MicroRNA-559 restrains gastric cancer progression via activating AKT signaling pathway by targeting TRIM14

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Primary research

Keywords: MiR-559, TRIM14, Gastric cancer, Multiplication, Cell cycle, Apoptosis

Posted Date: March 24th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-18874/v1>

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Abstract

Background: MicroRNAs (miRNAs) act as pivotal functions in gastric cancer (GC) carcinogenesis and progression. MiR-559 has been defined as a potential cancer suppressor gene in a few cancers. Nevertheless, the biological effect of miR-559 in human GC and the underlying molecular mechanism still unclear and need to be further illuminated.

Methods: Quantitative real-time PCR (qRT-PCR) was fulfilled for measuring the miR-559 expression level in GC. The dual-luciferase reporter was used to verify that tripartite motif-containing 14 (TRIM14) is a target gene of miR-559. The expression levels of TRIM14 were examined by qRT-PCR and Western blot in GC tissue specimens and cell lines. The effects of miR-559 on GC cell growth were detected with MTT and cell counting assays. Cell cycle and apoptosis were examined by using flow cytometry. Overexpression and siRNA further demonstrated the role of TRIM14 in GC.

Results: Our results revealed that miR-559 expression was dramatically downregulated in human GC tissue specimens and cell lines. MiR-559 overexpression restrained GC cell growth, and induced cell cycle G1-S phase arrest and apoptosis. MiR-559 inhibitors promoted cell multiplication and cell cycle G1-S transition, and inhibited apoptosis. MiR-559 level was negatively related to TRIM14 mRNA level in GC. The results showed that TRIM14 was affirmed to be a target gene of miR-559. MiR-559 overexpression downregulated the TRIM14 expression, and miR-559 inhibitors upregulated the TRIM14 expression. Particularly, knockdown of TRIM14 gave rise to the semblable cellular effects observed upon miR-559 overexpression. TRIM14 overexpression recapitulated the revulsive cellular and molecular effects by suppression of miR-559. Furthermore, both miR-559 overexpression and TRIM14 silencing led to the inhibition of the AKT signaling pathway. On the contrary, miR-559 suppression and TRIM14 overexpression activated the AKT signaling pathway, and inhibited Bax/Bcl-2 pathway.

Conclusions: The findings illustrate that miR-559 restrains cell multiplication via suppressing the AKT signaling pathway in human GC and induces cell apoptosis through the suppression of Bax/Bcl-2 signaling pathway via targeting TRIM14. The findings imply that miR-559 acts as a pivotal function in human GC and represents a latent novel target in GC therapy.

Background

Gastric cancer (GC) is regarded as the 4th most prevalent cancer and the 2nd main reason of cancer-relevant deaths in the world [1]. Its incidence and mortality are also dramatically higher in developing countries than that in developed countries, especially in East Asia and South Africa. There are over 90,000 new patients of GC and over 70,000 deaths each year in China, which ranks second in incidence rate and mortality [2]. Risk factors for GC include environmental factors, sex, blood group A, genetic factors and epigenetic factors [3]. Despite the significant achievements in the treatment, such as operative treatment, radiotherapy, chemotherapy, gene therapy, and adjuvant therapy, but the prognosis for GC is still pitiful with a five-year survival rate below 30% [4, 5]. Tumorigenesis and development are the multi-factor and

multi-step processes involving gene abnormal expressions, including the inactivation of antioncogenes and activation of oncogenes [6]. Many studies tried to discover the mechanism of GC, but the precise molecular pathways remain unclear. Further study on the carcinogenesis and progression of GC is imminently needed to develop new targeted therapies.

MicroRNAs (miRNAs) are small, endogenous non-coding RNA molecules consisting of approximately 20–24 nucleotides [7]. MiRNAs have been confirmed as a novel kind of gene transcription regulators through coupling on the 3'-untranslated regions (3'-UTR) of target mRNA [8]. Importantly, miRNAs modulate target gene expression via either translational suppression or mRNA degradation [9]. Previous studies have well demonstrated that miRNAs play pivotal roles in the many cellular processes such as cell multiplication, cell cycle, apoptosis, metabolism, migration, invasion and metastasis [10, 11]. Moreover, aberrant expressions of miRNAs are discovered in many cancers [12, 13]. Emerging evidences reveal that many miRNAs have significant regulatory functions in GC [14]. Up to date, a few researches discover that miR-559 is clinical implication and acts as an important role in breast cancer, papillary thyroid carcinoma and glioblastoma [15–17]. Whereas, the effect and molecular mechanism of miR-559 in GC carcinogenesis and progression is still unclear and demand to be further illuminated. Using bioinformatics software, we predicted that miR-559 could target tripartite motif-containing 14 (TRIM14). TRIM14 belongs to the TRIM family and locates at chromosome 9q22. TRIM proteins include one or two B-box motifs (B1 and B2), a RING (R) finger domain, and a coiled coil (CC) region [18]. These domains may exist or miss in diverse TRIM protein members, such as TRIM14 including B1-CC domains but lacking the R domain (an order of R-B1-B2-CC) [19]. It is reported that TRIM protein members may play oncogene or anti-oncogene to modulate different tumorigenesis and progression [20]. Accumulating evidences imply that TRIM14 acts as an oncogene role in colorectal cancer and breast cancer [21]. Nevertheless, the precise functions and the molecular mechanisms of TRIM14 in GC are still largely unknown.

In our current study, we explored the precise biological effect and mechanism of miR-559 in GC. It was discovered that the miR-559 expression level was remarkably reduced in GC tissue specimens and correlated with clinicopathological features, such as histologic classification, cancer magnitude and lymph node metastasis. Moreover, miR-559 dramatically suppressed MKN-45 and BGC-823 cell multiplication and cell cycle progression by the AKT signaling pathway by targeting TRIM14, and induced apoptosis through stimulating Bcl-2/Bax signalling. Knockdown of TRIM14 gave rise to the semblable biological effects observed upon miR-559 overexpression. TRIM14 overexpression promoted GC growth and inhibited apoptosis. Our results illustrate an anti-oncogenic function of miR-559 in GC, implying that miR-559 may represent a latent novel target in GC therapy.

Materials And Methods

Human GC tissue preparation

In the Department of Oncological Surgery, First Affiliated Hospital, Xi'an Jiaotong University, China, we collected seventy GC tissue specimens and matched adjacent normal tissue samples from sufferer who

were diagnosed, between August 2017 and November 2018. We gained the consent form from patients before tissue collection. The obtained tissue specimens were promptly refrigerated and stockpiled at -70 °C. The Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University authorized the researches.

Cell culture

Human normal gastric epithelial cell line (GES-1) and GC cell lines (BGC-823, MKN-45 and AGS) were obtained from the Cell Bank (Shanghai, China). The Cell Bank Cells have authenticated these cell lines. These cells were maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) including 10% fetal bovine serum (Gibco), streptomycin (100 µg/mL) and penicillin (100 U/mL), and were cultured at 37 °C in a thermotank under 5% CO₂ and 95% air.

Hsa-miR-559 vector construction

Hsa-miR-559 precursor expression vector (called after miR-559) and control empty vector (called after control) were constructed with chemosynthetic oligonucleotides and incorporated into the pcDNA6.2-GW/EmGFPmiR plasmid on the basis of the manufacturer's instructions. Full-length human TRIM14 gene DNA was cloned into the pCMV2-GV146 vector (Genechem Co. Ltd, Shanghai, China). Transfection was fulfilled through using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions.

Anti-miR-559/TRIM14 siRNA synthesis and transfection

MiR-559 inhibitors (named anti-miR-559), interfering RNA oligonucleotides, were synthesized by Gene Pharma (Shanghai, China). The sequence of anti-miR-559 was 5'- UUUUGGUGCAUAUUUACUUUA-3'. The useless siRNA was used as a control (named anti-miR-Control), and its sequence was 5'- CAGUACUUUUGUGUAGUACAA-3'. The miR-559 inhibitors were transfected into human GC MKN-45 and BGC-823 cells with Lipofectamine 2000. Small interfering RNAs (siRNAs) were used to silence or knock down the human TRIM14 gene. TRIM14 siRNA and negative control siRNA (named NC-siRNA) were designed and synthesized by GenePharma. The siRNAs were transfected with Lipofectamine 2000 and diluted to 70 nM for use in future experiment.

Dual-luciferase reporter assay

The binding site for miR-559 in the 3'-UTR of TRIM14 was constructed with synthetic oligonucleotides (Beijing AuGCT DNA-SYN Biotechnology Company, China) and inserted to the pmirGLO Dual-Luciferase expression vector (called after TRIM14-WT). The mutated 3'-UTR sequences of TRIM14 were also cloned and named TRIM14-MT. The pre-miR-559 plasmids and reporter plasmids (WT or MT) were cotransfected into HEK293T cells. The cells were collected and examined 24 hours after transfection. We measured the reporter activity with the Dual-Luciferase Assay System (Promega, Madison, USA).

Quantitative real-time PCR (qRT-PCR)

RNA was extracted from GC tissue specimens and cultured cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The SYBR Premix Ex Taq II Kit (Takara, China) was used to measure miR-559 expression and TRIM14 mRNA expression. qRT-PCR was performed. The data were normalized to RNU6B (U6) or GAPDH gene expression. The primer sequences contained the miR-559 reverse-transcription primer (5'-GTCGTATCCAGTGGCAGTGCTGGAGTCGGCAATTGCACTGGATACGACTTTTGGT-3'), miR-559 PCR forward primer (5'-ATCCAGTGGCAGTGCTGG-3'), miR-559 PCR reverse primer (5'-TGCTTAAAGTAAATATGC-3'), U6 reverse-transcription primer (5'-CGCTTCACGAATTTGCGTGTCTCAT-3'), U6 PCR forward primer (5'-GCTTCGGCAGCACATATACTAAAAT-3'), U6 PCR reverse primer (5'-CGCTTCACGAATTTGCGTGTCTCAT-3'), TRIM14 PCR forward primer (5'-GCAGAACTCAGCCAAGAA-3'), TRIM14 PCR reverse primer (5'-CTTGACTCTGCATTAGCCT-3'), GAPDH forward, 5'-GCCACATCGCTCAGACAC-3'; GAPDH reverse, 5'-GCCCAATACGACCAATCC-3'. The $2^{-\Delta\Delta Ct}$ method was used in the qRT-PCR analysis.

Cell proliferation assay

Human GC MKN-45 and BGC-823 cells were seeded into three 96-well plates (4-parallel wells/group) and cultured for 24 hours. Then, the GC cells were transfected with Control, miR-559, anti-miR-Control, anti-miR-559, NC-siRNA (70 nM), TRIM14 siRNA (70 nM), vector control or TRIM14 expression vector for 24, 48 or 72 h, respectively. Cell proliferation was examined using MTT assays (Sigma, St Louis, MO, USA) on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 492 nm.

Cell counting assay

To measure cell proliferation, 2.0×10^5 cells were plated in 6-well plates (3-parallel wells/group) and cultivated for 24 hours. MKN-45 and BGC-823 cells were transfected separately with control, miR-559, anti-miR-Control, anti-miR-559, NC-siRNA (70 nM), TRIM14 siRNA (70 nM), vector control and TRIM14 overexpression vector. The quantity of cells was counted at 24, 48 and 72 hours after transfection through using a Countess automated cell counter (Life Technologies Corp., Carlsbad, USA).

Cell cycle analysis

MKN-45 and BGC-823 cells were cultured in 24-well plates (3-parallel wells/group) and transfected for 24 hours. Next, the cells were collected for analysis by trypsinization and fixed in 70% ice-cold ethanol at 4 °C. The fixed cells were washed by using PBS and stained with 50 µg/ml propidium iodide and 50 µg/ml RNase A (DNase-free) for 10–15 min at room temperature. Finally, cell cycle distribution was detected by using fluorescence-activated cell sorting (BD Biosciences, USA), and the different cell cycle populations were analyzed with ModFit software (Bio-Rad Laboratories, Hercules, CA, USA).

Apoptosis assay

MKN-45 and BGC-823 cells were seeded into 12-well plates (3-parallel wells/group) and transfected for 48 hours. The cells were harvested and washed with PBS. We assessed cell apoptosis with an Annexin-V FITC Apoptosis Kit (Invitrogen, Thermo Fisher Scientific, USA) on the basis of the manufacturer's instructions. A flow cytometer (BD Biosciences, USA) was used to measure apoptotic cells. The changes of apoptosis were analyzed by using the ModFit software.

Western blot

Western blot was performed according to standard methods. Human GC tissue specimens and cultivated cells were lysed using RIPA lysis buffer (Invitrogen, Carlsbad, CA, USA) and centrifuged at 12,000 g. Next, we detected the protein concentration with the bicinchoninic acid (BCA) assay. The extracted protein was divided by 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). The nitrocellulose membranes were treated for 30 min in blocking solution including 5% nonfat dry milk and incubated with primary antibodies at 4 °C. The primary antibodies were including mouse monoclonal anti-TRIM14 (1:1,000, Cell Signaling Technology, USA), mouse monoclonal anti-AKT (1:1,000, Cell Signaling Technology, USA), mouse monoclonal anti-cyclin D1 (1:1,000, Santa Cruz, CA, USA), mouse monoclonal anti-p-AKT (1:1,000, Cell Signaling Technology, USA), mouse monoclonal anti-CDK2 (1:1,000, Santa Cruz, CA, USA), rabbit monoclonal anti-Bcl-2 (1:1,000; Cell Signaling Technology, USA), rabbit monoclonal anti-Bax (1:1,000, Santa Cruz, CA, USA), and mouse monoclonal anti-GAPDH (1:5,000, Santa Cruz, CA, USA). The membranes were subsequently treated by using ECL reagent (Pierce, Rockford, IL, USA) for chemiluminescence detection. The blots were scanned and recorded, and the band densities were analyzed using PDQuest software.

Statistical analysis

All experiments were fulfilled minimally in triplicate independently. The data were analyzed with SPSS 25.0 software (SPSS, Inc., Chicago, IL, USA). The data were represented as the means $\pm$ SEM from at least 3 experiments. One-way ANOVA and Student's t-test were used to analyze the statistical significance of differences between groups. Correlation analysis between miR-559 and TRIM14 in human GC tissues was performed with Pearson's correlation analysis. Values of $p < 0.05$ were deemed to indicate statistically significant differences.

Results

MiR-559 is dramatically downregulated in human GC and related to the clinicopathological features

To analyze the miR-559 level in human GC, we fulfilled qRT-PCR to detect miR-559 expression in clinical specimens (70 primary GC tissues and matched adjacent normal tissues) and GC cell lines (AGS, MKN-45 and BGC-823). The qRT-PCR assays revealed that miR-559 expression was observably reduced in 84.29% (59/70) of human GC tissues as compared to that in normal tissues (Fig. 1a, Table 1; $p < 0.01$). Further evaluation showed the relationship between miR-559 expression level and certain pathological characters of GC. It was discovered that lower miR-559 expression was correlated with histologic classification, cancer magnitude and lymph node metastasis (Table 1; $p < 0.01$). Nevertheless, miR-559 level was not related to age, venous invasion, gender, lymphatic invasion, T stage and TNM stage. Moreover, miR-559 expression was remarkably fewer in GC cell lines (AGS, MKN-45 and BGC-823) than in normal GES-1 cell

(Fig. 1b; $p < 0.01$). The findings indicated that miR-559 might be an effective marker and play as a cancer suppressor gene in human GC.

Table 1
Clinical significance of miR-559 expression in gastric cancer (n = 70).

Characteristics	Number of cases	miR-559 expression		p-value
		High (n = 11)	Low (n = 59)	
Age				0.752
≥ 60 years	27	4	23	
< 60 years	43	7	36	
Gender				0.829
Male	39	6	33	
Female	31	5	26	
Histology				0.002*
Well to moderate	46	10	36	
Poor	24	1	23	
Tumor size	32	8	24	0.009*
< 50 mm	38	3	35	0.003*
≥ 50 mm	40	2	38	0.653
Lymph node metastasis Yes	30	9	21	0.587
No	41	7	34	0.616
Lymphatic invasion	29	4	25	
Yes	22	4	18	
No	48	7	41	
Venous invasion				
Yes				
No				
T stage				
T1 + T2	46	7	39	
T3 + T4	24	4	20	
TNM Stage				0.509
I + II	37	6	31	
III + IV	33	5	28	

MiR-559 restrains GC cell proliferation, induces cell cycle arrest and apoptosis

To discover the potential function of miR-559 in GC, MKN-45 and BGC-823 cells were transfected with miR-559 vector, control vector, miR-559 inhibitor or negative control, respectively. qRT-PCR was performed to examine the miR-559 level after transfection. The results revealed that the miR-559 expression level was dramatically raised after transfection with miR-559 vector (Fig. 2a; $p < 0.01$). Whereas, there were no remarkable variations between cells transfected with anti-miR-559 and those with anti-miR-Control (Fig. 2b). MTT assay showed that miR-559 overexpression suppressed the growth of MKN-45 and BGC-823 cells at 48 and 72 h after transfection (Fig. 2c; $p < 0.01$), however, anti-miR-559 facilitated GC cell proliferation at 48 and 72 h after transfection (Fig. 2d; $p < 0.01$). Similar results were also discovered in the cell counting assay. MiR-559 overexpression inhibited MKN-45 and BGC-823 cell multiplication, but anti-miR-559 promoted cell multiplication (Fig. 2e, f; $p < 0.01$). Since cell cycle is related to the modulation of cell multiplication, we determined the processes by using a flow cytometer. Our findings revealed that miR-559 overexpression gave rise to a prominent amassing of the G0/G1 phase cells and a decreasing of the S and G2/M phase cells in MKN-45 and BGC-823 cells (Fig. 2g; $p < 0.01$); anti-miR-559 dramatically reduced the G0/G1 phase cells and enhanced the S and G2/M phase cells (Fig. 2h; $p < 0.01$). Analysis of cell apoptosis verified that the percentage of early apoptosis and late apoptosis cells was markedly added when miR-559 was overexpressed, and significantly reduced when anti-miR-559 was transfected (Fig. 2i, j; $p < 0.01$). Taken together, our results illuminated that miR-559 suppressed human GC cell growth, and caused G1-S cell cycle arrest and apoptosis.

MiR-559 directly targets TRIM14

TargetScanHuman 7.2 and RegRNA were applied for identifying plenty of potential target genes of miR-559. After analysis, TRIM14 was chosen for further research. The results revealed that miR-559 could target binding the 3'-UTR of the TRIM14 mRNA ranging from 246 to 268 bp (Fig. 3a). To verify whether miR-559 directly targets TRIM14, the reporter gene assay including the WT and MT 3'-UTR of TRIM14 was fulfilled. Reporter vectors and pre-miR-559 or the pmirGLO empty vectors were cotransfected into HEK293T cells. The luciferase activity of pre-miR-559/WT-TRIM14-UTR-transfected cells significantly decreased ($p < 0.01$), but there was no significant differences in pre-miR-559/MT-TRIM14-UTR-transfected cells (Fig. 3b), indicating that miR-559 directly targets binding the 3'-UTR of TRIM14. Then, we examined TRIM14 expression levels in GC. The results revealed that the expression of TRIM14 was remarkably increased at both the mRNA and protein levels in human GC tissues (Fig. 3c, d; $p < 0.01$). The influence of miR-559 on TRIM14 was evaluated in terms of the data acquired from qRT-PCR. There was an observably inverse relationship between TRIM14 and miR-559 (Fig. 3e; $r = -0.4710$, $p < 0.01$).

MiR-559 inhibits GC cell growth and induces apoptosis via the AKT and Bcl-2/Bax signaling pathways through targeting TRIM14

MiR-559 vector observably decreased the mRNA expression of TRIM14 in MKN-45 and BGC-823 cells, while anti-miR-559 memorably promoted TRIM14 mRNA expression (Fig. 4a, b; $p < 0.01$). Similar results were also found in protein expression (Fig. 4c, d). For exploring the possible mechanisms of miR-559 in GC progression, we detected the related protein expressions in the AKT signaling pathway and Bcl-2/Bax expressions. The findings displayed that miR-559 overexpression decreased the protein expressions of p-AKT, CDK2, Cyclin D1 and Bcl-2 in MKN-45 and BGC-823 cells, and upregulated Bax protein level in GC cells (Fig. 4c). Nevertheless, anti-miR-561 promoted the protein expressions of p-AKT, CDK2, Cyclin D1 and Bcl-2, and suppressed Bax protein level (Fig. 4d). Furthermore, the total AKT protein expression remained unchanged. Our findings suggested that miR-559 could regulate human GC cell growth, cell cycle transition and apoptosis via modulating the AKT and Bcl-2/Bax signaling pathways.

TRIM14 overexpression facilitated GC cell multiplication and cell cycle transition, and restrained apoptosis

To further illuminate that miR-559 performed a cancer suppressor gene function via TRIM14, we constructed a TRIM14 overexpression vector. In MKN-45 and BGC-823 cells, TRIM14 overexpression efficiently upregulated the TRIM14 mRNA (Fig. 5a; $p < 0.01$). MTT and cell counting assays showed that TRIM14 overexpression observably promoted cell growth in MKN-45 and BGC-823 cells (Fig. 5b, c; $p < 0.01$). TRIM14 overexpression observably reduced the G1 phase proportion and added the S and G2/M phase proportions in GC cell (Fig. 5d; $p < 0.01$). Moreover, TRIM14 overexpression markedly suppressed early and late apoptosis in MKN-45 and BGC-823 cells (Fig. 5e; $p < 0.01$). Next, the TRIM14 overexpression efficiency was analyzed. TRIM14 protein significantly upregulated in the TRIM14 overexpression vector cells; TRIM14 overexpression increased p-AKT, CDK2, Cyclin D1 and Bcl-2 protein expressions, and decreased Bax protein level in MKN-45 and BGC-823 cells. It was no significant difference of the total AKT protein level (Fig. 5f). Therefore, miR-559 could regulate GC cell multiplication and apoptosis by inhibiting TRIM14 via the AKT and Bcl-2/Bax signaling pathways.

Knockdown of TRIM14 suppresses GC cell multiplication and induces apoptosis

Since miR-559 modulated cell growth and apoptosis in GC cells, TRIM14 was verified as a direct target of miR-559. Thus, we silenced TRIM14 expression in MKN-45 and BGC-823 cells by using RNA interference to confirm its involvement in the cancer suppressor functions of miR-559. Knockdown of TRIM14 specifically decreased its mRNA expression level in MKN-45 and BGC-823 cells (Fig. 6a; $p < 0.01$). Knockdown of TRIM14 dramatically reduced cell activity at 48 and 72 hours after transfection (Fig. 6b; $p < 0.01$). Cell counting assay showed that knockdown of TRIM14 dramatically inhibited MKN-45 and BGC-823 cell proliferation (Fig. 6c; $p < 0.01$). Knockdown of TRIM14 remarkably increased the G0/G1 phase cells and decreased the S and G2/M phase cells in MKN-45 and BGC-823 cells (Fig. 6d; $p < 0.01$). Moreover, silencing of TRIM14 induced apoptosis in MKN-45 and BGC-823 cells (Fig. 6e; $p < 0.01$). Then, we measured the knockdown efficiency of TRIM14 siRNA at protein level. The results showed that the

protein expression of TRIM14 dramatically reduced in the siRNA group. TRIM14 siRNA reduced p-AKT, CDK2, Cyclin D1 and Bcl-2 protein expressions, and increased Bax protein expression in MKN-45 and BGC-823 cells (Fig. 6f). The findings were similar to the results after miR-559 overexpression, implying a same effect of TRIM14 knockdown and miR-559 overexpression.

Discussion

Growing evidence showed that the disorder expression of miRNAs plays pivotal roles in the processes of various tumorigenesis and progression [22, 23]. MiRNAs have been discovered to play either tumor suppressor genes or carcinogenic genes, according to the peculiar functions of the targeted gene. Increased expression of oncogenic miRNAs results in decreased anti-oncogene translation, which suppresses to the development and progression of cancer. Semblable effects are caused by reduced expression of tumor suppressor miRNAs leads to increased oncogene expression [24]. GC is a highly malignant tumor with complex pathogenesis. GC development is a typical multiple factors and multi-step process. More and more researchers reported that miRNAs participated in GC occurrence and development [25]. Abnormity of miR-559 has been reported for several different cancers. Even though recent evidences demonstrated the inhibitory effect of miR-559 on human cancers, such as breast cancer, papillary thyroid carcinoma and glioblastoma [16, 17], the functions of miR-559 in GC and the underlying mechanisms are still unknown. In this study, it was found that the expression of miR-559 was remarkably downregulated in both GC tissues and cell lines. The clinicopathological significance of miR-559 expression was analyzed. Our results showed that low miR-559 expression levels were observably associated with histologic classification, cancer magnitude and lymph node metastasis in GC patients, indicating that miR-559 may act a crucial role in GC diagnosis. The experiments further illustrated that miR-559 overexpression significantly suppressed GC cell proliferation by inhibiting G1-S phase transition and accelerated cell apoptosis, the suppression of miR-559 facilitated cell growth and G1-S transition, and restrained apoptosis. Our findings suggest that miR-559 acts as an anti-oncogene in GC and has the potency for being a novel diagnostic marker and a therapeutic target molecule.

Moreover, the miR-559 target analysis identified TRIM14 as a direct target of miR-559. TRIM14 belongs to the TRIM protein family, which includes more than 70 members. Dysregulation of these proteins are linked to different biological processes, such as cell proliferation, differentiation, apoptosis, angiogenesis, migration and invasion [26–28]. Previous studies have reported that TRIM14 is dramatically increased in lung cancer cells and promotes the retinoic acid-inducible gene-I-like receptor-mediated innate immune response against viral infections [29]. Valentina et al. found that TRIM14 overexpression promoted the increasing of a few innate immunity-related genes that participated in the regulation of NF- κ B and Wnt/ β -catenin signaling pathways in human HEK293T cells [30]. Su et al. discovered that TRIM14 was increased in tongue squamous cell carcinoma (TSCC), and promoted the progression of TSCC by regulating the NF- κ B signaling pathway [31]. Additionally, TRIM14 has been shown to promote cancer cell proliferation, chemoresistance, migration and invasion in breast cancer, colorectal cancer, osteosarcoma and gliomas [32–35]. Wang et al. reported that TRIM14 promote the migration and invasion of GC through regulating epithelial to mesenchymal transition [36]. However, the role of TRIM14 on GC cell

proliferation and the underlying mechanisms remain unclear. In this study, we discovered that TRIM14 was increased in GC tissues, which showed an inverse correlation between its mRNA level and miR-559 expression in GC tissues. Our findings suggested that miR-559 might participate in the progression of GC by targeting TRIM14. Further bioinformatic analysis demonstrated that there was a miR-559-binding site at the TRIM14 3'-UTR. The dual-luciferase reporter assay verified that miR-559 directly targeted TRIM14 by recognizing the 3'-UTR of TRIM14 mRNA and inhibited TRIM14 translation. Overexpression of TRIM14 promoted GC cell proliferation and induces G1-S phase transition, and inhibited apoptosis. On the contrary, silencing of TRIM14 suppressed cell proliferation and G1-S phase transition, and induced apoptosis. Our results further demonstrate that miR-559 functions as a tumor suppressor in GC by suppressing TRIM14 expression.

The AKT signaling pathway is one of the most common proliferation pathways in many cancers [37]. It is well known that dysregulation of the AKT signaling pathway is referred to tumorigenesis and progression, such as liver, lung, breast, thyroid, prostate, bladder, pancreatic, gastric, colorectal and cervical cancers [38, 39]. It is reported that the various clinicopathological characteristics of cancers are closely related to the activation of AKT signaling pathway [40]. AKT can modulate the function of plentiful substrates associated with cell cycle progression through direct phosphorylation of target molecules or indirectly regulating protein expressions [38, 41]. Previous studies show that the AKT downstream regulators, such as CDK2 and Cyclin D1, are momentous transcriptional factors in the G0/G1-S phase [42]. As we know, Cyclin A-CDK2 and Cyclin D-CDK4/6 protein kinase complexes are pivotal cell cycle regulators and may regulate cell cycle transition of the G1/G0-S phase [43]. It is reported that Cyclin D1 and CDK2 regulate cell proliferation and cell cycle in many human cancers [44, 45]. Our results demonstrated that miR-559 overexpression and TRIM14 siRNA downregulated CDK2 and Cyclin D1 expressions and induced cell cycle G1-S phase arrest through suppressing the AKT signaling pathway. On the contrary, anti-miR-559 and TRIM14 overexpression upregulated the expressions of CDK2 and Cyclin D1 and promoted G1-S phase transition by activating the AKT signaling pathway. Xu et al. reported that TRIM14 promoted cell proliferation and invasion in osteosarcoma through activating the AKT signaling pathway [32]. Our findings suggest that miR-559 may downregulate the expression of CDK2 and Cyclin D1, and induce G1-S phase arrest via inhibiting the AKT signaling pathway by targeting TRIM14.

The tumor growth is referred to cell proliferation and apoptosis. Apoptosis is an important indicator for anticancer therapy. A dysregulation between Bcl-2 and Bax can cause disorder of apoptosis, which result in oncogenesis and cancer progression. Bcl-2, a anti-apoptotic member, play protectors of the outer membrane and preserve its integrity by inhibiting the release of cytochrome c. On the other hand, Bax, a pro-apoptotic member, causes the release of cytochrome c and lead to mitochondrial dysfunction [46]. Therefore, a pivotal determinant of the intrinsic apoptosis pathway is the balance between Bax and Bcl-2 protein expressions [47]. Our findings demonstrated that miR-559 overexpression or TRIM14 siRNA induced GC cell apoptosis by modulating Bax/Bcl-2 signaling pathway; anti-miR-559 and TRIM14 overexpression suppressed apoptosis through controlling Bax/Bcl-2 pathway. The findings indicate that miR-559 may induce GC cell apoptosis through modulating Bax/Bcl-2 signaling pathway via TRIM14.

Conclusions

In summary, our study demonstrates that miR-559 plays as an anti-oncogene in GC. We find that miR-559 is markedly downregulated in human GC tissues and associated with the clinicopathologic features of GC sufferers. MiR-559 suppresses GC cell growth by restraining the AKT signaling pathway and induces cell apoptosis by modulating the Bax/Bcl-2 pathway via targeting TRIM14. The results indicate that miR-559 acts as a pivotal function in GC progression and may represent a latent novel target in GC therapy.

Abbreviations

Cyclin-dependent kinase 2: CDK2; Gastric cancer: GC; gastric epithelial cell line: GES-1; Quantitative real-time PCR: qRT-PCR; MicroRNA-559: miR-559; Mutation type: MT; small interfering RNA: siRNA; Tongue squamous cell carcinoma: TSCC; Tripartite motif-containing 14: TRIM14; Wild type: WT; 3'-untranslated region: 3'-UTR.

Declarations

Authors' contributions

HQ, LW and LZ designed the experiments; LW, BZ, GHW, LM and JBZ conducted the experiments; LM and YM provided research materials and methods; LW and BZ analyzed data; HQ, LW and LZ wrote the manuscript. All authors read and approved the final manuscript.

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Acknowledgments

We thank the members of the Key Laboratory of the China Ministry of Education for their interest and support of this research.

Competing interests

The authors declare that they have no conflict of interest.

Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Consent for publication

All authors are responsible for the submission of this article and accept the conditions of submission.

Ethical approval

Ethical approval was given by the Medical Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University.

Funding

The present study was supported by Shaanxi Province Natural Science Foundation (No. 2018JM5276).

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Figures

Figure 1

MiR-559 is downregulated in GC tissue samples and cell lines. a MiR-559 expression was markedly reduced in human GC tissues compared with that in adjacent normal tissues. b MiR-559 level was dramatically downregulated in human GC cell lines (AGS, MKN-45 and BGC-823) compared with that in normal gastric epithelial cells (GES-1). * $p < 0.01$.

Figure 2

MiR-559 inhibits human GC MKN-45 and BGC-823 cell proliferation and G1-S cell cycle transition, and induces apoptosis. a The miR-559 expression was determined in MKN-45 and BGC-823 cells after miR-559 overexpression. b The miR-559 expression was detected in MKN-45 and BGC-823 cells after anti-miR-559 treatment. c miR-559 overexpression suppressed cell activity at 48 and 72 hours after transfection. d Anti-miR-559 increased cell activity at 48 and 72 hours after transfection. e miR-559 overexpression restrained GC cell growth. f Anti-miR-559 facilitated GC cell proliferation. g The histogram revealed the proportion of cells in the G0/G1, S and G2/M phases after miR-559 overexpression. h The ratio of cells in the G0/G1, S and G2/M phases after anti-miR-559 treatment. i The data showed the ratios of early and

late apoptosis after miR-559 overexpression. j The data revealed the proportions of early apoptosis and late apoptosis after anti-miR-559 treatment. * $p < 0.01$, $n = 3$.

Figure 3

MiR-559 directly targets the TRIM14 gene. a Bioinformatics predicted reciprocities of miR-559 and its binding sites in the 3'-UTR of TRIM14. b Luciferase activity was measured by using the dual-luciferase assay. c TRIM14 mRNA expression was detected in GC tissues. d TRIM14 protein levels were examined by Western blotting. e MiR-559 and TRIM14 expressions were negatively correlated. The $2^{-\Delta\Delta C_t}$ values of miR-559 and TRIM14 were subjected to a Pearson correlation analysis ($n = 70$, $r = -0.4710$, $p < 0.01$). * $p < 0.01$.

Figure 4

MiR-559 modulates the AKT and Bcl-2/Bax signaling pathways in GC cells by targeting TRIM14. a TRIM14 mRNA was measured in MKN-45 and BGC-823 cells after miR-559 overexpression. b TRIM14 mRNA was determined in MKN-45 and BGC-823 cells after anti-miR-559 treatment. c MiR-559 overexpression suppressed the expressions of the TRIM14, p-AKT, Cyclin D1, CDK2 and Bcl-2 proteins,

and facilitated Bax expression in MKN-45 and BGC-823 cells. d Anti-miR-559 promoted the TRIM14, p-AKT, Cyclin D1, CDK2 and Bcl-2 protein expressions, and inhibited the Bax expression. *p < 0.001.

Figure 5

TRIM14 overexpression facilitates the proliferation and inhibits apoptosis in human GC cells. a TRIM14 overexpression increased TRIM14 mRNA levels in MKN-45 and BGC-823 cells. b MTT assay showed that TRIM14 overexpression promoted MKN-45 and BGC-823 cell activity at 48 and 72 h. c Cell counting assay revealed that TRIM14 overexpression promoted MKN-45 and BGC-823 cell growth. d Cell cycle was examined in MKN-45 and BGC-823 cells at 24 hours. e Apoptosis was detected in MKN-45 and BGC-823 cells at 48 hours. f The protein expressions of TRIM14, p-AKT, AKT, CDK2, Cyclin D1, Bcl-2 and Bax were measured after TRIM14 overexpression vector transfection. *p < 0.01, n = 3.

Figure 6

TRIM14 siRNA inhibits the proliferation and induces apoptosis in human GC cells. a The results revealed that the knockdown efficiency of TRIM14 siRNA in MKN-45 and BGC-823 cells. b TRIM14 siRNA decreased the activity of MKN-45 and BGC-823 cells at 48 and 72 hours. c TRIM14 siRNA inhibited MKN-45 and BGC-823 cell proliferation. d Flow cytometric analysis showed the percentage of the G0/G1, S and G2/M phases. G0/G1 phase cells increased after TRIM14 siRNA treatment, and S and G2/M phase cells were decreased. e The data revealed the percentage of early and late apoptosis after TRIM14 siRNA treatment. f The protein expressions of TRIM14, p-AKT, AKT, CDK2, Cyclin D1, Bcl-2 and Bax were examined after TRIM14 siRNA treatment. * $p < 0.01$, $n = 3$.