Upregulation of miRNA-301a-3p promotes tumor progression in gastric cancer by suppressing NKRF and activating NF-κB signaling

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Abstract. MicroRNA-301a (miRNA/miR-301a) and nuclear factor (NF)-κB signaling play important roles in tumor invasion, migration and progression. However, the role of miRNA-301a-3p in human gastric cancer (GC), and specifically in the activation of NF-κB signaling, remains unclear. The aim of the present study was to investigate miRNA-301a-3p expression in GC progression and the molecular mechanisms as regards the regulation of NF-κB signaling. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to detect miRNA-301a-3p expression in GC and paired normal tissues. The association between the expression of miRNA-301a-3p and patient pathological parameters and the prognosis of GC was statistically analyzed using an *in situ* hybridization (ISH) assay. An MTS assay and a Transwell assay were performed to evaluate the effects of miRNA-301a-3p on the proliferation, invasion and migration of GC cells. RT-qPCR and western blot analysis were used to analyze the association between miRNA-301a-3p and nuclear factor-κB repressing factor (NKRF) expression and the corresponding downstream NF-κB signaling molecules. A luciferase assay was used to verify the target effect of miRNA-301a-3p and NKRF. It was found that miRNA-301a-3p expression was significantly higher in 30 cases of primary GC compared with matched normal tissues. Additionally, the ISH assay indicated that the high expression of miRNA-301a-3p in GC was associated with tumor invasion depth, lymph node metastasis, lymph node invasion and tumor metastasis stage. Patients whose tumors had a higher miRNA-301a-3p expression level exhibited a poorer prognosis. The *in vitro* assay indicated that miRNA-301a-3p affected the proliferative and invasive ability of GC cells by targeting the expression of NKRF, which then affected NF-κB signaling. Therefore, it was hypothesize that miRNA-301a-3p promotes GC progression and affects the prognosis of patients with GC by targeting NKRF, which in turn, directly influences NF-κB activation.

Introduction

Gastric cancer (GC) is the third leading cause of cancer-related mortality worldwide following lung and liver cancer (1). Currently, the highest incidence and mortality rates of GC are observed in Eastern Asia and Latin America (2,3). The majority of GC-related deaths are caused by cancer cell invasion, metastasis and tumor recurrence (4). Thus, to gain a better understanding of the pathogenesis of GC, the underlying mechanisms of GC progression are worthy of further exploration.

The nuclear factor-κB (NF-κB) transcription factor plays a pivotal role in tumor inflammation, cell differentiation, proliferation and survival. In addition to its roles in immunity, NF-κB activity is frequently detected in a number of cancer types (5,6). During tumorigenesis and tumor development, tumor cells have been reported to respond to chronic inflammatory stimuli within the tumor microenvironment, which is dependent on NF-κB activation (7,8). Previous studies have demonstrated that NF-κB signaling is constitutively activated in a variety of human cancer types and is associated with tumor initiation, progression and metastasis (9-11). The NF-κB complex refers to a group of transcription factors (RelA, RelB, c-Rel, NF-κB1/p50 and NF-κB2/p52), which form homo or heterodimers and can upregulate or suppress the expression of a number of genes during cancer progression (11,12). In response to a variety of stimuli, the activation of NF-κB signaling promotes tumor cells to produce numerous tumorigenic factors, including the angiogenesis regulator, vascular

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endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-2, MMP-9, the chemokines, interleukin (IL)-1, IL-8, tumor necrosis factor (TNF), IL-6 and monocyte chemoattractant protein-1 (MCP-1), and the anti-apoptotic factors, Bcl-xL and cellular inhibitor of apoptosis proteins (cIAPs), which are essential to tumor progression (12). Helbig *et al* (13) found that NF-κB promoted breast cancer cell invasiveness by increasing CXCR4 expression. Furthermore, the aberrant activation of NF-κB signaling promotes lung tumorigenesis via the induction of angiogenesis-related factors, such as VEGF and IL-8 (14). In addition to these findings, accumulating evidence has indicated that the activation of NF-κB signaling is essential for the bone metastasis of prostate cancers (15,16). It has previously been demonstrated that the NF-κB signaling system is also deregulated in GC (17). Further research has revealed that RelA and NF-κB1/p50 are upregulated in GC and cancer cell lines and that the expression of these proteins in GC tissue is strongly associated with the abundance of other tumor- or metastasis-promoting markers, including signal transducer and activator of transcription (STAT)3, MMP-2 (18,19), cyclooxygenase (COX)2 and VEGF (20,21). In previous studies, the siRNA-mediated knockdown of RelA and NF-κB1/p50 exerted an anti-tumor effect both *in vitro* and *in vivo* (22,23). These results indicate that the NF-κB signaling pathway may serve as a therapeutic target for the treatment of GC. However, the underlying mechanisms of the constitutive activation of NF-κB signaling in GC remain poorly understood.

MicroRNAs (miRNAs or miRs), which are a series of small non-coding RNAs composed of 18-24 nucleotides, function in mRNA degradation and the post-transcriptional regulation of target genes by specific binding to their 3'-untranslated region (3'-UTR) (24,25). Abundant evidence has indicated that the aberrant expression of miRNAs affects the capacity of cancer cells to invade, migrate and metastasize (26,27). Moreover, miRNAs have also been reported to serve as a modulator of the NF-κB pathway. For example, miR-199a has been shown to activate the NK-κB pathway and to be associated with the tumor inflammatory microenvironment by regulating IKKβ (28). miR-146 also plays regulatory roles in the NF-κB pathway, as it negatively regulates the protein levels of IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) (29,30). miRNA-301a, which is located on chromosome 17q22, has been shown to be upregulated in a number of types of cancer, including hepatocellular carcinoma, pancreatic cancer, small cell lung cancer and breast cancer, which indicates a potential role for miRNA-301a in cancer development (31-34). In GC, Wang *et al* (35) reported that the high expression of miRNA-301a was associated with GC cell proliferation and invasion by targeting Runt-related transcription factor 3 (RUNX3). In a previous study by the authors, it was also found that the abnormal expression of miRNA-301a-3p in GC was associated with progression and a poor prognosis (36). However, the underlying biological processes and molecular mechanisms of action of miRNA-301a-3p in GC, particularly as regards the regulation of the NK-κB pathway, remain poorly understood.

In the present study, it was first found that the upregulation of miRNA-301a-3p in GC was associated with tumor progression and a worse prognosis. The function and molecular mechanisms of miRNA-301a-3p were also investigated. An *in vitro* assay indicated that the suppression of miRNA-301a-3p attenuated cancer cell growth and migration, as well as tumor progression. Additionally, the miRanda database was searched and it was found that NF-κB repressing factor (NKRF) was a candidate target gene of miRNA-301a-3p. A previous study indicated that NKRF was involved in the negative regulation of NF-κB (37). These results demonstrated that the upregulation of miRNA-301a-3p contributed to tumor progression in GC by regulating NKRF expression, which led to the induction of NF-κB activation and tumor growth. Therefore, this NF-κB activation mechanism may be a target for therapeutic intervention in GC.

Materials and methods

Samples and patients. All fresh GC tissue and paired adjacent non-cancerous samples were collected after obtaining written informed consent from 30 patients who underwent resection for GC at the Zhejiang Provincial People's Hospital from 2012 to 2013 (clinical characteristics are presented in Table SI). At the same time, a GC tissue microarray (TMA) containing 120 GC tissue samples was collected at the Zhejiang Provincial People's Hospital from 2012 to 2013 and used for the *in situ* hybridization (ISH) detection of miRNA-301a-3p (the characteristics of the cases are presented in Table I). The histological tumor type for all GC cases was diagnosed by 3 independent pathologists, and all cases were classified according to the American Joint Committee on Cancer (AJCC) classification (8th and 7th editions) of GC tumors (38,39). The matched normal gastric epithelial tissues, which were collected from an area at distance of >5 cm from the tumors, were also verified at the same time. None of the patients had received chemotherapy prior to surgery. The study design and method were approved by the Ethics Committee of Zhejiang Provincial People's Hospital. Upon admission, all patients or their relatives provided informed consent within the written treatment contract prior to their inclusion in the study. All patients were followed-up for >5 years or until December, 2018. The survival time was calculated from the date of surgery to the end of the follow-up period and/or the date of death. The age of the patients with GC ranged from 29 to 82 years (median age, 61.22 years). The clinicopathological characteristics of the patients with GC are summarized in Table I.

ISH detection of miRNA-301a-3p in a GC. For the ISH detection of miRNA-301a-3p in GC tissues, 5-μm-thick sections of the GC TMA were used. The sections were deparaffinized, rehydrated and subjected to ISH signal detection, as previously described (36). Briefly, a digoxin-labeled LNA-miRNA-301a miRCURY probe (Qiagen, Inc.) was used to detect miRNA-301a-3p expression in GC tissues. ISH was performed using a Dig Labeled Probe Detection kit I (POD) (MK1003, Boster Biological Technology) according to the manufacturer's instructions. For each sample, the immunoreactivity levels of miRNA-301a-3p were estimated under a light microscope by assessing the average signal intensity (on a scale of 0-3). The proportion of cells that indicated positive staining (0, <5%; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%) was independently estimated by 2 pathologists in the absence of clinical information, as previously described (36). The intensity and

Table I. Association between miRNA-301a expression and clinicopathological factors.

| Clinical parameters | miR-301a expression | | t/χ^2 | P-value |
	Low	High		
Age (years)	60.02±12.97	61.96±10.97	2.266	0.135
Sex			1.984	0.159
Male	30 (65.2%)	57 (77.0%)		
Female	16 (34.8%)	17 (23.0%)		
Location			4.347	0.114
Proximal	7 (15.2%)	12 (16.2%)		
Middle	24 (52.2%)	25 (33.8%)		
Distal	15 (32.6%)	37 (50.0%)		
Tumor size			4.971	0.026
≥5 cm	19 (41.3%)	46 (62.2%)		
<5 cm	27 (58.7%)	28 (37.8%)		
Histological type			0.509	0.917
Papillary adenocarcinoma	1 (2.2%)	2 (2.7%)		
Tubular adenocarcinoma	36 (78.3%)	56 (75.7%)		
Mucinous adenocarcinoma	2 (4.3%)	2 (2.7%)		
Signet-ring cell carcinoma	7 (15.2%)	14 (18.9%)		
Lauren classification			6.398	0.011
Diffuse type	25 (54.3%)	23 (31.1%)		
Intestinal type	21 (45.7%)	51 (68.9%)		
Differentiation			5.530	0.063
Well	5 (10.9%)	1 (1.4%)		
Moderately	10 (21.7%)	20 (27.0%)		
Poorly	31 (67.4%)	53 (71.6%)		
Invasion depth (T grade)			31.685	6.10E-07
T1	11 (23.9%)	2 (2.7%)		
T2	25 (54.3%)	19 (25.7%)		
T3	9 (19.6%)	45 (60.8%)		
T4	1 (3.2%)	8 (10.8%)		
Lymphatic metastasis (N grade)			17.880	4.66E-04
N0	25 (54.3%)	22 (29.7%)		
N1	0 (0.0%)	6 (8.1%)		
N2	19 (41.3%)	24 (32.4%)		
N3	2 (4.3%)	22 (29.7%)		
Distant metastasis (M grade)			8.588	0.003
M0	40 (87.0%)	46 (62.2%)		
M1	6 (13.0%)	28 (37.8%)		
TNM stage			32.346	4.42E-07
I	21 (45.7%)	5 (6.8%)		
II	17 (37.0%)	24 (32.4%)		
III	2 (4.3%)	17 (23.0%)		
IV	6 (13.0%)	28 (37.8%)		
TNM stage			21.687	3.21E-06
I + II	38 (82.6%)	29 (39.2%)		
III + IV	8 (17.4%)	45 (60.8%)		
Lymphatic invasion			3.487	0.062
Yes	11 (23.9%)	30 (40.5%)		
No	35 (76.1%)	44 (59.5%)		

Table I. Continued.

Clinical parameters	miR-301a expression		t/χ²	P-value
	Low	High		
Vascular invasion			10.042	0.002
No	25 (54.3%)	19 (25.7%)		
Yes	21 (45.7%)	55 (74.3%)		

All cases were classified according to the AJCC (2016, 8th version) pathological classification of gastric cancer. Invasion depth (T grade) grade T4 includes T4a and T4b. Lymphatic metastasis (N grade) grade N3 includes N3a and N3b. TNM grade I includes Ia and Ib, TNM grade II includes IIa and IIb, and TNM grade III includes IIIa, IIIb and IIIc.

percentage scores were subsequently multiplied to obtain a composite score; a score of 0 to 3 was defined as negative, while a score of 4 to 12 was defined as positive.

Cells and cell culture. Human GC cell lines (AGS, MKN-45, HGC-27 and GES-1) were purchased from the Cell Bank of Shanghai Institute of Cell Biology and cultured in RPMI-1640 (HyClone; GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) at 37°C in a humidified atmosphere containing 5% CO_2.

Transfection with miRNA inhibitor. The AGS and MKN-45 cells ($1x10^5$ cells per well) were seeded in 6-well plates. After 24 h, the cells were transfected with a micrOFF miRNA-301a inhibitor (miR21111890035-1-5, RiboBio), micrON hsa-miR-301a-5p mimic (miR10022696-1-5, RiboBio) and the corresponding negative controls at a final concentration of 50 nM using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, respectively. Following transfection for 48 h, the cells were collected for use in further assays, such as reverse transcription-quantitative polymerase chain reaction (RT-qPCR), cell proliferation assay, invasion assay and western blot analysis.

Inhibition of NF-κB activities by dehydroxymethylepoxyquinomicin (DHMEQ). DHMEQ is a potent NF-κB inhibitor and it was dissolved in dimethyl sulfoxide (DMSO) at 20 mg/ml and maintained in aliquots at -30°C. Prior to use in the MKN-45 cell culture to inhibit NF-κB activities, it was diluted with the medium to a final concentration of 15 μg/ml and treated for 24 h to abrogate constitutive NF-κB activity and downregulate NF-κB transcription.

Immunofluorescence assay. MKN-45 cells were seeded onto glass coverslips in 12-well plates at $1x10^4$ cells/well. After 24 h, the cells were pre-treated with 15 μg/ml of DHMEQ for 16 h. The cells were then washed twice with cold PBS (PBS) and fixed with formaldehyde for 20 min, and then permeabilized with 0.1% Triton X-100 in PBS for 20 min. After blocking with blocking buffer (1% BSA in TBST) for 30 min, the cells were then incubated with NF-κB p65 antibody (dilution, 1:500; cat. no. 8242, Cell Signaling Technology, Inc.) overnight. After washing with TBST 3 times, the cells were incubated the Cy3-labled secondary antibody (dilution, 1:5,000; cat.

no. A0516, Beyotime Institute of Biotechnology, Inc.) for 1 h at room temperature and counterstained with DAPI (cat. no. C1005, Beyotime Institute of Biotechnology, Inc.) for 5 min at room temperature. Fluorescence images were captured on a Leica SP2 confocal microscope (Leica Microsystems GmbH).

Luciferase reporter assays. The miRBase Targets (http://www.mirbase.org/), TargetScan Release 5.0 (http://www.targetscan.org/vert_72/) and PicTar databases (https://pictar.mdc-berlin.de/) were searched and found that NKRF may be a possible target of miRNA-301a-3p. The pYr-mirTarget-NKRF-3'UTR-WT (site1, site2 and site3) and the corresponding mutated luciferase vector, which contained the putative binding site or mutated site of miRNA-301a-3p, were purchased from Yinrun Biotechnology. 293 cells were purchased from the Chinese Academy Of Sciences Cell Bank and seeded in 96-well plates. The cells were then co-transfected with the wild-type (WT) or mutated (Mut) reporter plasmids using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Inc.) along with the miRNA-301a-3p inhibitor (100 nM). After 48 h, the luciferase activity was assessed using the DualGlo Luciferase Assay System (Promega Corp.) according to the manufacturer's instructions on a Sirius single tube luminometer (Berthold Technologies, GmbH & Co. KG,). The Firefly luciferase activity was normalized to *Renilla* luciferase activity for each transfected well. All transfection experiments were conducted in triplicate and were repeated independently 3 times.

To further confirm NKRF as a target gene of miRNA-301a-3p in GC cells, following the transfection of GC cells (AGS and MKN-45) with the miRNA-301a-3p inhibitor and mimic, the mRNA and protein expression levels of NKRF and downstream molecules in NF-κB signaling were also assessed by RT-qPCR and western blot analysis, respectively.

RNA isolation and RT-qPCR. Total RNA was isolated from the tissue samples and GC cells according to the protocol of the RNAsimple Total RNA kit [DP419; Tiangen Biotech (Beijing) Co., Ltd.]. Reverse transcription was performed using a One-step PrimeScript miRNA cDNA synthesis kit [D350A; Takara Biotechnology (Dalian) Co., Ltd]. and a PrimeScript™ RT reagent kit with gDNA Eraser [RR047A; Takara Biotechnology (Dalian) Co., Ltd.]. To detect the expression levels of miRNA-301a-3p, NKRF, NF-κB, VEGF, MMP-2 and MMP-9, RT-qPCR was performed on an

Table II. Sequences of the primers used in the present study.

Primer name	Sequence (5'-3')	Annealing temperature (˚C)
miRNA-301a-3p	CAGTGCAATAGTATTGTCAAAGC	58
U6B	CGCTTCACGAATTTGCGTGTCAT	58
GAPDH-F	ACAACTTTGGTATCGTGGAAGG	50-60
GAPDH-F	GCCATCACGCCACAGTTTC	50-60
NKRF-F	GTCAAAAACGCCACCTCTCAA	55
NKRF-R	CTCGCATGGAATTTGGAACCG	55
NF-κB-F	GGTGCGGCTCATGTTTACAG	58
NF-κB-R	GATGGCGTCTGATACCACGG	58
VEGF-F	AGGGCAGAATCATCACGAAGT	58
VEGF-R	AGGGTCTCGATTGGATGGCA	58
MMP-9-F	TGTACCGCTATGGTTACACTCG	56
MMP-9-R	GGCAGGGACAGTTGCTTCT	56
MMP-2-F	TACAGGATCATTGGCTACACACC	58
MMP-2-R	GGTCACATCGCTCCAGACT	58
CXCL8-F	ACTGAGAGTGATTGAGAGTGGAC	55
CXCL8-R	AACCCTCTGCACCCAGTTTTC	55
PTGS2-F	TAAGTGCGATTGTACCCGGAC	56
PTGS2-R	TTTGTAGCCATAGTCAGCATTGT	56
STAT3-F	ACCAGCAGTATAGCCGCTTC	58
STAT3-F	GCCACAATCCGGGCAATCT	58
IL-6-R	ACTCACCTCTTCAGAACGAATTG	55
IL-6-R	CCATCTTTGGAAGGTTCAGGTTG	55
c-FLIP-F	TGCTCTTTTTGTGCCGGGAT	55
c-FLIP-R	CGACAGACAGCTTACCTCTTTC	55
BCL2L1-F	GAGCTGGTGGTTGACTTTCTC	58
BCL2L1-R	TCCATCTCCGATTCAGTCCCT	58
BIRC2-F	GAATCTGGTTTCAGCTAGTCTGG	58
BIRC2-R	GGTGGGAGATAATGAATGTGCAA	58
CCL2-F	AGAATCACCAGCAGCAAGTGTCC	56
CCL2-R	TCCTGAACCCACTTCTGCTTGG	56

F, forward; R, reverse; NKRF, nuclear factor-κB repressing factor; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; STAT3, signal transducer and activator of transcription; IL, interleukin; BIRC2, baculoviral IAP repeat containing 2, also known as, c-IAP1; BCL2L1, BCL2 like 1, also known as, Bcl-xl; CXCL8, C-X-C motif chemokine ligand 8, also known as IL8; CCL2, C-C motif chemokine ligand 2, also known as MCP-1.

MX3000P system (Stratagene; Agilent Technologies, Inc.) using gene-specific primers with a SYBR Premix ExTaq kit [DRR081A; Takara Biotechnology (Dalian) Co., Ltd.]. All reactions were performed in triplicate. U6 (RNU6B) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal standards for normalization of the gene expression levels. The primers of candidate genes were selected from PrimerBank (https://pga.mgh.harvard.edu/primerbank/) and are listed in Table II. The qPCR reaction conditions were as follows: Initial denaturation (4 min at 95˚C) and then 40 cycles of denaturation at 95˚C for 20 sec, annealing at 58˚C for 20 sec, and extension at 72˚C for 20 sec. The relative expression levels were calculated using the $2^{-\Delta\Delta cq}$ method (40).

Cell proliferation assay. Cell proliferation was assessed using the Cell Proliferation MTS Assay kit (G3580, Promega Corp.) following the manufacturer's protocol. All cell lines (pre-transfected with the miRNA-301a-3p inhibitor, mimic, and the corresponding negative control cells) were seeded into 96-well plates with 3×10^3 cells in 200 μl culture medium per well. Following attachment, 20 μl of MTS reagents was added to each well every 24 h. Following an additional 4-h incubation, the absorbance was measured at 570 nm using a Tecan Infinite 200 microplate reader (Tecan Group Ltd. Switzerland.)

Migration and invasion assays. The migration assay was performed using Transwell plates containing membranes with 8 μm pores (3422; Corning Inc.). Cell invasion assays were performed using invasion chambers precoated with Matrigel (354480; BD Biosciences). The GC cells, which were pre-transfected with the miRNA-301a-3p inhibitor or mimic or 15 μg/ml DHMEQ, and the controls (2×10^5 for
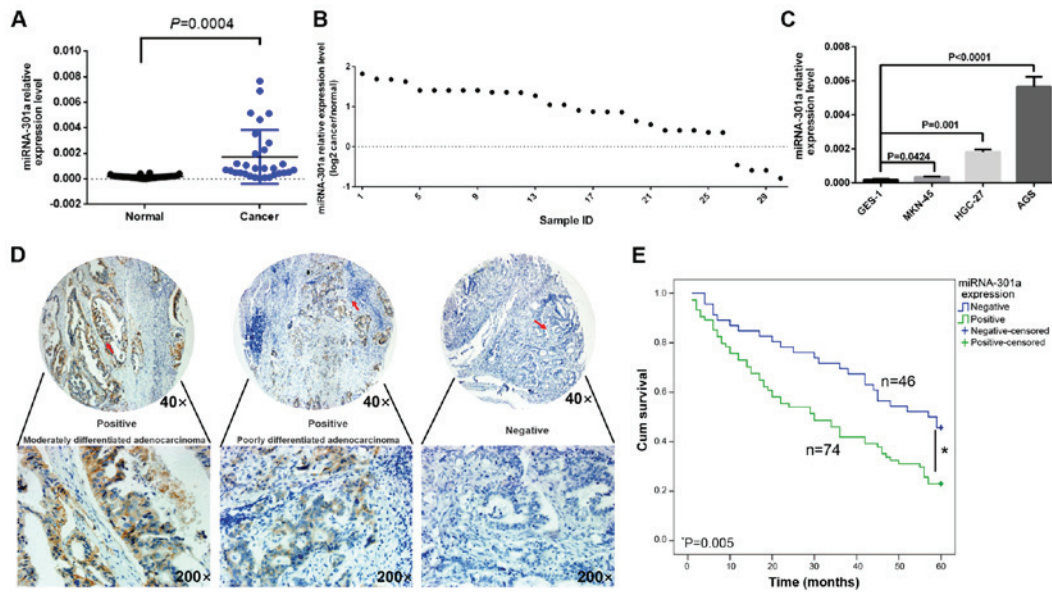
Figure 1. Expression of miRNA-301a-3p in GC tissue and Kaplan-Meier survival curves of patients with GC. (A and B) Expression of miRNA-301a-3p in 30 paired GC tissues and normal tissues. (C) Expression of miRNA-301a-3p in GC cell lines. (D) miRNA-301a-3p expression in GC tissues as shown by ISH assay. The magnification of the images in the top panel is x40 and that of the images in the bottom panel is x200. The bottom images are an enlarged version of the area indicated by the red arrow in the corresponding images in the top panels. (E) Kaplan-Meier survival curves of GC with miRNA-301a-3p expression (*P<0.05). GC, gastric cancer; ISH, in situ hybridization.

invasion assays and 5x10⁴ cells for migration assays) were resuspended in serum-free medium and seeded into the upper chamber. RPMI-1640 medium containing 20% FBS was added to the lower chamber as a chemoattractant. After 24 or 48 h, the cells were fixed and stained. Non-invading cells in the upper chambers were removed with cotton swabs. The number of migrating or invading cells that had attached to the lower surface was then counted in 5 random fields under an Olympus IX71 microscope (x200 magnification, Olympus Corp.).

Western blot analysis. Briefly, protein was extracted from the cells using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology). Sample protein concentrations were quantitated using the BCA method and 30 μg total protein per lane were separated by 10% SDS-PAGE and then transferred onto PVDF membranes. The PVDF membranes were blocked with 5% BSA for 2 h and then incubated with primary anti-human antibodies against NKRF (ab168829, at 1:1,000 dilution; Abcam), MMP-9 (EM1801-22, at 1:500 dilution, HuaBio Inc.), MMP-2 (ER40806, at 1:1,000 dilution, HuaBio Inc.), VEGF (ER30607, at 1:1,000 dilution, HuaBio Inc.), NF-κB-p65 (D14E12, #8242, at 1:2,000 dilution, Cell Signaling Technology, Inc.) and GAPDH (M1310-2, at 1:3,000 dilution; HuaBio Inc.) overnight at 4°C. The membranes were incubated with the corresponding horseradish peroxidase (HRP)-labeled goat anti-rabbit (A0208, at 1:10,000 dilution; Beyotime Institute of Biotechnology Inc.) or anti-mouse IgG antibody (A0216, at 1:10,000 dilution; Beyotime Institute of Biotechnology Inc.) for 1 h at room temperature. After washing, the WB signal was detected using an enhanced chemiluminescence system (Bio-Rad Laboratories, Inc.) and the densitometric analysis of the images was performed using Image Lab 6.0.1 software (Bio-Rad Laboratories, Inc.).

Statistical analyses. All statistical analyses were performed using Statistical Package for the Social Sciences version 13.0 (SPSS Inc.) and Prism software. Statistically significant differences in miRNA-301a-3p expression between cancer tissues and normal tissues were determined by a two-tailed paired Student's t-test. Data from the comparisons of multiple groups from the cell migration and cell invasion assays, luciferase assay, western blot analysis and RT-qPCR are expressed as the means ± SE, and the significant differences were determined by the one-way ANOVA S-N-K method and Tukey's post hoc test. The association between miRNA-301a expression and the patient clinicopathological characteristics was determined using the Chi-square test. Survival curves were plotted using the Kaplan-Meier method and were compared using the log-rank test. The significance of various survival-related variables was assessed by a Cox regression model in a multivariate analysis in this GC cohort. A P-value <0.05 (P<0.05) was considered to indicate a statistically significant difference.

Results

miRNA-301a-3p is upregulated in GC tissues and GC cell lines. The results of RT-qPCR indicated that miRNA-301a-3p expression in the GC tissues was significantly higher than that in the matched normal tissues (0.001727±0.002114 vs. 0.000172±0.00012, t=3.998, P<0.05) (Fig. 1A). Among these 30 cases of GC, only 4 cases were found to have a lower miRNA-301a-3p expression in the cancer tissues than in the adjacent non-cancerous tissue (Fig. 1B). Moreover, miRNA-301a-3p expression was increased in all 4 cancer cell lines compared with the normal gastric epithelial cell line, GES-1. Among these, the AGS cells exhibited the highest expression level of miRNA-301a-3p and the MKN-45 cells the lowest (Fig. 1C). These 2 cell lines were therefore selected for

Table III. Multivariate analysis as determined by Cox regression analysis in 120 patients with GC.

Clinicopathological parameters	95% CI		HR	P-value
	Lower	Upper		
Lauren classification	1.217	6.248	5.906	0.015
Differentiation	0.347	0.986	4.045	0.044
Distant metastasis	1.234	16.110	5.204	0.024
miRNA-301a expression	0.400	1.440	0.713	0.399

GC, gastric cancer; CI, confidence interval; HR, hazard ratio.

use in miRNA-301a-3p inhibition and mimic *in vitro* experiments, respectively.

Clinical significance of miRNA-301a-3p in GC. To further investigate the association between miRNA-301a-3p expression and patient clinicopathological characteristics, miRNA-301a-3p expression was validated independently by ISH assay using a TMA with 120 clinical GC tissue samples (Table I). Based on the miRNA-301a-3p immunoreactive scores, high levels of miRNA-301a-3p expression were detected in 74 (61.7%) of tumors, while low levels of miRNA-301a-3p expression were detected in 46 (38.3%) of tumors (Fig. 1D). miRNA-301a-3p expression was found to be associated with tumor size, Lauren classification, vascular invasion, invasion depth (T grade), lymphatic metastasis (N grade) and distant metastasis (P<0.05, Table I); however, it was not associated with sex, tumor location, age, histological type, lymphatic invasion and differentiation (P>0.05, Table I).

In patients with a tumor size ≥5 cm, the miRNA-301a-3p positivity rate was higher than that in patients with a tumor size <5 cm (70.8 vs. 50.9%, P<0.05). The miRNA-301a-3p positivity rate in GC patients with the intestinal type was higher than that in patients with the diffuse type (70.8 vs. 47.9%, P<0.05).

The miRNA-301a-3p positivity rate in patients with lymph node metastasis (71.2%, or 52/73) was higher than that in patients without lymph node metastasis (46.8%, or 22/47, P<0.05). The miRNA-301a-3p positivity rate in patients with distant metastasis (82.4%, or 28/34) was also higher than that in patients without distant metastasis (53.5%, or 46/86; P<0.05). In patients with GC, the miRNA-301a-3p positivity rate was significantly increased from 19.2% (grade I) to 82.4% (grade IV, P<0.05). Additionally, patients with vascular invasion also exhibited a higher positivity rate than those without vascular invasion (72.3%, 55/76 vs. 43.2%, 19/44, P<0.05, Table I).

High miRNA-301a-3p expression in GC is associated with a poor prognosis. The association between the miRNA-301a-3p expression level and the prognosis of patients with GC was also analyzed. In this cohort of patients in the TMA (n=120), the 5-year survival rate was 31.7%, and the mean survival time was 36.77±1.99 months. By contrast, the mean survival time of miRNA-301a-3p-positive patients was significantly shorter than that of miRNA-301a-3p-negative patients (32.31±2.53 vs. 43.94±2.94 months for miRNA-301a-3p-positive and -negative patients, respectively, P<0.05). The 5-year survival

rate of miRNA-301a-3p-positive patients (23.0%) was also significantly lower than that of miRNA-301a-3p-negative patients (45.7%, P<0.05, Fig. 1E). A Cox multivariate analysis showed that Lauren classification, differentiation and distant metastasis were independent prognostic factors, whereas miRNA-301a-3p expression was not an independent prognostic factor in this cohort of patients with GC (Table III).

miRNA-301a-3p expression affects GC cell proliferation, invasion and migration. To evaluate the possible role of miRNA-301a-3p in the proliferation and invasiveness of human GC cells, the AGS and MKN-45 cells were selected for the transfection assay as these 2 cell lines were found to have the highest or lowest endogenous miRNA-301a-3p expression out of all the cell lines tested (Fig. 1C).

The results of MTS assay revealed that when miRNA-301a-3p expression was inhibited in the AGS cells, they exhibited a significantly slower proliferation rate compared with the negative control cells (Fig. 2A, top panel; P<0.05). At the same time, as was expected, the results of migration and invasion assays indicated that following the inhibition of miRNA-301a-3p in the AGS cells, the migratory and invasive abilities were significantly decreased compared with the control cells (Fig. 2B; P<0.05). Furthermore, after increasing the miRNA-301a-3p expression in the MKN-45 cells, the proliferative, migratory and invasive abilities were significantly increased (Fig. 2A, bottom panel and C; P<0.05).

miRNA-301a-3p targets NKRF and involves NF-κB signaling. To investigate the possible mechanisms through which miRNA-301a-3p affects GC cell invasiveness, the miRBase Targets, TargetScan Release 5.0 and PicTar databases were searched and found that NKRF may be a possible target of miRNA-301a-3p. To clarify whether miRNA-301a-3p interacts directly with the 3'UTR region of NKRF, a binding site investigation was performed and it was found that the 3'UTR region of NKRF mRNA contained 3 miRNA-301a-3p binding sites (Fig. 3A). Thus, these 3 WT sequences of the human NKRF 3'UTR regions were inserted into a luciferase reporter vector and then co-transfected into 293 cells with the miRNA-301a-3p inhibitor. The results of the luciferase assay indicated that the relative luciferase activity from NKRF WT site 1 group was significantly increased in the presence of the miRNA-301a-3p inhibitor compared with the negative control (P<0.05, Fig. 3B), whereas other two WT sites (site 2 and site 3) of the NKRF
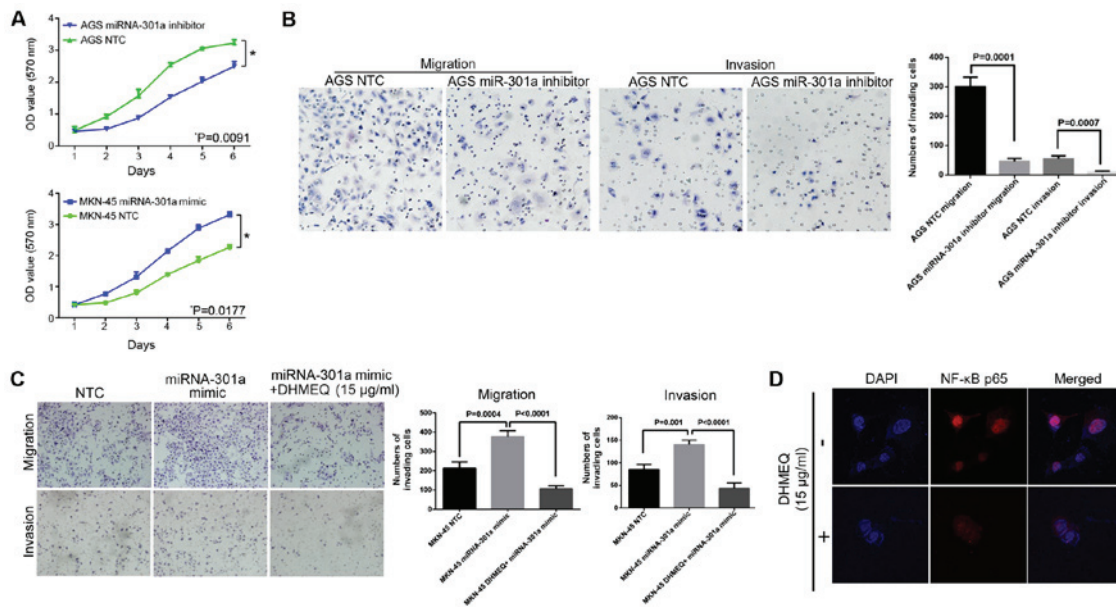
Figure 2. miRNA-301a-3p affects the proliferation and invasiveness of human GC cells. (A) MTS assay revealed that the decreased miRNA-301a-3p expression in AGS cells led to a significant reduction in the proliferative abilities of the GC cells, and increased the miRNA-301a-3p expression in MKN-45 cells led to a significant promotion of the proliferative abilities (*P<0.05). (B) Transwell migration and invasion assays revealed that the decreased miRNA-301a-3p expression in AGS cells led to a significant decrease in the number of invading cells. (C) The invasive and migratory abilities were increased following transfection with miRNA-301a-3p mimic in MKN-45 cells, whereas these promoting effects can be inhibited by DHMEQ. (D) NF-κB inhibitory effects of DHMEQ. Following treatment with 10 μg/ml of DHMEQ, the NF-κB nuclear activities were inhibited in MKN-45 cells. GC, gastric cancer; DHMEQ, dehydroxymethylepoxyquinomicin.

3'UTR exhibited no significant changes in luciferase activity. These data indicated that site 1 of the NKRF 3'UTR region may be a true binding site of miRNA-301a-3p. To confirm this finding, the corresponding mutated binding regions of site 1 of the NKRF 3'UTR (NKRF Mut site 1) were cloned into the luciferase reporter vector, which was subsequently co-transfected into the cells with the miRNA-301a-3p inhibitor. Finally, unlike the NKRF WT site 1 group, comparing with negative control group the miRNA-301a-3p inhibitor did not alter the activity of luciferase of NKRF MUT site 1 group (Fig. 3B). Thus, these results indicated that miRNA-301a-3p specifically and directly targeted NKRF by binding to the predicted site (site 1) of the NKRF 3'UTR region.

Furthermore, the results of RT-qPCR and western blot analysis also revealed that the NKRF expression levels were inversely associated with miRNA-301a-3p expression in GC cells at both the mRNA and protein level (Fig. 3C).

A previous study indicated that the NKRF protein binds to NF-κB by a direct protein-protein interaction, and in a cellular assay, NKRF inhibited NF-κB basal activity (37). Further research revealed that NF-κB signaling was activated in GC and cancer cell lines and that its expression in GC tissue was strongly associated with the abundance of other tumor- or metastasis-promoting markers, including STAT3, MMP-2, MMP-9, VEGF and others (21,22), which are essential for GC progression (10). Therefore, these downstream molecules of NF-κB signaling were also investigated in the present study. From the RT-qPCR screening results and validation by western blot analysis, it was confirmed that NKRF expression was significantly increased in the AGS cells transfected with the miRNA-301a-3p inhibitor, while the expression of NF-κB and its downstream effector molecules, such as

MMP-2, MMP-9 and VEGF was significantly decreased (Figs. 3C and D, and 4A). By contrast, when miRNA-301a-3p expression was increased in the MKN-45 cells, the expression of NKRF was significantly decreased, whereas the expression of NF-κB, MMP-2, MMP-9 and VEGF was significantly increased (Figs. 3C and D, and 4A). However, no significant changes were observed in the levels of other downstream molecules of the NF-κB pathway, such as STAT3, IL-6, IL-8 (as known as CXCL8), MCP-1 (as known as CCL2), COX2 (as known as PTGS2) and the pro- and anti-apoptotic factors, cIAP1 (as known as BIRC2), c-FLIP and BCL2L1 (as known as Bcl-xL), in GC cells in which miRNA-301a-3p expression was downregulated (data not shown).

In order to verify whether the NF-κB pathway was involved in the miRNA-301a-3p-induced invasiveness of GC, the specific NF-κB inhibitor, DHMEQ, was used. It was found that, after pre-blocking NF-κB activities in the MKN-45 cells, the promoting effects of miRNA-301a-3p mimic on cell invasion and migration were inhibited (Fig. 2C and D). Thus, these data indicate that miRNA-301a-3p is an important regulator of the candidate target gene NKRF, which participates in GC progression by regulating the NF-κB signaling axis.

Discussion

A number of miRNAs have been reported to function as oncogenes or tumor suppressors in cancer through their epigenetic regulation of target gene expression; these miRNAs form a complex regulatory network that influences cancer invasion, metastasis, drug resistance, stemness, EMT and signaling pathways (41,42). miRNA-301a has been reported to be upregulated in a number of types of cancer, which indicates that miRNA-301a
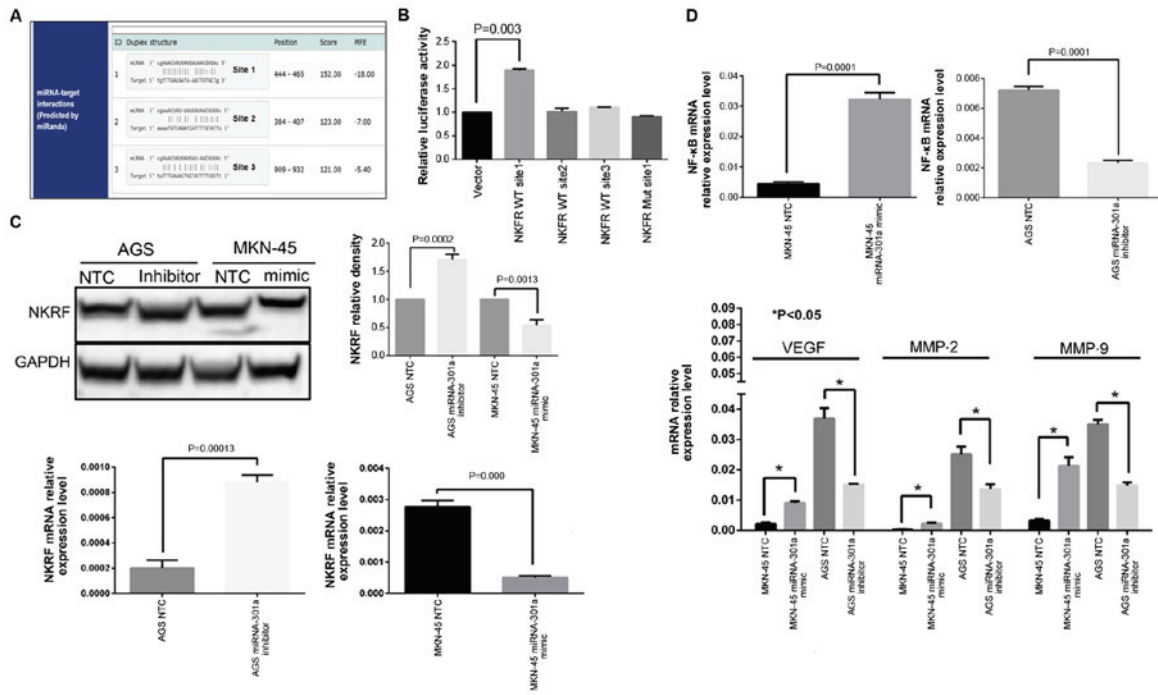
Figure 3. NKRF is a target of miRNA-301a-3p and involves NF-κB signaling. (A) Sequence alignment positions of the miRNA-301a-3p target sites in the NKRF 3'UTR. (B) pYr-MirTarget-NKRF-3'UTR luciferase assay result. (C) Changes in the levels of NKRF mRNA and protein after the GC cells were transfected with the miRNA-301a-3p inhibitor and mimic. (D) Significant changes in the mRNA levels of NF-κB and its downstream molecules after the GC cells were transfected with the miRNA-301a-3p inhibitor and mimic. (*P<0.05). GC, gastric cancer; NKRF, nuclear factor-κB repressing factor; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase.
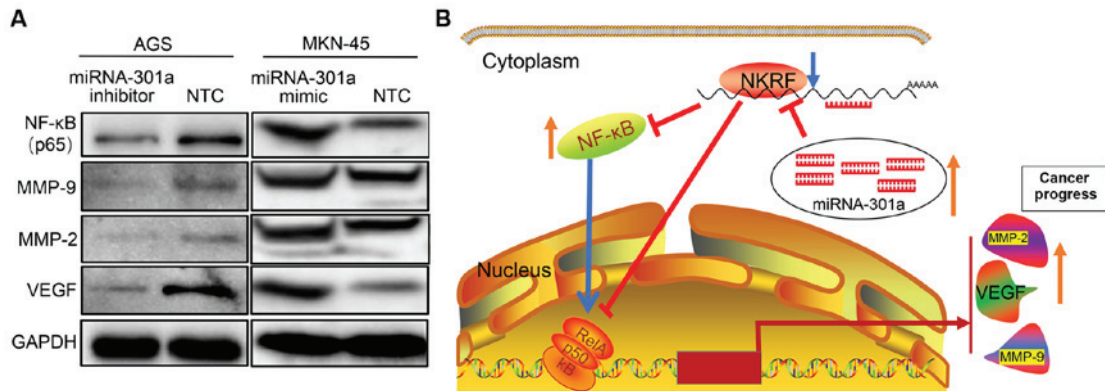

Figure 4. miRNA-301a-3p regulates NKRF and NF-κB downstream molecule expression. (A) Changes in the protein levels of NKRF, NF-κB, VEGF, MMP-2 and MMP-9 proteins after the GC cells were transfected with the miRNA-301a-3p inhibitor and mimic. (B) The hypothesized regulatory network of miRNA-301a-3p in GC. GC, gastric cancer; NKRF, nuclear factor-κB repressing factor; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase.

serves as an important tumor gene in cancer development (43-45). In the present study, miRNA-301a-3p expression was compared in 30 paired GC tissues and normal gastric tissues and the findings provide important evidence to support the upregulation of miRNA-301a-3p in GC tissue. Further analysis of clinical samples using the ISH method demonstrated that a high level of miRNA-301a-3p was associated with tumor size, differentiation, invasion depth, lymphatic metastasis, TNM stage, vascular invasion and poor prognosis. The *in vitro* Transwell and MTS assays also indicated that decreased miRNA-301a-3p levels in GC cells inhibited GC cell proliferative, invasive and migratory abilities. These findings support the hypothesis that miRNA-301a-3p may play a tumorigenic role in GC.

NF-κB comprises a family of transcription factors that are involved in the regulation of a wide variety of biological responses. NF-κB was first found to have multiple functions in the regulation of immune responses and inflammation; however, in recent years, increasing evidence has indicated that NF-κB may play a major role in oncogenesis (12). NF-κB and its downstream genes are involved in a number of processes that participate in the development and progression of cancer such as proliferation, migration and apoptosis (12). Aberrant or constitutive NF-κB activation, which affects apoptosis, proliferation and invasiveness, has been detected in several types of cancer (12). In recent years, numerous studies have focused on elucidating the functional consequences of NF-κB

activation, and miRNAs were found to be important regulators of the NF-κB signaling axis (46,47). In the present study, to explore the possibility that miRNA-301a-3p is involved in NF-κB activation in GC, a strategy was formulated with which to screen target genes of miRNA-301a-3p that could modulate NF-κB signaling. It was found that NKRF was the most potent target involved in NF-κB signaling regulation. NKRF is known as a transcriptional silencer protein that binds negative regulatory elements (NREs) specific for the suppression of NF-κB/Rel basal activity as well as several NF-κB regulated downstream genes (37). NKRF has also been shown to interact with NF-κB/p65 through a minimal-core sequence under basal or stimulated conditions (37,48). Therefore, multiple external factors that induce or suppress NKRF expression may contribute to NF-κB-driven transcriptional regulation, with important implications in inflammation and cancer. The luciferase assay in 293 cells demonstrated that miRNA-301a-3p epigenetically targets and regulates NKRF by binding to its 3'-UTR regions. This reverse targeted regulatory mechanism is supported by the finding that downregulation of miRNA-301a-3p expression in GC cells enhances NKRF gene expression at both the mRNA and protein levels. At the same time, to inhibit the expression of miRNA-301a-3p in GC cells, the NF-κB expression level was downregulated, whereas the NF-κB expression level was upregulated by miRNA-301a-3p mimic. Furthermore, the levels of a number of downstream molecules of NF-κB signaling that are involved in cancer invasion and progression, such as MMP-9, MMP-2 and VEGF, were also decreased according to miRNA-301a-3p inhibition. Substantial evidence has already validated that MMP-9, MMP-2 and VEGF are highly expressed in GC and are important for GC invasion and progression and are even associated with a poor prognosis (49). Thus, miRNA-301a-3p may promote GC cell invasion by affecting the expression of these genes by NF-κB signal regulation. Taken together, these results demonstrated that miRNA-301a-3p may promote GC invasion and progression by targeting NKRF expression and then involving the NF-κB signaling pathway. However, in order to increase the opportunity to suppress GC progression, additional research is required to determine the exact mechanisms underlying miRNA-301a-3p-mediated functions in GC cells.

In conclusion, the results of the present study indicated that the dysregulation of miRNA-301a-3p may play a role in the tumor progression and the prognosis of GC patients. Furthermore, these data suggested that miRNA-301a-3p may modulate GC cell invasion and progression by directly and negatively regulating NKRF and then activating the NF-κB pathway. Therefore, the restoration of miRNA-301a-3p expression or the targeted inhibition of NF-κB activity may be a potential therapeutic strategy for GC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

XH made a substantial contribution to the conception and design of the study. XX, YX, WL, NN and XL participated in the experimental design, conducted the experiment, and conducted data analysis and interpretation. JM and JX performed the *in situ* hybridization data analysis. XX, XH and XL wrote and modified the manuscript. HT, YX and XH analyzed and interpret the data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All fresh GC tissue samples were collected with the written informed consent of the 30 patients who underwent GC resection. The study design and method were approved by the Ethics Committee of Zhejiang Provincial People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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