Tumor-associated macrophages regulate gastric cancer cell invasion and metastasis through TGFβ2/NF-κB/Kindlin-2 axis

Zhu Wang1,2, Yang Yang1,2, Yancheng Cui1,2,3, Chao Wang1,2, Zhiyong Lai1,2, Yansen Li1,2, Wei Zhang1,2, Harri Mustonen3, Pauli Puolakkainen3, Yingjiang Ye1,2, Kewei Jiang1,2, Zhanlong Shen1,2, Shan Wang1,2

1Department of Gastroenterological Surgery, Peking University People’s Hospital, Beijing 100044, China; 2Laboratory of Surgical Oncology, Beijing Key Laboratory of Colorectal Cancer Diagnosis and Treatment Research, Peking University People’s Hospital, Beijing 100044, China; 3Department of Surgery, Helsinki University Central Hospital, and University of Helsinki, Helsinki 00290, Finland

Correspondence to: Shan Wang. Department of Gastroenterological Surgery, Peking University People’s Hospital, No. 11 Xizhimen South Street, Xicheng District, Beijing 100044, China. Email: shanwang60@sina.com; Zhanlong Shen. Department of Gastroenterological Surgery, Peking University People’s Hospital, No. 11 Xizhimen South Street, Xicheng District, Beijing 100044, China. Email: shanwang60@sina.com; Kewei Jiang. Department of Gastroenterological Surgery, Peking University People’s Hospital, No. 11 Xizhimen South Street, Xicheng District, Beijing 100044, China. Email: dr_jiangkewei@163.com.

Abstract

Objective: Recent studies have shown that tumor-associated macrophages (TAMs) play an important role in cancer invasion and metastasis. Our previous studies have reported that TAMs promote the invasion and metastasis of gastric cancer (GC) cells through the Kindlin-2 pathway. However, the mechanism needs to be clarified.

Methods: THP-1 monocytes were induced by PMA/interleukin (IL)-4/IL-13 to establish an efficient TAM model in vitro and M2 macrophages were isolated via flow cytometry. A dual luciferase reporter system and chromatin immunoprecipitation (ChIP) assay were used to investigate the mechanism of transforming growth factor β2 (TGFβ2) regulating Kindlin-2 expression. Immunohistochemistry was used to study the relationships among TAM infiltration in human GC tissues, Kindlin-2 protein expression, clinicopathological parameters and prognosis in human GC tissues. A nude mouse oncogenesis model was used to verify the invasion and metastasis mechanisms in vivo.

Results: We found that Kindlin-2 expression was upregulated at both mRNA and protein levels in GC cells cocultured with TAMs, associated with higher invasion rate. Kindlin-2 knockdown reduced the invasion rate of GC cells under coculture condition. TGFβ2 secreted by TAMs regulated the expression of Kindlin-2 through the transcription factor NF-κB. TAMs thus participated in the progression of GC through the TGFβ2/NF-κB/Kindlin-2 axis. Kindlin-2 expression and TAM infiltration were significantly positively correlated with TNM stage, and patients with high Kindlin-2 expression had significantly poorer overall survival than patients with low Kindlin-2 expression. Furthermore, Kindlin-2 promoted the invasion of GC cells in vivo.

Conclusions: This study elucidates the mechanism of TAMs participating in GC cell invasion and metastasis through the TGFβ2/NF-κB/Kindlin-2 axis, providing a possibility for new treatment options and approaches.

Keywords: Gastric cancer; tumor-associated macrophage; Kindlin-2; invasion and metastasis

Introduction

Gastric cancer (GC) is the fifth most frequently diagnosed cancer and the third most lethal cancer worldwide (1). Although many treatments for GC have been evaluated, the curative effect is still poor (2,3). Invasion and metastasis are
important factors that affect the prognosis of patients with GC (4). According to the American Surveillance, Epidemiology and End Results (SEER) database, the 5-year survival rate of GC patients is 62.5%, but the percentage of patients with local invasion and distant metastasis has been decreased to 27.0% and 3.4%, respectively (5). However, the molecular mechanism of GC invasion and metastasis remains unclear.

Chronic inflammation induced by inflammatory cells in tumors plays an important role in tumor progression (6). Tumor associated macrophages (TAMs) are an important component of the leukocyte infiltrate, and TAMs and related cell types in mouse and human tumors generally have an M2 phenotype, which is oriented towards promoting tumor growth, remodeling tissues, promoting angiogenesis and suppressing adaptive immunity (7). The M2 phenotype of macrophages may also regulate the invasion and metastasis of various tumors (8-10); however, the mechanisms involved in this process remain unclear. Kindlin-2, a FERM domain-containing protein that contributes to the maturation of focal adhesions through the recruitment of migfilin and filamin (11), is a newly discovered key molecule participating in β1 and β3 integrin activation (12). Our previous study found that Kindlin-2 protein expression was positively correlated with the infiltration depth and lymph node metastasis of GC at the mRNA and protein levels, but negatively correlated with prognosis (13). We also found that the invasive ability of GC cells enhanced by TAMs can be inhibited by interfering with Kindlin-2 expression. Therefore, we speculate that Kindlin-2 may play an important role in TAM-mediated process of GC invasion and metastasis and that this mechanism is worth studying.

This study, we first determined that transforming growth factor β2 (TGFβ2) secreted by TAMs increases the invasive ability of GC cells by promoting Kindlin-2 expression. Then, we investigated the mechanism of the TGFβ2/NF-κB/Kindlin-2 axis signaling pathway. Taken together, our findings provide new insights into the molecular mechanism and treatment of GC.

Materials and methods

Cell lines and cell culture

The human GC cell lines AGS, HGC-27, Hs-746T, and NCI-N87 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and the human monocyte cell line THP-1 was purchased from the National Infrastructure of Cell Line Resource. All cells were tested and authenticated for their genotypes by DNA fingerprinting. All cell lines were passaged for fewer than 6 months after reconstitution. For TAM/GC coculture, Transwell chambers (24-well plates, 0.4-μm pore size; Corning, New York, USA) were used. GC cells were seeded in the upper chambers and TAMs were placed in the lower wells; a volume of RPMI1640 medium containing 10% fetal bovine serum (FBS) (all from Thermo Fisher Scientific, Waltham, USA) sufficient to fill the lower wells and cover the cells in chambers was added. All experimental cells were cultured in RPMI 1640 medium supplemented with 10% FBS in an incubator with 5% CO2 at 37 °C.

Isolation of monocytes and macrophages

Mononuclear cells were isolated from the blood of healthy subjects via density gradient centrifugation (Ficoll-Paque, Amersham, Uppsala, Sweden). Three layers were present after density gradient centrifugation, and mononuclear cells located in the second cloudy layer were transferred to a clean tube, washed with a phosphate-buffered saline (PBS) + 10% acid citrate dextrose (ACD) solution and centrifuged twice. The cells were counted and 1.4×10^6 cells were placed on coverslips (Nalge Nunc International Corporation, Naperville, Germany) coated with Matrigel (Matrigel, BD Biosciences, San Jose, USA). The isolated cells were cultured in serum-free medium designed for macrophages (macrophage serum-free medium, Gibco, Paisley, UK) supplemented with granulocyte-macrophage colony stimulating factor [granulocyte-macrophage colony-stimulating factor (GM-CSF); 10 ng/mL, ImmunoTools, Oldenburg, Germany] and antibiotics at 37 °C with 5% CO2. Monocytes adhered to the Matrigel overnight and differentiated to macrophages due to GM-CSF, and the non-adherent mononuclear cells were removed from the medium next day. The monocytes were fully differentiated into macrophages after 6 d and then used for experiments. When cocultured with cancer cells, macrophages developed into TAMs expressing a special surface marker, CD163.

Quantitative flow cytometry

A total of 5×10^5 cells were stained with an isotype-matched control antibody or a relevant antibody for 1 h at 4 °C in the dark. For indirect immunofluorescence staining, CD163+ macrophages were initially incubated with an
unconjugated primary anti-CD163 antibody (Becton Dickinson, San Jose, USA) for 1 h at 4 °C, washed three times with Dulbecco’s phosphate-buffered saline (D-PBS), pH=7.4, and then incubated with a goat anti-mouse Alexa-Fluor 488-conjugated secondary antibody (1:200 dilution; Molecular Probes) for 1 h at 4 °C in the dark. The stained cells were washed twice with D-PBS, pH=7.4, resuspended in 400 μL of FACS-Flow buffer (Becton Dickinson, San Jose, USA), and kept on ice until analysis. All samples were analyzed using a FACS Calibur flow cytometer, and compensation for spectral overlap was ensured using FlowJo for Macintosh software (Version 8.3, TreeStar, San Carlos, USA). For CD163 density quantification, flow cytometric estimation of antibodies bound per cell (ABC) was performed using Quantibrite PE beads (Becton Dickinson, Heidelberg, Germany) as recommended by the manufacturer. After the cells were stained as described, a set of 4 precalibrated fluorescence-labeled beads were used for standardization before the samples were acquired. The Quantibrite PE beads were run at the same instrument settings used for the assay, and the linear regression obtained using the Quantibrite PE beads was used to convert the FL2 linear fluorescence staining of the cell population into the number of (CD163) PE molecules bound per cell. CD11b, CD68, CD86 and CD206 were detected via the same method and all antibodies were obtained from Becton Dickinson (San Jose, CA, USA).

RNA extraction and purification

Total RNA was extracted from tissue samples or cell lines using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and purified using an RNase-Free DNase Set (QIAGEN) according to the manufacturer’s protocol. The purity and concentration of the RNA were determined from OD260/280 readings using a spectrophotometer (NanoDrop ND-1000). RNA integrity was determined by 1% formaldehyde denaturing gel electrophoresis.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was used for reverse transcription, which was performed at 37 °C for 60 min with a Quantscript RT Kit (KR103; Tiangen Biotechnology, Beijing, China). All samples were quantified after PCR amplification using a RealMasterMix (SYBR Green) kit (FP202; Tiangen Biotechnology). The primer sequences are shown in Table 1. The PCR protocol was as follows: initial denaturation at 95 °C for 10 min, followed by 30 cycles at 95 °C for 10 s, annealing at 58.5 °C for 5 s, and extension at 72 °C for 8 s. PCR products were quantified via melting curves at 84 °C. All results were normalized to β-actin to ensure a uniform amount of RNA template.

RNA interference

Gene silencing was performed by transfecting AGS cells with siRNA oligonucleotides (GenePharma, Shanghai, China). The siRNA sequences are shown in Table 2. For transfections in 12-well plates, 1.0×10⁵ cells were seeded per well overnight, and then transfected with 80 nmol/L target siRNA or negative control sequence using Lipo2000

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer (5’ → 3’)</th>
<th>Reverse primer (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>TGCCAAAGGGATGCTAAGG</td>
<td>CTCCACAACCC TCTGCAC</td>
</tr>
<tr>
<td>IL-11</td>
<td>TGGTGAAGATGAAGCTG</td>
<td>ATGAAGCTTTGGAAGGAGGTTG</td>
</tr>
<tr>
<td>TNFSF7</td>
<td>TGCTGAGAATTGCCAG</td>
<td>GCACCGATGGACTGCAAA</td>
</tr>
<tr>
<td>MMP-9</td>
<td>AAGGTCGTCGGTGAGTT</td>
<td>AGTTGCCCGAGTTTACAGT</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>GAGCCGCGGAGCTGAGGG</td>
<td>GGCCCTCGAGGAGCAGAA</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>CAGACGAGACTACGCAGGG</td>
<td>TTAGACGATCGAGGCGAAA</td>
</tr>
<tr>
<td>β1-integrin</td>
<td>TTCCTGCTGCTGCTTGG</td>
<td>AGTTGGACGCGACCTGCTGG</td>
</tr>
<tr>
<td>NF-κB</td>
<td>AGTTGAGGGAGCTGGGGGGGG</td>
<td>GCCCTGGAAAAGTTCCTGCAACT</td>
</tr>
<tr>
<td>TGF-β</td>
<td>ACTGAGCTGCTGCTGCTGG</td>
<td>TGGTCCAGGAGCTAAATGTA</td>
</tr>
<tr>
<td>Kindlin-2</td>
<td>AAATGGTCACCTGGTCTGG</td>
<td>CTCTGCTTGGTCTTGGAC</td>
</tr>
<tr>
<td>β-actin</td>
<td>AAAGTGAAGAGACCTGCTG</td>
<td>AAGTGAGGAGCGGTTTGGT</td>
</tr>
</tbody>
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PCR, polymerase chain reaction; IL, interleukin; TNFSF7, tumor necrosis factor (ligand) superfamily, member 7; MMP, matrix metalloprotein; VEGF, vascular endothelial growth factor; TGF, transforming growth factor.

Transfection Agent (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. In 24–48 h after transfection, cells were harvested or incubated further for the following experiments.

**Western blot analysis**

GC cells and cocultured cells were lysed in radioimmuno-precipitation assay (RIPA) buffer (Thermo Fisher, Rockford, USA) containing 1% phenylmethane sulfonyl fluoride (PMSF), and the protein concentration was measured using a BCA protein assay kit (Beyotime, Shanghai, China). Fifty micrograms of protein was separated on an 8%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Beyotime, Shanghai, China) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, USA). The membrane was then blocked with 5% nonfat milk and incubated with anti-Kindlin-2 (1:5,000 dilution; cat. no.ab74030), anti-NF-κB (1:5,000 dilution; cat. no.ab16502) and GAPDH (1:1,000 dilution; cat. no. ab8245; all Abcam, Cambridge, UK) antibodies, followed by incubation at 37 °C for 1 h with a horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution, Beyotime, Shanghai, China). The membrane was stained with ECL Plus (Beyotime, Shanghai, China) according to the manufacturer’s instructions.

**Transwell matrigel invasion assay**

For the matrigel invasion assay, Transwell chambers (24-well plates, 8-μm pore size; Corning) were coated with 100 μL of Matrigel (5 mg/mL) (BD Biosciences, San Jose, USA). Briefly, a total of 1×10⁵ cells were seeded in the upper chambers in medium containing 5% nonfat milk and incubated with anti-Kindlin-2 (1:5,000 dilution; cat. no.ab74030), anti-NF-κB (1:5,000 dilution; cat. no.ab16502) and GAPDH (1:1,000 dilution; cat. no. ab8245; all Abcam, Cambridge, UK) antibodies, followed by incubation at 37 °C for 1 h with a horseradish peroxidase-conjugated secondary antibody (1:2,000 dilution, Beyotime, Shanghai, China). The membrane was stained with ECL Plus (Beyotime, Shanghai, China) according to the manufacturer’s instructions.

**Evaluation of immunohistochemical staining**

The immunohistochemical staining results were assessed by evaluating the mean score for both the intensity of staining and the proportion of tumor cells with an unequivocal positive reaction. Each section was assessed independently by 2 pathologists blinded to the patient data. Positive reactions were defined as those showing brown signals in the cytoplasm. The staining index was determined by multiplying the staining intensity score by the positive area score. The staining intensity score is as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The proportion of positive cells is defined as follows: 0, less than 5%; 1, 6%–25%; 2, 26%–50%; 3, 51%–75%; and 4, greater than 75%. For statistical analysis, we used the K-adaptive partitioning statistical (KAPS) algorithm in R software (Version 3.2.5; R Foundation for Statistical Computing, Vienna, Austria) to calculate the most efficient cut-off value. Cases with a score below the cut-off value were considered low expression, while cases with a score above the cut-off value are considered to show high expression.

**Luciferase reporter assay**

Two plasmid vectors were constructed: the dual-luciferase PGL-3 K2 wild-type (WT) vector containing the Kindlin-2 gene promoter (promoter region sequence from the transcription start site to 2,000 bp upstream) and the dual-luciferase PGL-3 K2 mutant (Mut) vector containing a mutated NF-κB promoter region (lacking the NF-κB binding site: −468 bp to −453 bp, CTGGGAATTTCTCTGG promoter region). AGS cells were seeded at 5×10⁴ cells/well in 24-well plates overnight, and then were cotransfected with PGL-3 K2 Mut, PGL-3 K2 WT, and empty PGL-3 vectors accordingly. Forty-eight hours after transfection, the cells were lysed using passive lysis buffer (Promega, Madison, USA), and the luciferase activity was measured by a GloMax20/20 luminometer (Promega) using the Dual Luciferase Reporter Assay System (Promega) and normalized to Renilla luciferase activity. The experiments were performed in triplicate.
Nude mouse oncogenesis model

This experiment was conducted at Crown Bioscience and received ethical approval from the Committee on the Ethics of Animal Experiments of Crown Bioscience [Crown Bioscience Institutional Animal Care and Use Committee (IACUC)]. The animal maintenance, handling and experimental procedures followed were in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.

Adult female BALB/c nude mice (18–22 g) were obtained from Anikeeper (Beijing, China). The animals were acclimatized to standard housing conditions (23±3 °C, 40%–70% relative humidity, 12 h light-dark cycle with lights on at 07:00), with free access to water and chow diet, for one week before the experiment. Each mouse was inoculated subcutaneously in the right front subaxillary region with tumor cells (1×10⁵) in 0.1 mL of PBS.

In the pre-experiment, six mice were divided into three groups, namely Kindlin-2 overexpression group, Kindlin-2 normal expression group and inhibitor alone (SB431542 adding) group (named group α, β and γ, respectively), and observed the tumor growth in different groups.

In the formal experiment, eighteen mice were randomly allocated to three study groups. Mice in group 01 and 03 were injected subcutaneously with pL/shGFP-NC (Kindlin-2⁻) cells, and mice in group 02 were injected subcutaneously with pL/shGFP-Kindlin-2 (Kindlin-2⁺) cells. Tumor growth was monitored twice a week using a caliper. Animals in group 01 and 02 were injected subcutaneously with TGFβ2 (0.1 μg/mouse; Cell signaling technology, Boston, USA) dissolved in 20 mmol/L citrate once a week, while group 03 mice received an injection of SB431542. When the tumor sizes reached 100–200 mm³ (1/2 length×width²), the tumor volume and body weight were recorded, and the first recorded date was d 0. When the average tumor size reached 2,000 mm³, tumors were harvested for subsequent histopathology and immunohistochemistry analysis.

Chromatin immunoprecipitation (ChIP) assay

A total of 5×10⁶ cells were cultured in each 10-cm dish and subjected to the following protocol: Rinse the cells twice with PBS. Add 5 mL 1% formaldehyde in PBS to the cells and incubate for 10 min at room temperature. Add 550 μL 1.25 mol/L glycine, swirl gently to mix and incubate for 5 min. Aspirate the supernatant and wash the pellet with PBS 2 times. Prepare the sonication buffer/protease inhibitors and add 0.5 mL of the mixture to each plate. After 1 min, scrape the cells with a sterile scraper. Pipet the suspension into a 2-mL Eppendorf tube and place the tube on wet ice for 10 min. Sonicate 10–15 times with 10 s pulses. Place the tube on dry ice followed by wet ice for 1–2 min after each pulse. Verify chromatin fragmentation by running 8 μL of the sample on a 1% agarose gel. Centrifuge the sample in a refrigerated microfuge for 10–15 min at the highest speed. Aliquot 25 μL of the sonicated sample as the input. Mix 250 μL sonicated sample with 555 μL dilution buffer containing protease inhibitor cocktail, and then add 50 μL of magnetic bead-coupled anti-rabbit IgG and incubate for 30 min at 4 °C with rotation. After absorbing the magnetic beads with a magnetic separation rack for 2 min, aliquot the supernatant into two groups: IP and neg. Add 5 μg of anti-NF-κB to the IP tubes and incubate with the neg tubes overnight with rotation. Adsorb the magnetic beads with the magnetic separation rack two times for 2 min each. Use ChIP elution buffers/protease K to elute the samples for 2 h at 62 °C. Add RNase and incubate for 20 min at 37 °C, followed by 10 min at 95 °C. Add a mixture of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuge the sample for 10–15 min at a speed of 10,783 r/min at 4 °C. Transfer the supernatant to fresh Eppendorf tubes and add 30 μg of glycogen, 1 mL of absolute ethyl alcohol, and 5 mol/L NaCl to a final concentration of 200 mmol/L. After incubating the samples for 30 min at −80 °C, centrifuge the samples for 20 min at a speed of 10,783 r/min at 4 °C to precipitate the DNA. Wash the DNA with 500 μL of 70% ethyl alcohol. After air drying the precipitate, dissolve it in 20 μL of 10 mmol/L Tris-HCl pH 8.0. PCR was used to evaluate the expression of Kindlin-2 in the three groups, and 8 μL of the PCR product was run on a 1% agarose gel.

Statistical analysis

Data analysis was performed using the SPSS software (Version 22.0, IBM Corp., New York, USA). Non-parametric tests were used to compare Kindlin-2 expression in tumor tissues and paraneoplastic tissues. Differences in Kindlin-2 expression and clinicopathologic variables were compared using the Fishers’ exact test or the Cochran-Armitage trend test in the case of ordered categories. The overall survival (OS) time was calculated from the date of surgery to the last follow-up assessment or the day of death. The median survival follow-up was 46.6 (range, 2.0–99.0) months. Univariate analysis was conducted using
the Kaplan-Meier method, and survival times were compared using the log-rank test. The Cox proportional hazards model was used for multivariate analysis. P<0.05 on both sides were considered statistically significant.

Results

**TMAs increased invasive ability of GC cells through TGFβ2**

THP-1 monocytes were used for macrophages differentiation. THP-1 monocytes were differentiated into M1 macrophages with 6 h PMA stimulation followed by lipopolysaccharide (LPS) + interferon (IFN)-γ treatment for 18 h, while M2 macrophages were collected after stimulation with PMA for 6 h followed by interleukin (IL)-4 + IL-13 treatment for 18 h. The expression of surface antigens, such as CD163 (86.45±2.13 % vs. 34.05±5.56, P<0.001), CD206 (76.82±2.84 % vs. 16.53±5.97, P<0.001), and CD11b (90.69±1.89 % vs. 12.48±3.39, P<0.001), was significantly higher in M2 macrophages than in M1 macrophages. In contrast, CD86 (6.95±2.79 % vs. 18.78±2.72, P<0.001) expression was significantly lower in M2 macrophages than in M1 macrophages, and the expression of CD68 (75.89±4.74 % vs. 92.70±1.61, P=0.061) was not significantly different between two groups (Figure 1A). In the following studies, we used M2 macrophages, and unless otherwise stated, TAMs specifically target M2 macrophages.

We examined the Kindlin-2 mRNA expression and the invasive ability of GC cells in two groups: a TAM/GC coculture group and a conventional GC cell-only group. The relative Kindlin-2 mRNA expression level of TAM/GC and GC groups were (258.3±8.2)% and (100.0±6.5)%, respectively (P<0.001) and the related cell invasive ability of these two groups were (258.3±8.2)% and (100.0±6.5)%, respectively (P<0.001). After downregulating Kindlin-2 expression in GC cells using siRNA and incubating GC cells with TAMs, the relative Kindlin-2 mRNA expression [0.22±0.06 vs. 4.74±0.35 for TAM/GC siRNA-Kindlin-2 (siK2) and TAM/GC groups, respectively, P<0.001] and invasive ability [(212.67±14.38)% vs. (258.33±8.22)%, P=0.029] promoted by TAMs were significantly decreased (Figure 1D–F).

To investigate the intrinsic mechanism, we identified four cytokines significantly increased in the TAM/GC coculture group using cytokine chips (Human Common Cytokines PCR Array, QIAGEN) and RT-qPCR [normalized to the GC alone group: tumor necrosis factor (ligand) superfamily, member 7 (TNFSF7), 39.8±1.1, P<0.05; TGFβ2, 16.9±0.4, P<0.05; IL-8, 28.8±2.3, P<0.05; IL-11, 31.2±1.7, P<0.05] (Figure 1B). Moreover, the TGFβ receptor inhibitor (SB431542) reduced the relative expression of Kindlin-2 mRNA (relative to the TAM/GC group: 0.6±0.0, P=0.001), β1-integrin (0.5±0.0, P<0.001), matrix metalloprotein (MMP)-9 (0.7±0.0, P=0.006), vascular endothelial growth factor (VEGF)-A (0.4±0.0, P=0.004), and VEGF-C (0.3±0.0, P<0.001) (Figure 1C).

Next we explored the effect of TGFβ2 on the invasion and metastasis of GC cells, RT-qPCR was used to examine the Kindlin-2 mRNA expression in GC cells among six different groups. TGFβ2 treatment for 24 h significantly increased Kindlin-2 mRNA expression in GC cells (5.33±0.32 vs. 1.00±0.18, P<0.001), similar to the effect observed in the TAM/GC coculture group. The TGFβ receptor inhibitor SB431542 decreased the Kindlin-2 mRNA upregulation in the TAM/GC cell coculture group (4.74±0.35 vs. 3.77±0.32, P=0.017). Similar results were acquired using another TGFβ receptor inhibitor, LY2157299 (Supplementary Figure S1A). Furthermore, SB431542 further reduced Kindlin-2 mRNA expression in the TAM/GC (siK2) group (0.15±0.02 vs. 0.22±0.03, P=0.020) (Figure 1D). Moreover, consistent results were obtained for Kindlin-2 protein expression (Figure 2B).

The transwell invasion assay was used to examine the invasive ability of GC cells. TGFβ2 treatment for 24 h significantly increased the cell invasive ability of GC cells [183.0±10.9] % vs. [100.0±6.5] %, P<0.001], similar to the effect observed in the TAM/GC coculture group. The TGFβ receptor inhibitor SB431542 decreased the GC cells invasive ability of the TAM/GC cell coculture group [(258.3±8.2) % vs. (226.7±11.1) %, P=0.011]. After adding SB431542 to the TAM/GC (siK2) group, the invasive ability of the GC cells [(205.2±10.2) % vs. (130.2±0.03) %, P=0.001] got decreased further (Figure 1E,F). Moreover, consistent results were obtained in the migration assay (Supplementary Figure S1C), and the cell wound scratch assay also showed similar results (Supplementary Figure S1B).

**TGFβ regulated expression of Kindlin-2 through transcription factor NF-κB**

Using bioinformatics software, we analyzed possible regulatory factors that could affect the expression of Kindlin-2 gene and the target range located from 2,000 bp upstream to 2,000 bp downstream of its transcription start site. There were eight transcription factors predicted by
Figure 1 Tumor-associated macrophages (TAMs) increased invasive ability of gastric cancer (GC) cells through transforming growth factor β2 (TGFβ2). (A) CD163, CD206 and CD11b expression levels were significantly higher in M2 macrophages than in M1 macrophages (CD163, 86.45±2.13 vs. 34.05±5.56, P<0.001; CD206, 76.82±2.84 vs. 16.53±5.97, P<0.001 and CD11b, 90.69±1.89 vs. 12.48±3.39, P<0.001). CD86 was significantly higher in M1 macrophages than in M2 macrophages (CD86, 6.95±2.79 vs. 18.78±2.72, P<0.001), and CD68 expression was not significantly different between the two groups (CD68, 75.89±4.74 vs. 92.70±1.61, P=0.061); (B) Tumor necrosis factor (ligand) superfamily, member 7 (TNFSF7), TGFβ2, interleukin (IL)-8 and IL-11 expression levels were significantly increased in the TAM/GC coculture group (TNFSF7, 39.8±1.1, P<0.05; TGFβ2, 16.9±0.4, P<0.05; IL-8, 28.8±2.3, P<0.05; IL-11, 31.2±1.7, P<0.05); (C) Kindlin-2, β1 integrin, matrix metalloprotein (MMP)-9, vascular endothelial growth factor (VEGF)-A and VEGF-C expression levels decreased by SB431542 under TAM/GC condition (Kindlin-2 mRNA, 0.6±0.0, P=0.001; β1-integrin, 0.5±0.0, P<0.001; MMP-9, 0.7±0.0, P=0.006; VEGF-A, 0.4±0.0, P=0.004 and VEGF-C, 0.3±0.0, P<0.001); (D) Comparison of Kindlin-2 mRNA expression among GC, TAM/GC, TAM/GC(siK2), TGFβ2/GC, TAM/GC+SB431542, and TAM/GC(siK2)+SB431542 groups (GC, 1.00±0.18; TAM/GC, 4.74±0.35; TAM/GC(siK2), 0.22±0.03; TGFβ2/GC cells, 5.33±0.32; TAM/GC+SB431542, 3.77±0.32 and TAM/GC(siK2)+SB431542, 0.15±0.02, P<0.001); (E,F) Invasion assay comparing the cell invasive ability among GC, TAM/GC, TAM/GC (siK2), TGFβ2/GC, TAM/GC+SB431542, and TAMs/GC(siK2)+SB431542 groups [GC, (100.0±6.5)%; TAM/GC, (258.3±8.2)%; TAM/GC(siK2), (205.2±10.2)%; TGFβ2/GC cell, (183.0±10.9)%; TAM/GC+SB431542, (226.7±11.1)% and TAM/GC(siK2)+SB431542, (130.2±0.03)%, P<0.001)]. * P<0.001. LPS, lipopolysaccharide; IFN, interferon.
TFSEARCH (with a threshold score of 95 points) and seven transcription factors predicted by JASPSCAN (score of 100 points) that might regulate Kindlin-2 gene transcription (Figure 2A). NF-κB was displayed in both analyses, and NF-κB could participate in many chronic inflammatory and cancer signaling pathways (14); thus, NF-κB was selected for subsequent research.

To elucidate the role of NF-κB in the GC process, we designed four different groups of GC, TGFβ2/GC, TAM/GC, and TAMs/GC+SB431542 to detect the expression of NF-κB mRNA. We found that both TGFβ2/GC (1.35±0.07 vs. 1.00±0.14, for the TGFβ2/GC and GC groups, respectively, P=0.012) and the TAM/GC coculture (1.55±0.09 vs. 1.00±0.14, for the TAM/GC and GC groups, respectively, P=0.002) significantly increased the NF-κB expression. Furthermore, SB431542 reduced the expression of NF-κB under the TAM/GC coculture condition (0.74±0.08 vs. 1.55±0.09, P<0.001). Consistent results were obtained in the protein level of NF-κB (Figure 2B).

![Figure 2](image)

**Figure 2** Transforming growth factor β2 (TGFβ2) regulated expression of Kindlin-2 through transcription factor NF-κB. (A) Transcription factor predicting bioinformatics software, TFSEARCH and JASPSCAN were used to predict the probable transcription factors by different standards (TFSEARCH, with a threshold score of 95 points; JASPSCAN, score of 100 points); (B) Comparison of Kindlin-2 and NF-κB expression at the mRNA and protein levels among the gastric cancer (GC), TGFβ2/GC, TAM/GC, and TAMs/GC+SB431542 groups. (NF-κB mRNA: GC, 1.00±0.14; TGFβ2/GC, 1.35±0.07; TAM/GC, 1.55±0.09 and TAM/GC+SB431542, 0.74±0.08, P<0.001); (C) Comparison of Kindlin-2 and NF-κB expression at the mRNA and protein levels among the GC(siNC), TGFβ2/GC(siNC), GC(siNF-κB), and TGFβ2/GC(siNF-κB) groups. [GC(siNC), 0.98±0.04; TGFβ2/GC(siNC), 2.57±0.14; GC(siNF-κB), 0.54±0.04 and TGFβ2/GC(siNF-κB) 0.68±0.04, P<0.001]; (D) Comparison of the cell invasive ability among the GC(siNC), TGFβ2/GC(siNC), GC(siNF-κB), and TGFβ2/GC(siNF-κB) groups [GC(siNC), (100.7±4.9)%; TGFβ2/GC(siNC), (179.7±15.4)%; GC(siNF-κB), (41.3±4.9)% and TGFβ2/GC(siNF-κB), (66.7±7.5)%, P<0.001] (×400). *, P<0.05; **, P<0.001.
NF-κB siRNA was used to knock down NF-κB expression, and Kindlin-2 expression was examined in four groups of GC cells: GC-negative control siRNA (siNC), TGFβ2/GC (siNC), GC+NF-κB siRNA (siNF-κB) and TGFβ2/GC (siNF-κB) groups. The results showed that the expression of Kindlin-2 decreased appreciably after NF-κB knockdown [0.54±0.04 vs. 0.98±0.04 for the GC(siNF-κB) and GC(siNC) groups, respectively, P<0.001], or after TGFβ2 treatment, and that the expression of Kindlin-2 increased in TGFβ2/GC(siNC) group (2.57±0.14 vs. 0.98±0.04, P<0.001) and the TGFβ2/GC(siNF-κB) group (0.68±0.04 vs. 0.54±0.04, P=0.008) (Figure 2C). Moreover, consistent results were observed for the invasion experiments: the invasive ability of GC cells decreased by NF-κB knockdown [(100.7±4.9)% vs. (41.3±4.9)%, P<0.001] or TGFβ2 treatment, and the invasive ability increased by TGFβ2 in both TGFβ2/GC(siNC) group [(179.7±15.4)% vs. (100.7±4.9)%, P<0.001] and TGFβ2/GC(siNF-κB) group [(66.7±7.5)% vs. (41.3±4.9)%, P=0.005] (Figure 2D).

**Binding sites of transcription factor NF-κB in Kindlin-2 gene promoter region**

To accurately define the binding sites, we constructed two plasmid vectors: a dual-luciferase PGL-3 K2 WT vector containing the WT Kindlin-2 promoter region (promoter region sequence from the transcription start site to 2,000 bp upstream) and a dual-luciferase PGL-3 K2 Mut vector containing a mutated Kindlin-2 promoter region (lacking the NF-κB binding site: −468 bp to −453 bp promoter region) (Figure 3A). Then we transfected these vectors into GC cells and detected the luciferase/Renilla fluorescein relative light unit (RLU) ratio after 48 h. Compared with that in the K2 WT group, the dual-luciferase activity ratio in the PGL-3 K2 WT group increased significantly (1.227±0.084 vs. 0.999±0.143, P=0.010), while this ratio decreased in the PGL-3 K2 Mut group (0.795±0.084 vs. 0.999±0.143, P=0.016) (Figure 3B). Furthermore, the ChIP assay using anti-NF-κB confirmed that NF-κB can bind to Kindlin-2 promoter region (Figure 3C, Supplementary Figure S2A,B).

**CD163 and Kindlin-2 were overexpressed in GC tissues, and high expression of CD163 and Kindlin-2 was positively associated with TNM stage and poor prognosis in GC patients**

To establish the pathological significance and clinical relevance of Kindlin-2 in GC patients, immunohistochemical staining assays for CD68, CD163, TGFβ2 and Kindlin-2 were performed on tissue microarrays containing 180 tissues from 100 GC patients (80 pairs of cancer/normal tissues, along with another 20 cancer tissues), with a median survival time of 46.6 (range, 2.0–99.0) months. The immunohistochemical staining score, accounting for both the intensity of staining and the proportion of tumor cells with an unequivocal positive reaction, was calculated for each tissue. The expression of CD163 (42.00% vs. 26.25%, P=0.029) and Kindlin-2 (54.00% vs. 21.25%, P<0.001) was higher in GC tissues than in normal tissues, while the protein level of CD68 (46.00% vs. 57.50%, P=0.125) and TGFβ2 (35.00% vs. 30.00%, P=0.478) was not significantly different between the two groups (Figure 4A, Table 3).

The KAPS algorithm in R software was used to calculate the most efficient cut-off values for the expression of four proteins (15–17), and we found that CD163 (cut-off value=3, P=0.016), TGFβ2 (cut-off value=3, P=0.007) and Kindlin-2 (cut-off value=4, P<0.001) could significantly distinguish the outcomes of these GC patients. A univariate analysis showed that high expression of CD163 (P=0.019) and Kindlin-2 (P=0.025) was positively associated with higher tumor burden as defined by TNM stage (Table 4).
Figure 4 CD163 and Kindlin-2 were overexpressed in gastric cancer (GC) tissues and high CD163 and Kindlin-2 expression was positively associated with TNM stage and poor prognosis in GC patients. (A) Immunohistochemical staining for CD68, CD163, TGFβ2 and Kindlin-2. (×60, ×200); (B) CD163 and Kindlin-2 expression levels were higher in GC tissues than in normal tissues, while CD68 and TGFβ2 expression levels were not significantly different between the two groups, as determined by comparing the immunohistochemical staining scores (CD163, 42.00% vs. 26.25%, P=0.029; Kindlin-2, 54.00% vs. 21.25%, P<0.001; CD68, 46.00% vs. 57.50%, P=0.125 and TGFβ2, 35.00% vs. 30.00%, P=0.478); (C) Reduced overall survival (OS) was positively associated with high expression levels of CD163, TGFβ2 and Kindlin-2 in GC patients (CD163, cut-off value=3, P=0.016; TGFβ2, cut-off value=3, P=0.007 and Kindlin-2, cut-off value=4, P<0.001), *, P<0.01; **, P<0.05.
Furthermore, a Kaplan-Meier survival analysis indicated that reduced OS was positively associated with high expression levels of CD163 (P=0.016), TGF\(\beta\)2 (P=0.007) and Kindlin-2 (P<0.001) in GC patients (Figure 4C), and the multivariate analysis revealed that Kindlin-2 (P<0.001) was an independent prognostic parameter for OS (Table 5).

Invasion mechanisms were verified in vivo in a nude mouse oncogenesis model

Stably transfected Kindlin-2 knockdown cell lines were generated containing green fluorescence. The expression of Kindlin-2 was significantly higher in the stably transfected group 02 (Kindlin-2\(^{-}\)) than the negative control group 01 (Kindlin-2\(^{+}\)) (1.00±0.02 vs. 0.23±0.09, P<0.001) (Figure 5A).

### Table 3 Comparative analyses of four protein expression levels in tumor and normal tissues

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<tr>
<th>Protein</th>
<th>Cases (n)</th>
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<th>High [n (%)]</th>
<th>(\chi^2)</th>
<th>P</th>
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TGF\(\beta\)2, transforming growth factor \(\beta\)2.

### Table 4 Correlation analyses of CD163 and Kindlin2 expression in relation to clinicopathological characteristics of 100 GC patients

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<th>Characteristics</th>
<th>Cases (n)</th>
<th>CD163 expression</th>
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GC, gastric cancer.
The pre-experiment revealed that the tumorigenesis ability increased by Kindlin-2 overexpression and decreased by TGFβ inhibitor (Supplementary Figure S3A). In formal experiment, Kindlin-2 knockdown cells (group 02), as well as Kindlin-2 expressing cells (group 01 and group 03), were injected into the subcutaneous tissues of nude mice. Group 01 and group 02 mice received a subcutaneous injection of TGFβ2 once a week, while group 03 mice received an injection of SB431542. Tumor volumes and body weight were measured and recorded three times per week (Figure 5B). When the average tumor size reached 2,000 mm$^3$, the mice were sacrificed, and the volume and weight of tumors were examined. Tumors formed in all mice, but tumors in group 01 (TGFβ2/Kindlin-2+ group) were larger (2,251.38±469.10 mm$^3$ vs. 1,530.63±627.78 mm$^3$, P=0.045; 2,251.38±469.10 mm$^3$ vs. 380.43±23.28 mm$^3$, P<0.001) and heavier (1,468.35±358.73 mg vs. 948.10±443.81 mg, P=0.047; 1,468.35±358.73 mg vs. 468.19±45.11 mg, P<0.001) than other groups (group 02, TGFβ2/Kindlin-2− group; group 03, SB431542/Kindlin-2+ group) (Figure 5C). The tumors were also examined by hematoxylin and eosin (HE) staining (Figure 5D), and immunohistochemistry (IHC) staining in the border of tumors showed that the expression of several invasion-related markers was significantly different in group 01 compared with group 02 (Supplementary Figure S3B). Two mice in group 01 exhibited suspected nodes in the lung, but they were not confirmed as metastatic tumors by HE staining (Supplementary Figure S3C,D).

**Discussion**

TAMs are abundant in the tumor microenvironment and play important roles in tumor progression (18). Recent
Figure 5 Invasion mechanisms were verified *in vivo* in a nude mouse oncogenesis model. (A) Green fluorescence was observed under a fluorescence microscope in the stably transfected Kindlin-2 knockdown group. Kindlin-2 expression was significantly higher in the stably transfected group than in the negative control group (×400); (B) Tumor volumes and body weights were measured and recorded three times per week; (C) When the average tumor size reached 2,000 mm$^3$, the mice were sacrificed, and the volume and weight of tumors were examined. Tumors formed in all mice, but the tumors in group 01 (TGFβ2/Kindlin-2+ group) were larger (2,251.38±469.10 mm$^3$ vs. 1,530.63±627.78 mm$^3$, P=0.045; 2,251.38±469.10 mm$^3$ vs. 380.43±23.28 mm$^3$, P<0.001) and heavier (1,468.35±358.73 mg vs. 948.10±443.81 mg, P=0.047; 1,468.35±358.73 mg vs. 468.19±45.11 mg, P<0.001) than those in the other groups (group 02, TGFβ2/Kindlin-2− group; group 03, SB431542/Kindlin-2+ group); (D) Tumors were examined by hematoxylin and eosin (HE) staining (×400). TGFβ2, transforming growth factor β2.
studies have shown that TAMs develop from macrophages stimulated with CSF-1, CCL2, VEGF, IL-10, IL-6 and other factors secreted by tumor cells (19-22); in turn, TAMs inhibit the immune response and promote tumor angiogenesis (23). Moreover, chronic inflammation mediated by TAMs is closely related to tumor invasion and metastasis (24). Schmieder et al. found that M2 macrophages play a positive role in tumor progression (25) and Yan et al. demonstrated that high level of TAM infiltration is related to aggressive characteristics and is an independent prognostic factor in GC (26). In our study, we successfully induced the differentiation of M1 macrophages and M2 macrophages. We identified M2 macrophages as TAMs by flow cytometry: CD163, CD206, CD11b are highly expressed on M2 macrophages (27,28), and CD86 is substantially expressed on M1 macrophages (29).

TGFβ2 belongs to multifunctional transforming growth factor superfamily that regulates cell growth, differentiation and matrix production (30,31). High TGFβ2 expression correlates with malignancy in many cancers (32). NF-κB, a multisubunit eukaryotic transcription factor consisting of either homo- or heterodimers of various members of the Rel family, such as p50, p52, p65 (RelA), c-Rel and RelB (33), participates in a major pathway mediating a complex of matrix metalloproteinases and inflammatory factors and plays important roles in DNA transcription, cytokine production, cell survival, and inflammatory responses (34,35). Furthermore, several studies have demonstrated that NF-κB is involved in many signaling pathways active in various cancers (36-38). Our previous study found that the expression of TGFβ2 was significantly increased when GC cells were cocultured with TAMs. Our current study reported that TGFβ2 could increase the invasive ability of GC cells through promoting Kindlin-2 expression. Furthermore, we explored the binding sites of the transcription factor NF-κB in the Kindlin-2 gene promoter region using dual-luciferase and ChIP assays. Several studies have shown that Kindlin-2 interacts with numerous signaling pathways, such as the Wnt/β-catenin (39), TGFβ/Smad (40) and TGFβ-HOXB9-paxillin signaling pathway (41). To the best of our knowledge, this study first reported the role of TGFβ2/NF-κB/Kindlin-2 signaling axis influencing cancer progression.

The deregulation of Kindlin-2 is associated with prognosis in various human cancers (42,43). Our study examined four typical markers of tumor microenvironment to establish their pathological significance and clinical relevance in GC patients. We found that CD163 and Kindlin-2 protein levels were higher in GC tumor tissues than normal tissues and that CD163 (cut-off value=3), TGFβ2 (cut-off value=3) and Kindlin-2 (cut-off value=4) could most significantly distinguish the outcomes of these GC patients. We noticed there was no significant difference in TGFβ2 between tumor and normal tissues, and the difference in Kindlin-2 expression was modest, although both had remarkable effects on survival time. To explain these observations, we noticed that the data used for the survival related analysis were only the scores for TGFβ2/Kindlin-2 in tumor tissues, and the expression analysis compared the difference between tumor and normal tissues. Moreover, the analysis of different expressions focused mainly on determining whether the marker could be used for diagnosis, while the survival analysis aimed to determine whether the marker could be used for judging prognosis; interaction might exist between them, but the relationship was not absolute. A future in-depth study with a larger sample size will be required to explore the potential reasons. Through univariate analysis, we found that high expression of CD163 and Kindlin-2 was positively associated with higher TNM stage and reduced OS. Additionally, the multivariate analysis indicated that Kindlin-2 was an independent prognostic parameter for OS. Hence, we demonstrated that Kindlin-2 could be an appropriate biomarker for GC detection and outcome prediction.

Integrins are obligate heterodimers composed of α and β subunits (44). Many studies have shown that integrins can considerably affect the survival, growth, proliferation, differentiation, invasion and metastasis of tumor cells through various pathways (45,46). Kindlin-2 has been reported to play a crucial role in the activation of β1 and β3 integrins through enhancing integrin phosphorylation (47). Several studies have suggested that integrin expression is related to NF-κB activation. For example, Lamb et al. (48) showed that the upregulation of integrin α6β1 increased NF-κB activation in prostate cancer. In addition, Kiefel et al. (49) found that the interaction of integrin α5 with the L1 cell adhesion molecule resulted in the constitutive activation of NF-κB in pancreatic adenocarcinoma cells. We found that NF-κB can promote the expression of Kindlin-2. If Kindlin-2 can phosphorylate integrins and subsequently activate NF-κB, a regulatory feedback mechanism might exist among NF-κB, Kindlin-2 and integrin. However, further study is needed to confirm this hypothesis.
Conclusions

We report a novel finding that Kindlin-2 is highly expressed in GC and is positively associated with GC cell invasion and metastasis. Further findings demonstrated that TAMs participate in the progression of GC through the TGFβ2/NF-κB/Kindlin-2 axis, and that Kindlin-2 is a novel putative target for the treatment of GC. This work strengthens the general link between Kindlin-2 expression and malignancy, thus creating new options and approaches for further investigation.

Acknowledgements

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

References


48. Lamb LE, Zarif JC, Miranti CK. The androgen receptor induces integrin α6β1 to promote prostate tumor cell survival via NF-κB and Bcl-xL independently of PI3K signaling. Cancer Res 2011;71:2739-49.


**Figure S1** Comparison of Kindlin-2 expression or cell invasive ability among different groups. (A) Comparison of Kindlin-2 mRNA expression among GC, TAM/GC, TAM/GC(siK2), TGFβ2/GC, TAM/GC+LY2157299, and TAM/GC(siK2)+LY2157299 groups of cells. (GC, 0.98±0.04; TAM/GC, 4.54±0.08; TAM/GC(siK2), 0.32±0.02; TGFβ2/GC, 5.14±0.11; TAM/GC+LY2157299, 0.28±0.12 and TAM/GC(siK2)+LY2157299, 0.27±0.01, P<0.001); (B) Scratch assay comparing the cell invasive ability among GC, TAM/GC, TAM/GC(siK2), TGFβ2/GC, TAM/GC+SB431542, and TAMs/GC(siK2)+SB431542 groups of cells [GC, (8.3±2.1)%; TAM/GC, (83.3±3.9)%; TAM/GC(siK2), (43.2±7.2)%; TGFβ2/GC, (44.3±1.9)%; TAM/GC+SB431542, (78.3±2.9)% and TAMs/GC (siK2)+SB431542, (9.3±2.0)%, P<0.001] (x100); (C) Using migration assay to compare the cell invasive ability among GC, TAM/GC, TAM/GC(siK2), TGFβ2/GC, TAM/GC+SB431542, and TAMs/GC(siK2)+SB431542 groups of cells [GC, (5.8±1.1)%; TAM/GC, (84.3±2.9)%; TAM/GC(siK2), (40.2±7.4)%; TGFβ2/GC, (38.3±3.7)%; TAM/GC+SB431542, (58.3±3.1)% and TAMs/GC(siK2)+SB431542, (9.6±1.7)%, P<0.001] (x400). GC, gastric cancer; TGFβ2, transforming growth factor β2; TAM, tumor-associated macrophage. *, P<0.05.
Figure S2 Amplification curve and melting curve of chromatin immunoprecipitation (ChIP) assay. (A) Amplification curve of ChIP assay; (B) Melting curve of ChIP assay.
Figure S3 Evaluation of tumorigenesis abilities in preliminary experiments, and study of pulmonary nodules in formal experiments. (A) Different tumorigenesis abilities were tested in three groups, Kindlin-2 overexpression group, Kindlin-2 normal expression group and SB431542 adding group (named group $\alpha$, $\beta$ and $\gamma$, respectively); (B) Immunohistochemistry (IHC) staining in the border of tumors showed several invasion-related marker proteins that were significantly different in group 01 compared with group 02 ($\times100$); (C) Two suspected lung metastatic tumors were found in group 01 mice; (D) The suspected lung metastatic tumors were examined by hematoxylin and eosin (HE) staining ($\times100$, $\times400$).