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The effect of miR-224 down-regulation on SW80 cell proliferation and apoptosis and weakening of ADM drug resistance

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Abstract. – **OBJECTIVE:** **Glycogen synthase kinase-3β (GSK-3β) can phosphorylate and degrade β-catenin, and negatively regulates Wnt/β-catenin signal pathway. MiR-224 up-regulation is associated with colorectal cancer (CRC). Bioinformatics analysis showed complementary binding sites between m[illegible] and GSK-3β. This study investigated if [illegible] plays a role in mediating GSK-3β expr[illegible]on, Wnt/β-catenin pathway activity, CRC cell [illegible]eration, apoptosis as well as drug sensitivi[illegible] Adriamycin (ADM).**

MATERIALS AND METHOD[illegible]al lucife[illegible]ase gene reporter assay de[illegible] the regulatory relationship betwe[illegible]miR-22[illegible]d GSK-3β. Expression of miR-[illegible] GSK-3[illegible]β-catenin, and Survivin was me[illegible]l in [illegible] epithelium NCM460, [illegible]RC [illegible]W4[illegible]d drug-resistant SW[illegible]ADM c[illegible]. Flow cytometry measure[illegible]poptosis u[illegible]DM with an IC50 concen[illegible]of SW480 ce[illegible]llowed by CCK-8 ana[illegible]s [illegible]l proliferation. SW480/ADM cells we[illegible]e treate[illegible]h miR-224 inhibitor and/or pS[illegible]R-GSK-3β, [illegible]d by analysis of the expr[illegible]sions of GSK-3β, [illegible]enin and Survivin, [illegible] apoptosis, and cell proliferation by EdU [illegible]ning.

[illegible]TS: [illegible]-224 targeted and inhibited GSK-[illegible]sion. In [illegible]480/ADM cells, GSK-3β expre[illegible]and ce[illegible]poptosis rate were low[illegible]an the [illegible]S[illegible]0 cells, whilst miR-224, [illegible]in, and [illegible]vin expression or proliferatio[illegible]ere highe[illegible]than those in SW480 cells. Tra[illegible]ection of miR-224 inhibitor and/or pSi-co[illegible]gnificantly increased GSK-3β expression [illegible]W480/ADM cells, and decreased [illegible]tenin and Survivin expression, leading to [illegible]d proliferation potency, enhanced cell apo[illegible]is and suppressed ADM resistance.

[illegible]ONCLUSIONS: MiR-224 up-regulation is as[illegible]ciated with AD[illegible] resistance of CRC cells. Sup[illegible]ssion of mi[illegible]24 expression up-regulated G[illegible]3β express[illegible], inhibited Wnt/β-catenin signa[illegible]way acti[illegible]y and Survivin expression, as well [illegible] ADM resistance of CRC SW480 cells.

[illegible]s:
[illegible], GSK-3β, Wnt/β-catenin, Adriamycin, Col[illegible]rectal carcinoma, Drug resistance.

Introduction

Colorectal carcinoma (CRC) is the most common malignant tumor in digestive tract worldwide and frequently located at the junction between the rectum and sigmoid colon. CRC is frequently occurred in 40-50 years aged population, and males have 2-3 folds higher incidence than females[1]. CRC has an insidious onset, with only uncomfortable, indigitation, and stool occult blood. As disease gradually progresses, stool habitat may change, accompanied with abdominal pain, stool blood, lesions and intestinal obstruction, plus anemia, fever and body weight loss. Although major advancement has been made regarding the diagnosis and treatment of CRC, its overall treatment efficacy has not been improved, without significant changes in patient survival or prognosis. In terminal stage with distal metastasis, 5-year survival rate was less than 20%[2].

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β-catenin is the core regulatory protein in canonical Wnt/β-catenin signal pathway. Abnormal upregulation of β-catenin can over-activate Wnt/β-catenin signal transduction and is closely associated with occurrence, progression, and metastasis of various tumors[3-5]. Various studies[6-9] showed that higher activity of Wnt/β-catenin is correlated with the drug resistance of various tumor cells. Glycogen synthase kinase-3β (GSK-3β) is an important factor in Wnt/β-catenin signal pathway, in which GSK-3β is located upstream of β-catenin, responsible for its phosphorylation and degradation, thus blocking Wnt/β-catenin signal pathway transduction for antagonizing its effects on proliferation facilitation and apoptosis inhibition. So, it plays a tumor suppressor role in the occurrence of various tumors[10-12]. Recent reports[13-15] showed important roles of abnormal expression of GSK-3β in the onset, progression and metastasis in CRC.

MicroRNA is a group of newly discovered non-coding single-stranded RNA in eukaryotes with 22-25 nucleotides length and can modulate more than one-third of human genes expression via targeted degradation of mRNA or inhi[illegible] its translation. Abnormal expression or fu[illegible] miRNA plays an important role in tumor[illegible]genesis[16,17]. Multiple investigations[18,19] showe[illegible]nificantly elevated miR-224 expression in CR[illegible] mor tissues, and its expression level was correla[illegible] with treatment sensitivity and [illegible] prognosis. Bioinformatics analysis sh[illegible] co[illegible]mentary binding sites between m[illegible]24 and [illegible]UTR of GSK-3β mRNA. This st[illegible] 224 plays a role in mo[illegible]lating [illegible] expression, Wnt/β-catenin path[illegible] activity, [illegible] cell proliferation, apoptosis a[illegible] as drug sens[illegible] against Adriamycin (A[illegible]).

[illegible]aterials and [illegible]ods

Ma[illegible] Reage[illegible] and Materials

N[illegible] epitheli[illegible] cell line NCM460 was purchas[illegible] Scien[illegible] (Carlsbad, CA, USA). [illegible] CR[illegible] SW480 was purchased from [illegible] (Manas[illegible], USA). Roswell Park Memo[illegible] Institute-1640 (RPMI 1640) culture medium [illegible] purchased from Gibco (Waltham, MA, [illegible]) [illegible] bovine serum (FBS) was purchased [illegible] Gemini Bio Product (Woodland, Northern [illegible]nia, USA). RNA extraction kit GenElute™, To[illegible]A Purification Kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescent quantitative PCR reagent TransScrip Green One-Step qRT-PCR SuperMix was purchase[illegible] Quanshijin Bio (Beijing, China). MiR[illegible] miR-214 inhibitor, miR-NC and t[illegible]ection re[illegible]agent riboFECT™ CP was pur[illegible]ed from Ruibo Bio (Changzhou, China). [illegible] anti-human GSK-3β and β-catenin was purchas[illegible] Active Motif (Carlsbad, CA, US[illegible]). Mouse [illegible]man Survivin and β-actin w[illegible] purchased fro[illegible] System (Minneapolis, [illegible]N, USA). HRP co[illegible]gated secondary a[illegible] wa[illegible]urchased from Cell Signaling Techno[illegible]vers, MA, USA). RIPA lysis buff[illegible]nd BC[illegible]in qu[illegible]fication kit, Annexin [illegible] cell apoptosis [illegible] BeyoECL Plus hyper[illegible] ECL develop[illegible] kit were all purchase[illegible]om [illegible]ime Bio (Nantong, China). pSicoR plasmid wa[illegible]chased from BioVector (Beiji[illegible] China). Cell [illegible]ation test kit Click-iT[illegible] Alexa Fluor 488 [illegible]ow Cytometry Assay [illegible] was purchased from Molecular Probes (Eu[illegible]ne, OR, USA). [illegible]ciferase activity kit (Dual-Glo [illegible]iferase Assay [illegible]ystem) and pMIR luciferase re[illegible] plasmid[illegible]ere purchased from Promega (Ma[illegible] WI, [illegible]SA). CCK-8 cell viability assay kit was pu[illegible]ased from Dojindo (Kumamoto, Ja[illegible]). Adriamycin (ADM) was purchased from [illegible]ma (Tokyo, Japan).

Cell Culture

SW480 and NCM460 cells were kept in RPMI 1640 medium containing 10% FBS and 1% streptomycin, and in an incubator with 5% CO_2 at 37°C. Culture medium was changed every 2-3 days. After paving all dishes, cells were passed at 1:4 ratios. Cells at log-growth phase were used for further experiments.

Generation of SW480/ADM Cell Model and Drug-Resistant Index

Cells at log-growth phase were treated with 0.5 μg/mL ADM as the starting concentration. 24 h later, the culture medium was changed to remove dead cells. When cells showed stable growth for 2-3 weeks, ADM drug concentration was then gradually elevated to 1 μg/mL, 2 μg/mL, 4 μg/mL. Those cells that can normally grow at 4 μg/mL ADM were maintained for repeated passage to establish ADM-resistant cancer cell line SW480/ADM.

SW480 and SW480/ADM cells were inoculated into 96-well plate and treated for 48 h with gradient concentrations of ADM (0, 1, 2.5, 5, 10, 20, 40, 80 and 160 μg/ml). Six replicates were recruited at each concentration. The CCK-

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8 reagent was then added for measurement of the absorbance values at 450 nm (A450) of each well after 4 h incubation. Inhibition rate (%) = (1-A450 (drug treatment group))/A450 (control group) x 100%. The IC_{50} value was calculated as the drug concentration required for inhibiting 50% cell growth using SPSS software. Resistance index (RI) = IC50 of SW480/ADM cells/ IC50 of SW480 cells.

Flow Cytometry Analysis of Cell Proliferation

Cultured cells were re-suspended. After 2 h incubation in 10 µM EdU, cells were further cultured for 48 h, and were digested by trypsin, and centrifuged in PBS containing 1% bovine serum albumin (BSA) for washing them. 100 µL Click-iT was added for 15 min room temperature incubation and was washed with PBS containing 1% BSA. 100 µL Click-iT saponin was added for 10 min permeabilization, followed by the addition of 500 µL reaction buffer containing PBS, $CuSO_4$, Alexa Fluor 488, buffer additive for 30 min incubation under dark at room temperature. 3 mL Click-iT saponin-based permeabili[illegible] and wash reagent were used for centri[illegible] and washing. Cells were then re-suspe[illegible] in 500 µL Click-iT saponin-based permeabili[illegible]n and wash reagent. Next, they were tested fo[illegible] proliferation using FC 500 MCL/MPL flow [illegible]tometry (Beckman Coulter, F[illegible]A, USA).

Dual Luciferase Activ[illegible] Assay

Full-length fragment [illegible]-ty[illegible] forms of 3'-UTR of [illegible]K-3β [illegible]s amplified and was dual dige[illegible] and liga[illegible]o a pMIR plasmid for trans[illegible]ng competent [illegible]a. Positive clones wi[illegible]o[illegible]equences we[illegible] screened by sequencin[illegible] and we[illegible]med as pMIR-GSK-3β-wt and [illegible]MIR-GSK-3β[illegible] RiboFECT™ CP was use[illegible]o co-transfect p[illegible]GSK-3β-wt (or pMIR[illegible]K-3β-mut) and miR-224 mimic (or miR-224 [illegible]bitor o[illegible]R-NC) into SW480 cells. After 48 h[illegible]at[illegible], the du[illegible]luciferase activity was measur[illegible]ual-Gl[illegible]ciferase Assay System.

[illegible]ructio[illegible]SK-3β Ov[illegible] Expressio[illegible] Plasmid

[illegible] fragment of GSK-3β gene was amplified a[illegible]d for the length by gel electrophore[illegible]. After dual enzymatic digestion, the fragment [illegible]ated into a pSicoR plasmid for transforming [illegible]teria. Positive clones were amplified to extract recombinant plasmids containing targeted fragments. Sequencing was performed to confirm correct insertion of target fragments. The[illegible] plasmids with correct insertion were na[illegible] coR-GSK-3β. Empty plasmid pSic[illegible] blank wa[illegible] used as the control group.

Cell Transfection and Groupi[illegible]

In vitro cultured SW48[illegible]DM ce[illegible] assigned into five trans[illegible]on groups: [illegible] transfection group, m[illegible]-224 in[illegible]ibitor trans[illegible]tion group, pSico[illegible]k tr[illegible]ection group, pSicoR-GSK-3β group, [illegible]-224 in[illegible]tor + pSicoR-GSK-3[illegible]oup. D[illegible]transf[illegible]on, 100 µL 1 X ribo[illegible]T™ CP buff[illegible]d to dilute 3 µL pSic[illegible]k, pSicroR-G[illegible]β, miR-NC, and miR[illegible]4 in[illegible]. After room temperature incubation for 5 m[illegible]µL riboFECT™ CP reagent[illegible] added for a g[illegible]mixture for 0-15 min a[illegible] temperature. ri[illegible]ECT™ CP reagent [illegible]xture was added into the culture medium for [illegible]h continuous[illegible]ubation. Cells were collected [illegible]ssays.

qR[illegible] fo[illegible]ne Expression

GenE[illegible] Total RNA Purification Kit was [illegible]ed to extract cell RNA following the manual [illegible]. TransScript Green One-Step qRT-[illegible]CR [illegible]perMix was used to test gene relative expression level by one-step qRT-PCR. In a 20 µL reaction system, 1 µg template RNA, 0.3 µM forward and 0.3 µM reverse primer, 10 µL 2 X TransStart Tip Green qPCR SuperMix, 0.4 µL One-step RT Enzyme Mix, 0.4 µL Passive Reference Dye II and RNase-free water were added. qRT-PCR conditions were: 45°C for 5 min, 95°C 30 s, followed by 40 cycles each containing 94°C for 5 s and 60°C for 30 s. ABI ViiA™7 Real-time fluorescent quantitative PCR cycler was used to measure gene expression.

Western Blot

Proteins were extracted by radioimmunoprecipitation assay (RIPA) lysis buffer and were quantified using bicinchoninic acid (BCA) test kit. 40 µg samples were separated in 8-10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separating gel and 5% condensing gel. After transferring to the polyvinylidene difluoride (PVDF) membrane, the membrane was blocked by 5% defatted milk powder at room temperature. Primary antibody (GSK-3β at 1:3000 dilutions, β-catenin at 1:2000, Survivin at 1:200, and β-actin at 1:10000) was added for 4°C overnight incubation. HRP-conjugated secondary antibody (1:20000) was

added for 60 min incubation at room temperature. After three times of PBST rinsing, BeyoECL Plus working buffer prepared from equal volume mixture of solution A and solution B was added onto the blotting membrane for even distribution. After 2-3 min incubation at room temperature, BeyoECL Plus working solution was discarded along with excess water in filter paper. The membrane was placed between two fresh-keeping films for testing.

Cell Apoptosis Assay

Cells were digested and collected. After rinsing in PBS, cells were re-suspended in 100 µL Annexin V Binding Buffer. 5 µL FITC Annexin V was firstly added, followed by addition of 5 µL PI. After 15 min room temperature incubation, 400 µL Annexin V Binding Buffer was added. FC 500MCL/MPL flow cytometry was used to measure cell apoptosis.

Statistical Analysis

SPSS 18.0 (SPSS Inc., Chicago, IL, US... ...as used for data analysis. All measu... were presented as mean ± stand... deviation (SD). Student *t*-test was used t... ompare measurement data between group... < 0.05 was considered statistically significant.

R... lts

MiR-224 Targeted a... ...bited GSK-3β Expr... ...on

Online pr... ...tion of mic... ...g showed complemen... ...nding sites be... ...en miR-224 and 3'-U... of G... ...β mRNA (Figure 1A). Dual luciferase gene repo... ...ssay showed that transfecti... ...f miR-224 mi... ...inhibitor significantly ... ...d or increased rel... ...ve luciferase activity

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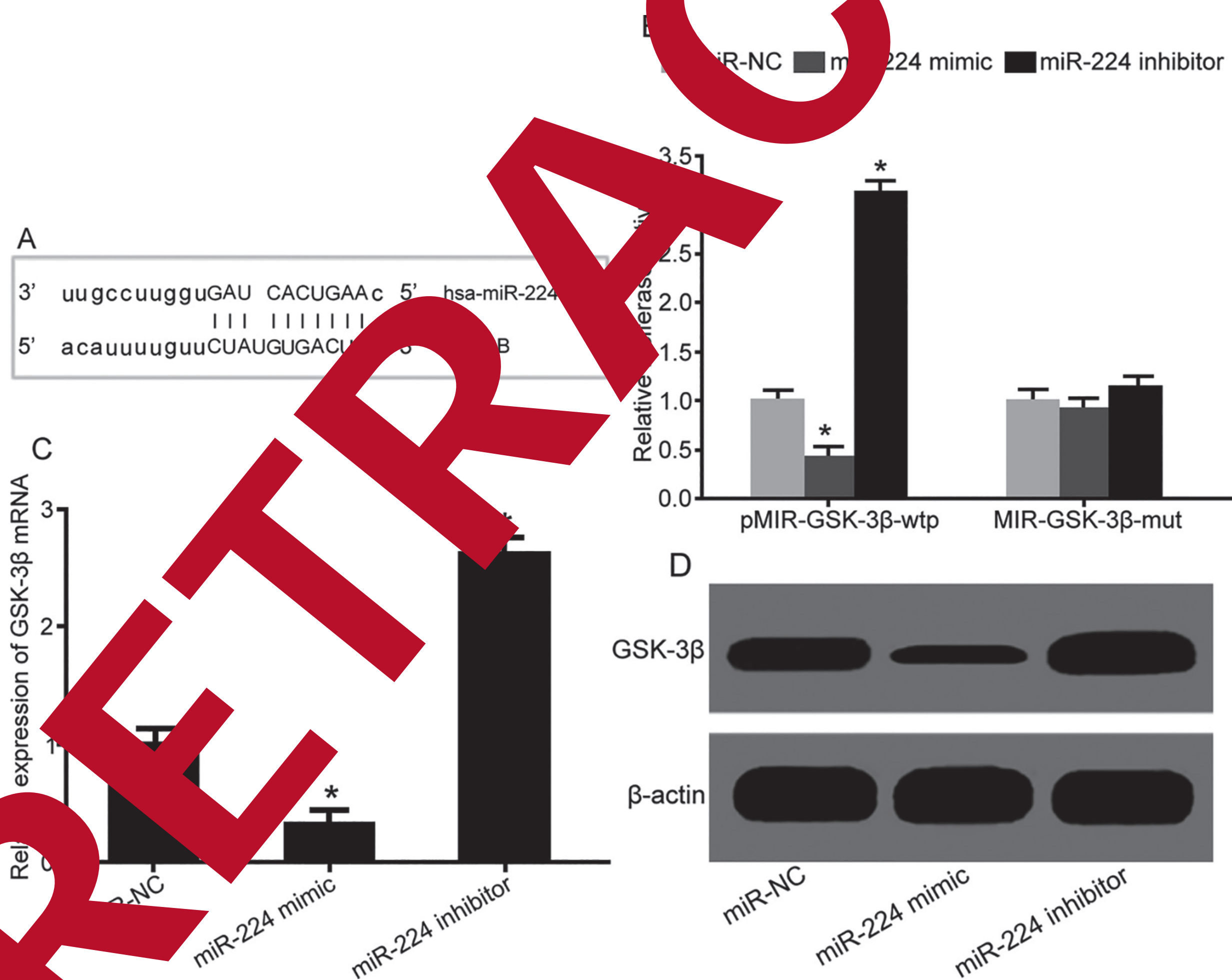

...1. ***A***-miR-224 targeted and inhibited GSK-3β expression. ***A,*** Binding sites between miR-224 and 3'-UTR of GSK-3β mR... ***B,*** Dual luciferase reporter gene assay. ***C,*** qRT-PCR for GSK-3β mRNA expression. ***D,*** Western blot for GSK-3β protein expression. *, $p < 0.05$ compared with miR-NC group.

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inside SW480 cells (Figure 1B), indicating that miR-224 could target and regulate GSK3β mRNA expression. Moreover, compared with miR-NC transfection, miR-224 mimic transfection showed significantly decreased GSK-3β mRNA (Figure 1C) or protein level (Figure 1D), whilst miR-224 inhibitor transfection group showed significantly elevated GSK-3β mRNA (Figure 1C) or protein expression (Figure 1D).

Elevated miR-224 Expression and Lower GSK-3β Expression in Drug-Resistant Cells SW480/ADM

SW480 cells had IC_{50} value of 2.51 μg/mL for ADM, whilst SW480/ADM cells had IC_{50} value of 28.74 μg/mL. RI of SW480/ADM cells against parental cell line SW480 was 11.45. qRT-PCR results showed significantly elevated expression of miR-224, β-catenin and Survivin mRNA in SW480 cells compared to those in NCM480 cells, whilst GSK-3β mRNA expression was decreased. Compared with SW480 cells, SW480/ADM cells showed higher miR-224, β-catenin and Survivin mRNA expression, with lower GSK-3β mRNA expression (Figure 2A). Western blot showed elevated β-catenin and Survivin protein expression in SW480 cells, which had lower GSK-3β protein expression compared to those in NCM480 cells. SW480/ADM cells showed significantly elevated β-catenin and Survivin expression plus

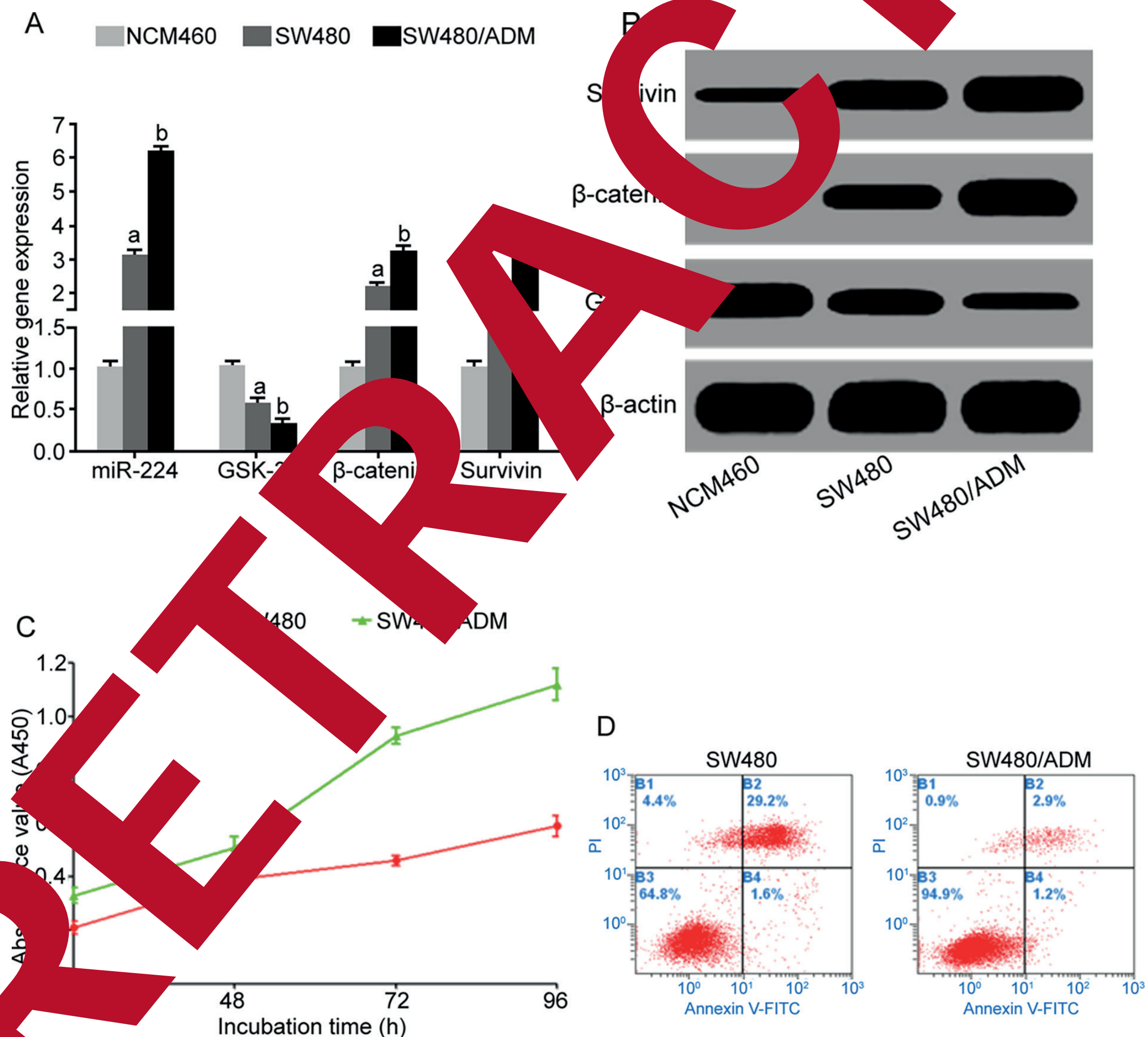


Figure 2. Elevated miR-224 expression and lower GSK-3β expression in drug resistant cells SW480/ADM. ***A,*** qRT-PCR for gene expression. ***B,*** Western blot for protein expression. ***C,*** CCK-8 for cell proliferation. ***D,*** Flow cytometry for cell apoptosis. a, $p < 0.05$ comparing between SW480 and NCM460 cells; b, $p < 0.05$ comparing between SW480/ADM and SW480 cells.

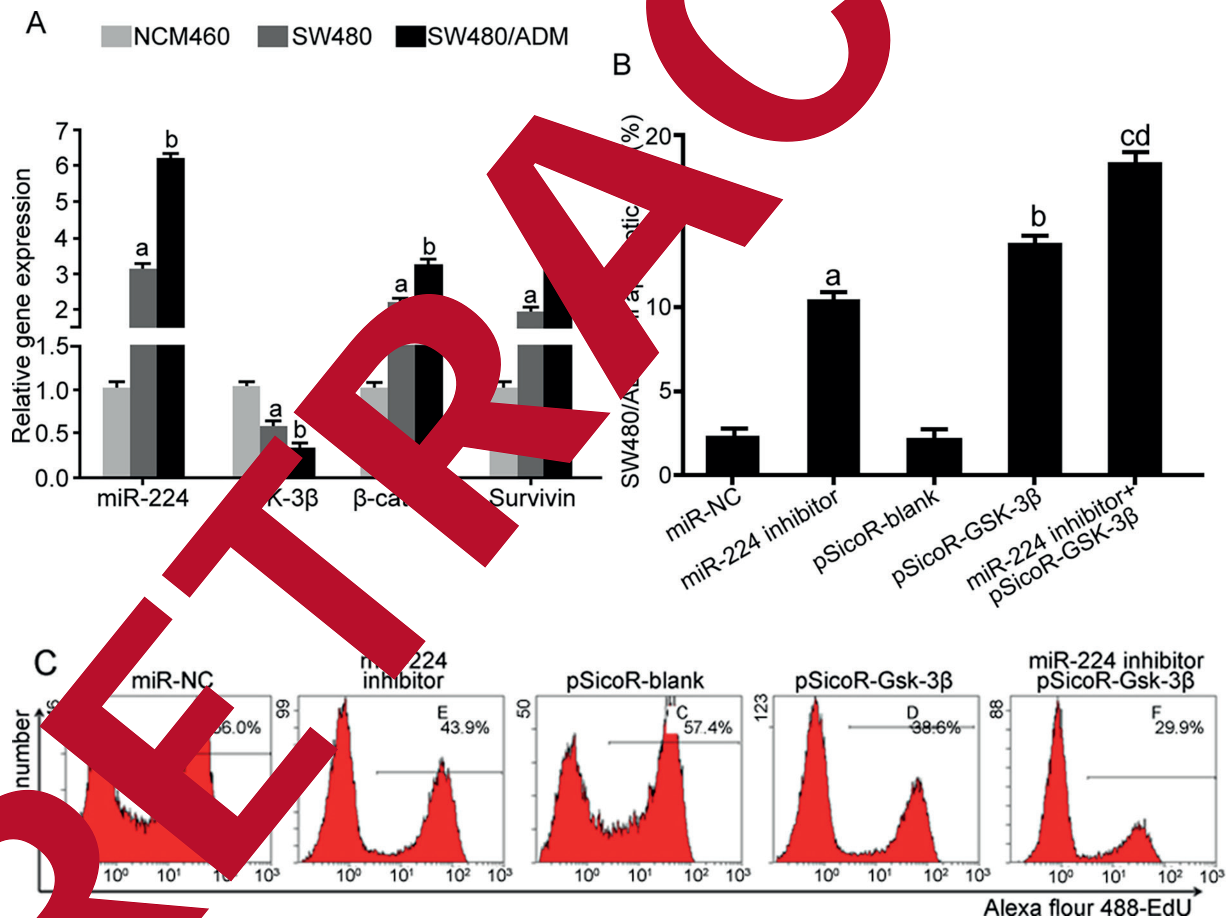
lower GSK-3β protein expression compared with SW480 cells (Figure 2B). Under 2.51 μg/mL ADM treatment, SW480 cells showed a significantly lower proliferation potency than SW480/ADM cells (Figure 2C), whilst apoptotic rate was significantly higher (Figure 2D).

MiR-224 Down-Regulation Induced SW480/ADM Cell Apoptosis and Enhanced ADM Sensitivity

Under 4 μg/mL ADM treatment, SW480/ADM cells showed lower apoptotic rate (Figure 3B), whilst proliferation potency was potentiated (Figure 3C). After transfection of miR-224 inhibitor and/or pSicoR-GSK-3β, GSK-3β expression was remarkably elevated in SW480/ADM cells, whilst β-catenin and Survivin expression was decreased (Figure 3A), accompanied by elevated cell apoptotic rate (Figure 3B) and weakened proliferation potency (Figure 3C), plus reduced drug re[illegible]ce of ADM.

Discussion

Wnt/β-catenin signal p[illegible]way is a[illegible]pathway using β-catenin a[illegible]e core molec[illegible] [illegible]nonical Wnt/β-catenin [illegible]gnal pathway is hi[illegible]y conserved in evolu[illegible]nd i[illegible]dely involved in regulating var[illegible]ous b[illegible] effects i[illegible]uding cell proliferati[illegible] cycle, [illegible]popt[illegible]. β-catenin is the c[illegible] regulatory p[illegible] canonical Wnt/β-cate[illegible]al pathway. [illegible] abnormally elevated [illegible]ateni[illegible]pression can over-activate Wnt/β-catenin sign[illegible]hway and is closely associ[illegible] with the occ[illegible]e, progression, and



[illegible]ure 3. MiR-224 down-regulation induced SW480/ADM cell apoptosis and enhanced ADM sensitivity. ***A,*** Western blot for [illegible]n expression. ***B,*** Flow cytometry for cell apoptosis. ***C,*** EdU staining for cell proliferation. a, $p < 0.05$ comparing between [illegible] inhibitor and miR-NC groups; b, $p < 0.05$ comparing between pSicoR-GSK-3β and pSicoR-blank groups; c, $p < 0.05$ com[illegible]g between miR-224 inhibitor + pSicoR-GSK-3β and miR-224 inhibitor groups; d, $p < 0.05$ comparing between miR-224 inhibitor + pSicoR-GSK-3β and pSicoR-GSK-3β groups.

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metastasis of various tumors such as prostate cancer[4], breast carcinoma[5], and pancreatic cancer[3]. Over-activation of Wnt/β-catenin pathway is closely associated with drug resistance, poor treatment response, and post-surgery recurrence of multiple tumors including leukemia[7], pancreatic cancer[9], lung cancer[8], and CRC[6]. In the negative feedback regulatory mechanism of Wnt/β-catenin signal pathway, β-catenin can be phosphorylated by GSK-3β in the complex composed of axin, adenomatous polyposis coli (APC), and GSK-3β. It can be further degraded by β-transducin repeat-containing protein (β-TrCP) induced ubiquitin/proteasome pathway, thus maintaining a low level in cytoplasm. So, it cannot facilitate the target gene transcription and expression until entry into the nucleus for binding with T-cell factor/lymphoid enhancing factor (TCF/LEF)[20,21]. As a negative regulator factor, GSK-3β can antagonize the transcriptional facilitating role of Wnt/β-catenin on various downstream target genes including Survivin, cyclin D, and Bcl-2, thus inhibiting Wnt/β-catenin signal pathway induced pro-proliferation and anti-apoptotic effects, and playing a tumor suppressor role in the occu[illegible] of multiple tumors such as breast canc[illegible] cavity cancer[11], and non-small cell lung c[illegible]r[12]. Various researches showed that decrease[illegible]pression or function of GSK-3β was assoc[illegible] with the drug resistance of vario[illegible] tumor c[illegible] such as lung cancer[8] and brea[illegible][22]. Recen[illegible] investigations[13-15] revealed t[illegible] impor[illegible] regulatory role of GSK-3β abn[illegible]l expres[illegible]n in the occurrence, progression [illegible]tast[illegible]. Multiple studies[18,19] s[illegible]ed s[illegible]tly elevated miR-224 expressio[illegible] CRC tu[illegible]ssues, and the correlation b[illegible]en its express[illegible]el and treatment res[illegible] or patient [illegible]ognosis. Bioinformati[illegible] analys[illegible]ed complementary binding si[illegible] between m[illegible]4 and 3'-UTR of GSK-3β [illegible]NA. This work [illegible]igated if miR-224 pl[illegible] a role in mediating GSK-3β expression, Wn[illegible]atenin [illegible]hway activity as well as ADM drug[illegible] of ADM[illegible]

Dua[illegible]rase ge[illegible]reporter assay showed [illegible]transfe[illegible] of [illegible]R-224 mimic or miR-224 i[illegible]or sign[illegible]y decreased or increased rela[illegible] luciferase activity in SW480 cells, and su[illegible] or potentiated GSK-3β expression i[illegible]lls, demonstrating the targeted [illegible]ulatory relationship between miR-224 and [illegible]β. Results of this report showed significa[illegible] elevated miR-224 expression in CRC cells SW480 compared with normal human colon epithelial cells NCM460, whilst GSK-3β expression was significantly lower, ind[illegible] possible tumor suppressor role [illegible] in CRC, and the correlation bet[illegible]n GSK-3β down-regulation induced by ab[illegible]nally elevated miR-224 and CRC occurre[illegible] Adamopoulos et al[18] showed significantly [illegible]d miR-224 expression was obser[illegible] in CRC [illegible] tissues compared with adj[illegible]nt tumor tiss[illegible] higher expression i[illegible]ated higher recu[illegible]t rate and worse sur[illegible]r pr[illegible]osis. Arndt et al[23] found signi[illegible]ed miR[illegible]4 expression in CR[illegible]umor [illegible] and [illegible] correlation with T[illegible] stage. Bru[illegible] revealed abnormall[illegible]d miR-224 e[illegible]sion in colon canc[illegible]umo[illegible]es compared with normal colon tissues. Fu et [illegible]so showed abnormally elev[illegible] miR-224 ex[illegible]n in colon tumor ti[illegible]ao et al[26] revea[illegible] abnormally elevat[illegible] miR-224 expression in CRC patient tumor [illegible]sues, and its [illegible]rrelation with CRC progres[illegible] and progn[illegible]. This study suggested that m[illegible]24 up-reg[illegible]tion might participate in the CR[illegible]s, which was consistent with previous [illegible]es conducted by Arndt et al[23], [illegible]unet et al[24] and Fu et al[25].

[illegible]/ADM cells had significantly high[illegible]IC[illegible] values than SW480 cells, plus higher proliferation activity and apoptosis resistance, indicating successful induction of ADM-resistant SW480 cells. Compared with SW480 cells, SW480/ADM cells had significantly elevated miR-224 expression, and lower GSK-3β expression, indicating that GSK-3β down-regulation was induced by abnormally elevated miR-224 expression and drug resistance of CRC. In studies on miR-224 and tumor cell drug resistance, Zhao et al[27] found significantly elevated miR-224 expression in cisplatin-induced ovarian carcinoma drug-resistant cell line compared with parental cell line, and the induction of ovarian cancer cell apoptosis resistance and cisplatin resistance via targeted inhibition on PPKCD expression. Wang et al[28] revealed that compared with cisplatin sensitive lung cancer cells, those tumor cells with cisplatin drug resistance showed significantly elevated miR-224 expression, which can target and suppress p21 expression to decrease lung cancer cell apoptosis as well as suppress cisplatin sensitivity. In this study, miR-224 up-regulation is a facilitating factor for tumor cell resistance, which was consistent with the results obtained by Zhao et al[27] and Wang et al[28]. Further study showed that af-

ter transfection of miR-224 inhibitor and/or pSicoR-GSK-3β, SW480/ADM cells showed significantly elevated GSK-3β expression, plus lower β-catenin or Survivin expression, enhanced cell apoptosis rate, inhibited proliferation activity and reduced drug resistance of ADM. Liao et al[26] showed that miR-224 could facilitate cell cycle transition from G1 to S phase via inhibiting PHLEPP1 and PHLPP2 expression, thus facilitating proliferation of CRC cell lines SW620 and HCT116 proliferation *in vivo*. Lin et al[29] found that miR-224 could enhance *in vitro* invasion potency and metastatic ability of CRC cells via targeting SMAD4 expression. Zhang et al[19] revealed that over-expression of miR-224 could facilitate proliferation of CRC cells SW480 to enhance its migration and invasion potency. This work revealed the association between miR-224 up-regulation and malignant biological features of CRC tumor cells. Amankwatia et al[30] showed that miR-224 down-regulation significantly enhanced the sensitivity of colon cancer HCT116 cells on drug sensitivity of chemotherapy reagent 5-fluorouracil (5-FU). Our investigation showed that miR-224 down-regulation ca[illegible] pair drug resistance of CRC cells, as su[illegible] by Amankwatia et al[30]. Besides, miR-2[illegible] this study also revealed that GSK-3β was a[illegible]portant regulatory factor for ADM resistanc[illegible] tumors, and miR-224 up-regulation can enha[illegible] Wnt/β-catenin signal pathwa[illegible]get gen[illegible] Survivin expression via ta[illegible]ted in[illegible]tion on GSK-3β expression, thus [illegible]ibiting [illegible]M drug sensitivity of CRC cell[illegible]h [illegible] reported before. Ho[illegible]er, [illegible]nvestigated the targeted relati[illegible]ip betwe[illegible]R-224 and GSK-3β in *in vi*[illegible]odel, plus re[illegible]ry role in chemothera[illegible]sistance. Whe[illegible]er miR-224 plays sim[illegible]ar regu[illegible] role *in vivo* has not been conf[illegible]ed. In the fu[illegible] *in vivo* studies of miR-22[illegible]ight be performed [illegible]ugh collecting sampl[illegible]rom patients with chemotherapy sensitivi[illegible]d resi[illegible]ce for measuring the expression[illegible]iR-[illegible]4 and G[illegible]K-3β.

[illegible]nclusions

M[illegible]224 up-regulation is associated with A[illegible]e of CRC cells. Inhibition of miR-[illegible] can up-regulate GSK-3β expression, reduce [illegible]catenin signal transduction and target ge[illegible]vivin expression, thus suppressing ADM drug resistance of CRC cell line SW480.

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Conflict of Interest

The Authors declare that [illegible] have no c[illegible]nflict of inter[illegible]s.

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