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Targeting of GSK-3β by miR-214 to facilitate gastric cancer cell proliferation and decrease of cell apoptosis

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Abstract. – OBJECTIVE: Wnt/β-catenin pathway regulates cell proliferation and apoptosis. GSK-3β degrades β-catenin and negatively regulates Wnt/β-catenin pathway. A previous study indicated that the GSK-3β expression was significantly reduced in gastric cancer, along with the increase of miR-214 expression. Bioinformatics analysis revealed complementary binding between miR-214 and 3'-UTR of GSK-3β mRNA. This study investigated the regulatory role and related mechanism of miR-214 in the proliferation and apoptosis of gastric cancer cells.

PATIENTS AND METHODS: Gastric cancer tissues were collected from [illegible] and the expressions of miR-214, GSK-3β, β-catenin were determined. Dual luciferase reporter gene assay was used to study the regulatory role between miR-214 and GSK-3β. Expressions of miR-214, GSK-3β, β-catenin and survivin in GES-1 and MKN-28 cells were [illegible]. Flow cytometry was used to measure cell proliferation and apoptosis. [illegible] cultured MKN-28 cells were treated with miR-214 inhibitor and/or pSicoR-GSK-3β. Levels of GSK-3β, β-catenin and survivin were detected, cell apoptosis was evaluated by flow cytometry and proliferation was tested by EdU staining.

RESULTS: Compared to normal gastric mucosa, the levels of miR-214 and β-catenin were elevated and the expression of GSK-3β was decreased in gastric cancer tissues. Compared to GES-1 cells, the expressions of miR-214, β-catenin and survivin in MKN-28 cells were upregulated along with downregulation of GSK-3β expression. The proliferation was enhanced whilst apoptosis was suppressed. After the transfection of miR-214 inhibitor and/or pSicoR-GSK-3β, GSK-3β expression was induced in MKN-28 cells while β-catenin and survivin expressions were inhibited, along with the increase of cell apoptosis.

CONCLUSIONS: MiR-214 decreases GSK-3β expression and promotes the pathogenesis of gastric cancer. The inhibition of miR-214 reduces the proliferation of gastric cancer cells via upregulation of GSK-3β and suppression of Wnt/β-catenin signal pathway, which provides fundamental support for the future therapy of gastric cancer.

Key Words: MiR-214, GSK-3β, Wnt/β-catenin pathway, Gastric cancer, Cell proliferation, Apoptosis.

Introduction

Gastric cancer (GC) is a type of malignant tumor commonly found in digestive tract worldwide[1]. GC relatively presents insidious onset without significant symptoms at early stage, but progresses rapidly at terminal stage. Due to high malignancy, potency of invasion and metastasis, GC severely threatens patient's health and life quality[2]. β-catenin represents a critical protein in canonical Wnt/β-catenin signal pathway, and plays an important role in the activation of Wnt/β-catenin signal pathway. Of note, up-regulation of β-catenin induces abnormal activation of Wnt/β-catenin signal pathway, and is closely correlated with onset, progression and prognosis of multiple tumors such as breast cancer[3], pancreatic carcinoma[4], colon cancer[5] and endometrial carcinoma[6]. Besides, β-catenin, glycogen synthase kinase-3β (GSK-3β) belongs to the Wnt/β-catenin signal pathway, in which GSK-3β can phosphorylate and degrade β-catenin protein to suppress the activation of

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Wnt/β-catenin signal pathway, serving as tumor suppressor in tumor pathogenesis. Previous studies showed that the decrease of GSK-3β was closely correlated with onset, progression and drug resistance of colon cancer[7], prostate carcinoma[8], and breast cancer[9]. Also, the reduction of GSK-3β was found to be closely correlated to GC pathogenesis, tumor growth and progression[10]. MicroRNA is a kind of single stranded small molecule non-coding RNA with the length of 22-25 nucleotides, and is widely distributed in multiple tissues and cells. MicroRNA is involved in cell proliferation, differentiation, tissue development and organ formation[11]. The role of microRNA in tumor pathogenesis and progression has become research interests[12,13]. Multiple studies[14,15] found that miR-214 expression in GC tissues was significantly elevated, and it was closely correlated with tumor progression, patient's treatment sensitivity and prognosis. *In silico* analysis by bioinformatics showed complementary binding sites between miR-214 and GSK-3β. This study aimed to study the role of miR-214 in affecting GC cell proliferation or apoptosis.

Materials and Methods

Major Reagent and Materials

Human GC cell line MKN-28 and no[illegible] human gastric mucosal epithelial cell line GE[illegible] were purchased from Jilin B[illegible]chnology Co., Ltd. (Changchun, Jilin, [illegible]na). [illegible]ell Park Memorial Institute-1640 [illegible]MI-164[illegible] medium was from HyClone (Sou[illegible]n, U[illegible] [illegible]tal bovine serum (FBS) was [illegible] Bio-Products (West [illegible]ramento[illegible] USA). Penicillin streptomy[illegible]was from Gi[illegible]ckville, MD, USA). To[illegible] extraction ki[illegible]asyPure RNA Kit an[illegible]uore[illegible] quantitative PCR kit TransScript Green One[illegible] qRT-PCR SuperMix were [illegible]ovided from Tra[illegible] Biotech (Beijing, C[illegible]). Lipofectamine 200[illegible] transfection reagent [illegible]as purch[illegible]sed from Invitrogen (Carlsbad, CA[illegible]), m[illegible]NC, miR-214 mimic and miR-214 i[illegible]ere fro[illegible]RiboBio (Guangzhou, Guangdo[illegible]ina). [illegible] cell proliferation flow [illegible]etry te[illegible]s offered from Sigma-Aldri[illegible]t. Louis, [illegible], USA). Mouse anti-human GSK[illegible], β-catenin and survivin antibodies were fro[illegible]Cambridge, MA, USA). Rabbit [illegible]human [illegible]actin and horseradish peroxidase [illegible]P)-conjugated secondary antibody were from B[illegible] (Hercules, CA, USA). pGRE-luc lucifer[illegible] reporter plasmid was purchased from Junrui Biotech (Shanghai, China). Light [illegible] luciferase reporter gene assay system [illegible] Active Motif (Carlsbad, CA, USA)[illegible]adioimm[illegible]noprecipitation assay (RIPA) lys[illegible]uffer and bicinchoninic acid (BCA) protei[illegible]ntification kit were purchased from Beyotime [illegible], China). Over-expression plasmid [illegible]icoR w[illegible]hased from Addgene (Cambri[illegible], MA, US[illegible] Annexin V and PI d[illegible] were purchased [illegible] BioLegend (San Die[illegible]CA, US[illegible]

Clinical Inform[illegible]

A total of [illegible]GC patie[illegible] m[illegible]s and 13 females) wh[illegible]eived treatme[illegible]ongqi Hospital Affil[illegible]udanjiang Me[illegible]al University from M[illegible]2016 t[illegible]mber 2016 were recruited. GC tissues were co[illegible]during surgery. Another 2[illegible]al gastric mu[illegible]issues were collect[illegible] [illegible] gyroscope as co[illegible]rol group.

This study was approved by Ethics Commit[illegible] in Hongqi H[illegible]ital Affiliated to Mudanjiang [illegible]dical Univers[illegible] and all the enrolled objects ha[illegible]ned infor[illegible]d consent.

Cell Cu[illegible]

MKN-28 and GES-1 cells were all cultured in [illegible]0 medium containing 10% fetal bovine [illegible]rum (FBS) and 1% streptomycin-penicillin in a 37°C chamber with 5% CO_2.

Dual Luciferase Reporter Gene Assay

Using HEK293T genomic DNA as the template, full-length fragment of wild type or mutant forms of 3'-UTR of GSK-3β gene was amplified and were cloned into pGRE-luc plasmid. Recombinant plasmid was used to transform DH5α competent cells. Positive clones with correct sequences were screened out by sequencing and were named as pGRE-GSK-3β-wt and pGRE-GSK-3β-mut. Lipofectamine 2000 was used to co-transfect GRE-GSK-3β-wt (or pGRE-GSK-3β-mut) and miR-214 mimic (or miR-NC) into HEK293T cells. After 48 h incubation, dual luciferase activity was measured by LightSwitch™ Luciferase Assay System.

Construction of GSK-3β Over-Expression Plasmid

CDS domain fragment of GSK-3β gene was amplified and determined for the length by gel electrophoresis. After dual enzymatic digestion, the fragment was ligated into pSicoR-GFP plasmid for transforming bacteria. Positive clones were amplified to extract recombinant plasmids

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containing targeted fragments. Sequencing was performed to confirm correct insertion of target fragments. Those plasmids with correct insertion were named as pSicoR-GSK-3β. Empty plasmid pSicoR-blank was used as the control group.

Cell Transfection and Grouping

In vitro cultured MKN-28 cells were assigned into five transfection groups: miR-NC transfection group, miR-214 inhibitor transfection group, pSicoR-blank transfection group, pSicoR-GSK-3β group, and miR-214 inhibitor + pSicoR-GSK-3β group. At 72 h after transfection, assays were performed.

qRT-PCR for Gene Expression

TransScript Green One-Step qRT-PCR SUperMix was used to test gene relative expression level using RNA extracted by EasyPure RNA kit. In a 20 μL reaction system were added 1 μ template RNA, 0.2 μM forward and 0.2 μM reverse primer, 10 μL 2XTransStart Tip Green qPCR SuperMix, 0.4 μL One-step RT Enzyme Mix, 0.4 μL Passive Reference Dye II and RNase-free water. qRT-PCR conditions were: 45°C for [illegible], 95°C 30 s, followed by 40 cycles each co[illegible] 94°C 5 s and 60°C for 30 s. ABI 7500 R[illegible]ime fluorescent quantitative PCR cycler was u[illegible] to measure gene expression (Thermo Fisher [illegible]entific, Waltham, MA, USA). Primer seque[illegible]es were: miR-214 P_F: 5'-GGA[illegible]ACG CA[illegible] CAG TCA-3'; miR-214 P_R: [illegible]CAG[illegible]GAGGC TCCGT GGT-3'; U6 P_F[illegible]-ATTGC[illegible]AACGA TACAG AGAAG ATT[illegible]6 P_R: [illegible] GCTTC ACGAA TTTG-3[illegible] P_F: [illegible] GTC GCCAT CAA[illegible]AAGTA[illegible]3'; GSK-3β P_R: 5'-GCGTC T[illegible]TT GGCTC[illegible]A T-3'; β-catenin P_F: 5[illegible]T ACACA G[illegible]G AT-GCT GCT-3'[illegible]cate[illegible]: 5'-GCAGT TTTGT CAGTT CAGGG A-3'; [illegible]in P_F: 5'-AGGAC CACCG[illegible]TCT CTACA[illegible] survivin P_R: 5'-AA[illegible] TGGCT CGTTC T[illegible]AGT G-3'; β-actin P[illegible]-GAAC[illegible] CTAAG GCCAA C-3'; β-actin P_R: [illegible]TCA[illegible]CAC GATTT CC-3'.

Western [illegible]

[illegible]teins w[illegible]cted by radioimmunoprecip[illegible]n assay ([illegible]PA) lysis buffer, and 50 μg sam[illegible]s were separated in 10% sodium dodecyl sul[illegible]amide gel electrophoresis (SDS-P[illegible]E). A[illegible] transferring to the polyvinylidene [illegible]oride (PVDF) membrane, the membrane w[illegible]cked by phosphate-buffered saline and twee[illegible]0 (PBST) containing 5% defatted milk powder for 60 min at room temperature. Primary antibody (GSK-3β at 1:300, β-cateni[illegible], survivin at 1:200, and β-actin at 1:8[illegible] was add[illegible] for 4°C overnight incubation. H[illegible]eradish peroxidase (HRP)-conjugated se[illegible]ry antibody (1:5000) was added for 60 min in[illegible]n at room temperature. After 3 times [illegible]f PBS[illegible]g, enhanced chemiluminesce[illegible] (ECL) rea[illegible] added for development[illegible]

Flow Cytometry [illegible]ll [illegible]optosis

Following m[illegible]ual [illegible]tion, c[illegible] were re-suspended [illegible] 100 μL [illegible]in[illegible] Binding Buffer. 5 μL[illegible]C Annexin V [illegible]stly added, followed [illegible]ition of 10 μL [illegible]ropidium iodide (PI)[illegible]fter [illegible]emperature incubation for 15 min, 400 μL An[illegible]V Binding Buffer were add[illegible]ICS XL-MCL [illegible]ytometry was used t[illegible]measure cell apoptosis (Beckman Coulter, [illegible]ea, CA, USA).

[illegible]w Cytomet[illegible] for Cell Proliferation

[illegible] Flow Cy[illegible]metry Kit was used to test cell proli[illegible]ls were incubated in culture medium containing 10 μM EdU at 37°C for 2 h. [illegible]lls were inoculated into 60 mm culture dish [illegible] incubated for 72 h. Cells were then [illegible]gest[illegible] and collected, and were fixed in paraformaldehyde. 500 μL test buffer containing phosphate-buffered saline (PBS), catalyst solution, 6-FAM azide and buffer additive were added for 30 min dark incubation at room temperature. After centrifugation and re-suspending in 500 μL wash reagent, EPICS XL-MCL flow cytometry was used to test cell proliferation (Beckman Coulter, Brea, CA, USA).

Statistical Analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were presented as mean±standard deviation (SD). Student *t*-test of Mann-Whitney U test was used for comparing measurement data between groups. A statistical significance was defined when $p < 0.05$.

Results

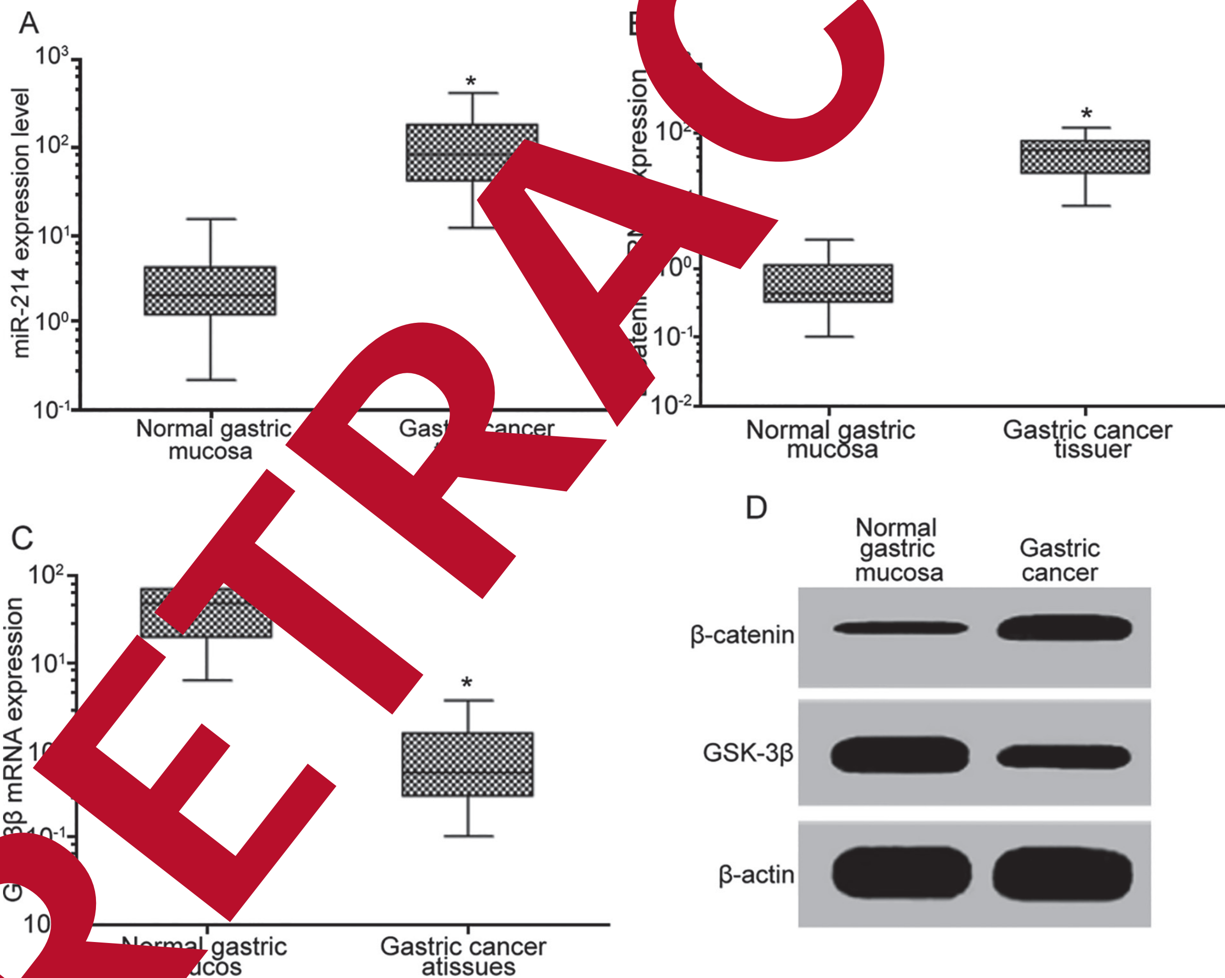
Up-regulation of miR-214 and β-catenin, and Down-Regulation of GSK-3β in GC Tissues

qRT-PCR results showed that, compared to normal gastric mucosal tissues, the levels of

miR-214 and β-catenin in GC tissues were significantly elevated while GSK-3β mRNA level was statistically reduced (Figure 1A-C). Similarly, Western blot detection also indicated that the expression of GSK-3β protein in GC tissues was decreased than that in normal gastric mucosal tissues, whilst β-catenin protein expression was increased (Figure 1D).

MiR-214 Expression in GC Cells was Correlated with Lower GSK-3β and Higher Survivin Expression

Flow cytometry revealed that the proliferation potency of MKN-28 cells was significantly higher than that of GES-1 cells (Figure 2A), whilst the basal apoptotic rate was lower comparatively (Figure 2B). qRT-PCR results showed that, compared to GES-1 cells, the expressions of miR-214, β-catenin, and survivin mR[illegible]s in MKN-28 cells were significantly hi[illegible] GSK-3β mRNA expression was re[illegible]rkably [illegible]creased (Figure 2C). Concomi[illegible]ly, Western blot results unraveled signific[illegible] lower GSK-3β protein expression in MK[illegible]ells compared to GES-1 cells, al[illegible] with [illegible]crease of β-catenin protein ex[illegible]sion (Figu[illegible] [illegible] *silico* target gene pred[illegible]on with microR[illegible] showed the existen[illegible]f bindi[illegible] sites between miR-214 and 3'-UT[illegible] [illegible]SK[illegible] mRNA [illegible]Figure 2E). Additionall[illegible] dual [illegible]se repo[illegible] assay illustrated tha[illegible]fter the t[illegible]tio[illegible]ith miR-214 mimic[illegible] relative luc[illegible] enzymatic activity i[illegible]3 cells was si[illegible]ificantly decreased [illegible]gure [illegible] demonstrating the targeted regulation betw[illegible]R-214 and 3'-UTR of GSK[illegible]RNA.



[illegible]**ure 1.** miR-214 and β-catenin expression, and lowered GSK-3β expression in gastric cancer tissues. ***A,*** qRT-PCR for miR-[illegible]ression in GC and normal gastric mucosal tissues. ***B,*** qRT-PCR for β-catenin mRNA expression in GC and normal ga[illegible]cosal tissues. ***C,*** qRT-PCR for GSK-3β mRNA expression in GC and normal gastric mucosa. ***D,*** Western blot for GSK-[illegible]nd β-catenin protein expression in GC and normal gastric mucosa. *$p < 0.05$ compared to normal gastric mucosa.

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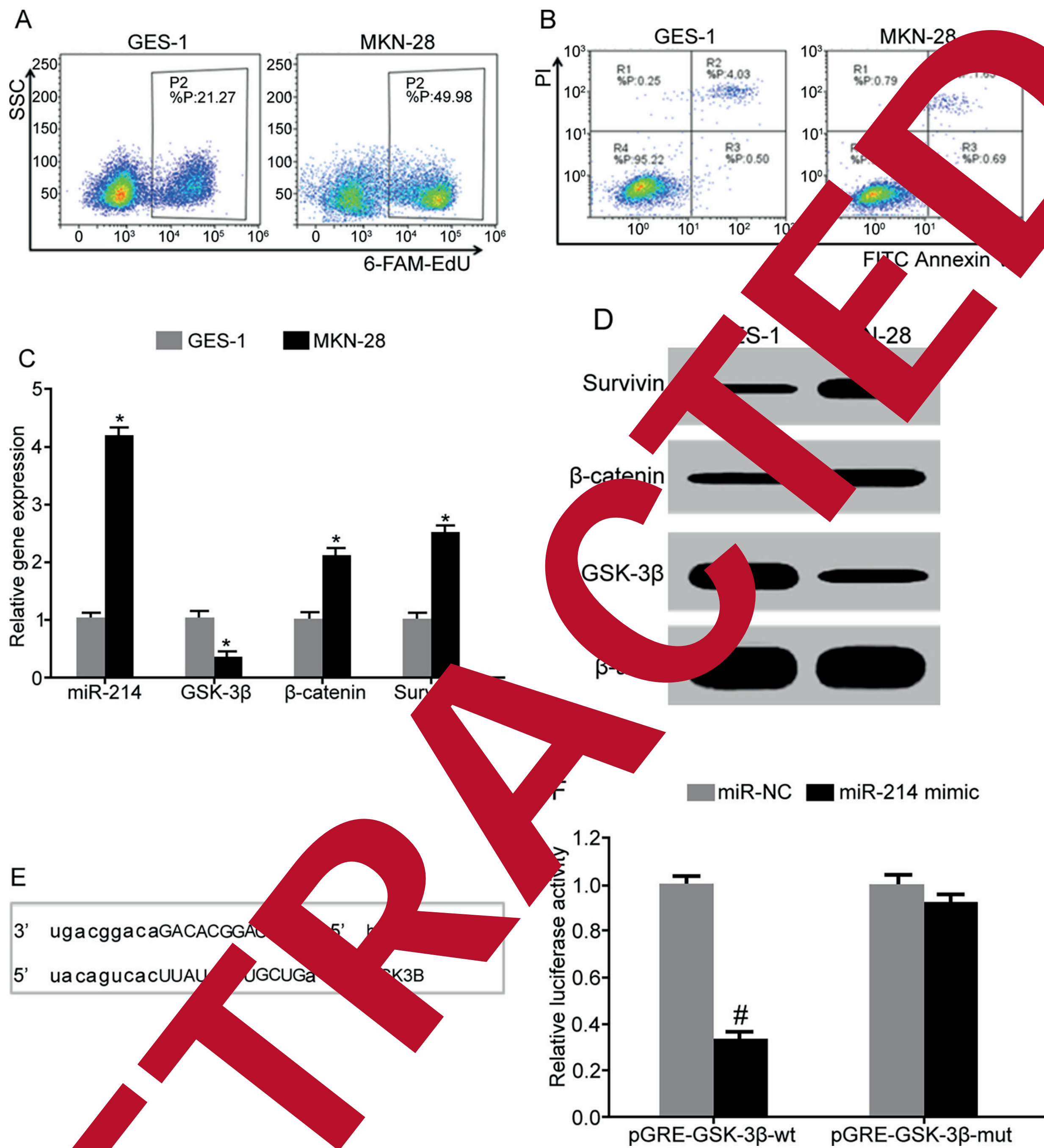

Fig[illegible] Up-reg[illegible]on of miR-214 was correlated with lower GSK-3β or survivin expression. ***A,*** Flow cytometry for GES-1 and [illegible] cel[illegible]oliferation. ***B,*** Flow cytometry for GES-1 and MKN-28 cell apoptosis. ***C,*** qRT-PCR for gene expression. ***D,*** Wester[illegible]rotein exp[illegible]on. ***E,*** Binding sites between miR-214 and GSK-3β mRNA. ***F,*** Dual luciferase reporter gene assay. *$p <$ [illegible]paring [illegible]ween MKN-28 and GES-1 cells; #$p < 0.05$ comparing between miR-214 mimic and miR-NC.

Mi[illegible]14 Inhibition Up-Regulated GSK-3β[illegible], Facilitated MKN-28 Cell [illegible]ptosis [illegible]nd Inhibited Proliferation

[illegible] order to further validate the mechanism of [illegible] on the proliferation of gastric cancer cells, [illegible]e evaluated the expressions and apoptosis by the transfection of miR-214 inhibitor and/or pSico-GSK-3β. Our data indicated that, after the transfection, significantly rising expression of GSK-3β, downregulation of β-catenin and survivin expressions in MKN-28 cells were observed in MKN-28 cells (Figure 3A). More-

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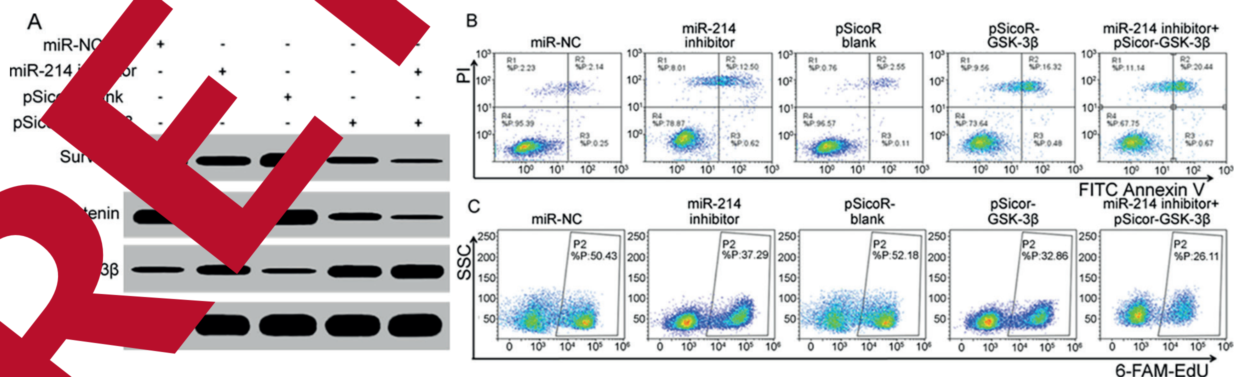
over, the increase of cell apoptosis and weakened proliferation potency were shown after the treatment of miR-214 inhibitor and/or pSico-GSK-3β (Figure 3B, 3C).

Discussion

Wnt/β-catenin signal pathway is one of the highly conserved pathways during the evolution, and is closely correlated with tissue/embryonic development, body growth, immune response, and tumor formation[16]. Canonical Wnt/β-catenin signal pathway is featured with cytoplasmic deposition, stable expression and nuclear translocation of β-catenin. In contrast to β-catenin, GSK-3β negatively regulates Wnt/β-catenin signal pathway, and hydrolyzes β-catenin protein via phosphorylation[17,18]. Survivin is the most potent apoptotic inhibitor in IAPs family, and can retard cell apoptosis via suppressing activity of Caspase-3 and Caspase-7[19]. Survivin also plays an important role in facilitating mitosis[20]. Several studies showed the involvement of survivin as an important target gene in Wnt/β-c[illegible]in signal pathway[21,22]. GSK-3β negatively [illegible] signal pathways including PI3K/AKT, [illegible]κB, ERK1/2-MAPK besides Wnt/β-catenin pa[illegible], thus affecting various cellular processes suc[illegible] cell metabolism, proliferation, apoptosis, [illegible]ferentiation and motility[23]. D[illegible]lation o[illegible] GSK-3β or up-regulation of [illegible]aten[illegible]cilitates occurrence of various tum[illegible],5,7,8. Mi[illegible]14 plays an essential role in the o[illegible]d de[illegible] multiple tumors via [illegible]e ca[illegible] key proteins and s[illegible]aling pa[illegible][24]. In this work, we found t[illegible] compared t[illegible] in normal gastric mu[illegible]sues, the exp[illegible]ions of miR-214, β-catenin expression and GSK-3β were significantly altered, indicating the p[illegible] of miR-214, β-catenin and GSK-3β [illegible]GC path[illegible]genesis. Volinia et al[25] showed s[illegible]ficantly elevated miR-214 expression in [illegible]atient tumor tissues compared to normal g[illegible] mucosa. Ueda et al[26] also found hi[illegible]er mi[illegible]xpression in GC tumor tissu[illegible]ompared to [illegible] tissues, and demonstr[illegible]d close correlati[illegible]tween miR-214 up-r[illegible]ation a[illegible]tumor infiltration depth, lymph [illegible]et[illegible]sis and [illegible]linical phase, which ca[illegible] be tr[illegible] predict[illegible] index for unfavorab[illegible] prognosis[illegible] et[illegible]7 found that GC tiss[illegible]had about 7.1[illegible]gher miR-214 expre[illegible] adjacent tiss[illegible]es, and miR-214 was [illegible]nifica[illegible]orrelated with infiltration depth, vein invasion, [illegible]h node metastasis and TN[illegible]e. Yang et a[illegible]wed that those GC p[illegible] with higher miR-214 expression pre[illegible]nted worse prognosis, indicating tumor facili[illegible]ing role of m[illegible]14 in GC pathogenesis. This [illegible]y also showe[illegible]bnormally elevated miR-214 e[illegible]ion in G[illegible]issues, suggesting its correlation [illegible]thogenesis, as consistent with Volinia et al[illegible] and Ueda et al[26]. Hirakawa et al[29] [illegible]served lower GSK-3β expression in GC tissues [illegible]to normal gastric mucosal epithelium. [illegible]udy, GSK-3β expression level was abnormally decreased, indicating tumor suppressing role of GSK-3β in GC pathogenesis, as similar with Hirakawa et al[29]. Yang et al[28] found more than 4-fold increase of miR-214 expression in GC cell lines such as SGC-7901, BGC-823. Xin et al[30] demonstrated significantly elevated miR-214 expression in highly metastatic GC cell lines GC9811-P and MKN-28M compared to low metastatic GC cell lines GC9811 or MKN-28NM, indicating the correlation between miR-214 up-reg-



Fig[illegible] miR-214 inhibition increased GSK-3β expression, facilitated MKN-28 cell apoptosis and inhibited their proliferation. ***A,*** We[illegible]n blot for protein expression. ***B,*** Flow cytometry for cell apoptosis. ***C,*** EdU staining for cell proliferation.

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ulation and malignant biological features of GC cells. Xin et al[30] showed that over-expression of miR-214 could facilitate proliferation, migration and clonal formation of low metastatic GC cells GC811 via targeted inhibition of tumor suppressor gene PTEN, thus enhancing its malignant biological features. The down-regulation of miR-214 expression in highly metastatic GC9811-P cells remarkably elevated PTEN expression and weakened malignant biological features. Yang et al[28] showed targeted regulation between miR-214 and PTEN in GC cell lines SGC-7901 and BGC-823. Ko et al[10] found that knockout of GSK-3β expression remarkably accelerated GC tumor growth, enhanced HIF-1 and VEGF expression for angiogenesis, thus facilitating GC progression. Zhou et al[31] showed that chemical inhibitor to antagonize GSK-3β effects could enhance tumor stem cell property of GC cell lines SGC7901 and MGC803. Ngo et al[32] observed that weakened GSK-3β effects enhanced GC cell migration or invasion potency. Dar et al[33] found that weakened GSK-3β function could elevate expression of β-catenin and downstream target gene Cyclin D1 and c-myc expression in GC cells, thus facilitating GC cell cycle and potentiating the proliferation [illegible]. This study revealed that miR-214 up-regulation played a role in decreasing GSK-3β expression or GC pathogenesis; down-regulation of miR-214 could enhance GSK-3β expression, weaken Wnt/β-catenin signal pathway [illegible] facilitate GC cell apoptosis and suppress the proliferation. However, the limitation in our report still exists and the clinical effect of miR-214 req[illegible] validation with animal models.

Conclusions

Our data demonstrated [illegible] the down-regulation of miR-124 suppresses the proliferation of gastric cancer cells by inducing GSK-3β expression [illegible] inhibiting Wnt/β-catenin signal pathway, which offers an academic basis for a future therapy for gastric cancer.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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