. THP-1 cells were cultured to 70% confluence on the glass side. Then, after washing twice with 1 × PBS, we fixed the cells with 4% paraformaldehyde for 1 h at 25°C. Next, we permeabilized cells with 0.2% Triton X-100 (#X100, Sigma) in 1 × PBS at 37 °C for 15 min and closed by 10% normal goat serum at room temperature for 1 h. Subsequently, we incubated the cells with an anti-Mannose Receptor antibody (Abcam, ab64693, dilution 1 μg/mL) overnight at 4°C. Then, after
PBS-washing, we incubated the cells with the goat anti-mouse IgG Alexa 488 conjugated fluorescence secondary antibody (R37120, dilution 1/1000) for 1 h at room temperature. Ultimately, we stained the cells with DAPI and observed under a microscope.

**Wound healing assay**

We incubated AGS and HGC-27 cells (3 \( \times \) 10^5 cells/well) in 6-well plates with a complete medium that include extracellular matrix molecule (10 μg/mL) until the cell monolayer was formed. We have used a pipette tip (200 μL) to scratch a straight line on plate bottom. Next, we cultured AGS and HGC-27 cells with a medium (serum-free) for 1 day. Finally, we observed the wound-healing distance of both typed cells and gauged them by an inverted microscope (Carl Zeiss, Oberkochen, Germany).

**Tube formation assays**

HUVECs (2×10^4) were inoculated onto the 96-well plate that were coated with Matrigel, and the supernatant of CAFs was added. After culturing for 8 h, the tubular structure of HUVECs was observed under the inverted microscope.

**Statistical analysis**

Data were represented by the mean ± standard deviation of three independent experiments. GraphPad Prism 7.0 (GraphPad Software, USA) was used for statistical analysis. Multiple group differences were analyzed using a one-way ANOVA. Two group comparisons were performed using a Student’s t-test. P<0.05 was indicated statistical significance.

**RESULTS**

**Overexpression of KIF26B in GC and its poor prognosis**

We examined kinesin superfamily expression levels in normal and tumor tissues and identified 18 DEGs (differentially expressed genes), of which 17 showed up-
regulation, and 1 showed down-regulation (Fig.1A). We listed the differentially expressed genes of KIFs and quantified them, and then found that the most significant difference in expression of KIF26B was observed in GC tissues (Supplementary Table). According to survival analysis, higher KIF26B expression resulted into poor prognosis (Fig.1B). Therefore, we chosen KIF26B as the follow-up study gene. Subsequently, we conducted a pan-cancer analysis on KIF26B and found that KIF26B was up-regulated in the vast majority of cancers, with markedly significant differences in stomach adenocarcinoma (Fig.1C). QRT-PCR demonstrated that KIF26B expression was higher in GC tissues than that in Normal tissues (Fig.1D). Immunohistochemistry results presented high KIF26B (located in cytoplasm) expression in GC tissues from patients (Fig.1E). Western blotting indicated that KIF26B expression in HGC-27/AGS cells was markedly higher compared with that in GES-1 cells (Fig.1F). A significant relation between high levels of KIF26B and infiltration depth, distant metastasis, lymph node metastasis, and TNM staging (T1+T2 vs T3+T4) was observed (Table 1).

**KIF26B’s effect on tumorigenesis, lung metastasis, and abdominal metastasis**

We determined a xenograft tumor nude mouse model. We learned from xenograft tumor experiment results that knockdown of KIF26B could inhibit tumor growth of GC (Fig.2A-C). Immunohistochemical results demonstrated that KIF26B and Ki67 (located in nucleus) expressions were both decreased after KIF26B suppression in tumor tissues from mice, indicating that there was an inhibition on the proliferative activity of GC cells after knocking down KIF26B (Fig.2D). In addition, the GC cells in the sh-KIF26B group also decreased their metastasis to the pulmonary lymph node compared with sh-NC group (Fig.2E-F). Meanwhile, knockdown of KIF26B also inhibited the metastasis of GC cells to the peritoneum (Fig.2G-H). From this, it was demonstrated that knockdown of KIF26B can inhibit the occurrence of tumors, lung metastasis, and abdominal metastasis of GC in vivo.
High expression of KIF26B may affect the activation or infiltration of GC related fibroblasts

KIF26B is highly correlated with the infiltration degree of CAFs, followed by endothelial cells (Fig.3A-B). We found a positive correlation between expressions of KIF26B and PD-1 (PDCD1) and PD-L1 (CD274) (Fig.3C). To further verify the correlation between KIF26B and the CAFs, we have utilized Timer 2.0 database. We confirmed that KIF26B was significantly correlated with CAFs biomarkers, such as ACTA2 (R=X, P=X), FAP, ITGB1, PDPN, and THY1 (Fig.3D). In the meanwhile, immunohistochemistry experiments have found that both KIF26B and α-SMA (activation biomarker of CAFs) could be detected simultaneously in GC tissue from patients (Fig.3E). Next, the results of qRT-PCR confirmed that α-SMA/ACTA2 mRNA in metastatic GC tissues were markedly higher while comparing with non-metastatic GC tissues (Fig.3F). Summarizing the above experimental results, it is found that high expression of KIF26B may affect the activation and infiltration of CAFs, thereby influencing the metastasis of GC.

KIF26B affects the activation of fibroblasts to form the CAF phenotype

For future researches, we incubated primary human foreskin fibroblasts (HFF) with the supernatant of sh-KIF26B and sh-NC-transfected HGC-27 cells. The transfection’s efficacy was confirmed by western blotting (Fig.4A). Next, qRT-PCR and ELISA assays were used for detecting chemokines (CXCL12) in HFF cells, and KIF26B knockdown decreased CXCL12 expression, indicating reducing the chemotaxis of HFF cells (Fig.4B-C). The transwell assay also demonstrated that suppression of KIF26B could decline HFF’s invasive ability (Fig.4D). Finally, we detected the biomarkers of CAFs, including α-SMA, FAP, ITGB1, PDPN, and THY1, and inhibition of KIF26B decreased the expression levels of CAFs biomarkers (Fig.4E). It has been proven that KIF26B can promote the activation of fibroblasts to form the CAF phenotype.
**KIF26B enhances crosstalk between CAFs and macrophages, thereby mediating M2 polarization of macrophages**

A positive relation between KIF26B expression and the number of M2 macrophages was found (Fig.5A-B). Immunohistochemical results uncovered that KIF26B and CD163+ expressions were both increased in GC tissues (high infiltration) from patients, indicating that KIF26B expression was related positively with the CD163+ expression (Biomarker of M2 macrophage) (Fig.5C). We transfected primary CAFs with sh-KIF26B, collected the supernatant and incubated THP-1 cells (induced by 100 ng/mL). Immunofluorescence assay detected CD206 (Surface biomarker of M2 macrophage) in THP-1 cells and indicated that the fluorescence intensity in the sh-KIF26B group was decreased markedly compared with sh-NC group (Fig.5D). Subsequently, we counted macrophages using flow cytometry and found the number of M2 macrophages was a decreased in the sh-KIF26B group comparing with sh-NC group (Fig.5E). In addition, data analysis found a significant positive correlation between KIF26B and chemokine CXCL12 expression, and its corresponding receptor CXCR4 (Fig.5F-G). In summary, KIF26B in GC cells may affect the M2 polarization of macrophage through CXCL12 secreted by CAFs.

**KIF26B regulates M2 polarization of macrophages through fibroblast activation, thereby regulating invasiveness, angiogenesis, and EMT processes of GC cells**

We collected the supernatant of CAFs that transfected with sh-NC, sh-KIF26B, pcDNA-NC and pcDNA-KIF26B as a conditioned medium to incubate GC cells (AGS and HGC-27 cells). Overexpression of KIF26B enhanced the migration and invasiveness of HGC-27 and AGS cells when comparing with the negative control group, while knockdown of KIF26B could reduce the migration and invasiveness of AGS and HGC-27 cells (Fig.6A-B). Tube formation assay have shown that the tube forming ability of cells was enhanced when KIF26B is overexpressed, and it was inhibited after KIF26B knockdown (Fig.6C). We detected EMT related protein expressions in HGC-27 and AGS cells, such as N-cadherin, E-cadherin, MMP2, and MMP9 (Fig.6D). While comparing
with the control group, the sh-KIF26B group had lower N-cadherin, MMP2, and MMP9 expressions, while the pcDNA-KIF26B group had higher N-cadherin, MMP2, and MMP9 expression levels, while the expression level of E-cadherin was opposite to other proteins (Fig.6D). M2 polarized macrophages’ infiltration quantity in the pcDNA-KIF26B group was much higher comparing the control group, while the infiltration quantity of M2 polarized macrophages in the sh-KIF26B group was lower (Fig.6E). Based on the above experimental results, it was revealed that KIF26B can regulate M2 polarization of macrophages through fibroblast activation, thereby regulating invasiveness, angiogenesis, and EMT processes of GC cells.

DISCUSSION

GC is the one of the most serious cancer around the globe [28, 29]. For this, exploring GC progression mechanisms and developing new treatments have become necessary.

By analyzing TCGA database, we discovered overexpression of KIF26B in most tumors. Especially, highly expressed KIF26B was found in GC tissues and cells. Through survival analysis, we found that higher KIF26B expression is associated with poor GC prognosis. The clinical data analysis showed a high level of KIF26B significant association with invasion depth, lymph node metastasis, and TNM stage of tumor. Similar to our results, Teng et al mentioned in their article published in 2018 that the level of KIF26B is significantly increased in breast cancer cells and tissues, and KIF26B level is positively related to the tumor size, TNM grading and differentiation of breast cancer patients [30]. Furthermore, data from Wang et al suggest that KIF26B causes the occurrence of colorectal cancer and acts as a possible therapeutic target for CC (colorectal cancer) [31]. Higher expression of KIF26B is observed in hepatocellular carcinoma tissues and cell-lines [32]. Also, Increased KIF26B expression is related to poor-survival and differentiation, and advanced TNM [32].

GC metastasis is the main cause of death for patients [33], and the 5-year survival rate of patients in china with metastatic GC is less than 10% [34, 35]. Therefore, improving understanding of the mechanism of gastric cancer metastasis will have a great
significance for the prevention and treatment of gastric cancer. We found that knockdown of KIF26B decreased the occurrence, lung metastasis, and abdominal metastasis of GC. Previous research shows that KIF26B has an impact on the metastasis of breast cancer [30] and GC [13]. These are good supports for our results.

CAFs are an essential component of various tumors, such as GC [36, 37]. CAFs aid in the invasion and metastasis of tumors during their occurrence and development [38]. The interaction mechanism between GC cells and CAFs is still unclear. Previous studies have found that knockdown of KIF26B inhibits the activation of renal fibroblasts [39]. We indicated that the high expression level of KIF26B promoted the activation and infiltration of CAFs through data analysis and the experiments on GC tissues and cell lines. Also, we found a positive relation between KIF26B expression and M2 macrophages activation. Cai et al. show that the fibroblast activating protein is associated with the invasion of M2 macrophages in gastrointestinal cancer [40]. Our subsequent experimental findings are similar to those of Cai et al., where KIF26B enhances crosstalk between tumor fibroblasts and macrophages, thereby mediating the M2 polarization of macrophages. More specifically, we found that KIF26B in GC cells may affect macrophage M2 polarization through CXCL12 secreted by CAFs.

The invasion [41], tube formation [42, 43], and EMT process [44] of tumor cells is related to the cancer progression. Our research results indicated that KIF26B could regulate M2 polarization of macrophages through fibroblast activation, thereby regulating tumor cell migration, invasion, angiogenesis, and EMT processes and completing the regulation of GC development and metastasis.

**CONCLUSION**

Although advances in basic and clinical studies have reduced the mortality rate of GC over the past decade, its prognosis remains poor, possibly due to incomplete diagnosis, high metastasis rate, and high chemotherapy resistance. We found that higher KIF26B expression could promote CAFs activation, thereby mediating macrophage M2 polarization and affecting the occurrence, lung metastasis, and
abdominal metastasis of GC. This study provides useful insights for exploring new mechanisms of GC and delaying its progression.
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