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

C.-Q. LIANG1, Y.-M. FU1, Z.-Y. LIU1, B.-R. XING1, Y. JIN2, J.-L. HUANG3

1Department of Emergency Medicine, the Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China
2Department of Pathology, the Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China
3Department of Gastrointestinal Surgery, the third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China

Caiqian Liang and Yongmei Fu contributed equally to this work

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 miR-224 and GSK-3β. This study investigated if miR-224 plays a role in mediating GSK-3β expression, Wnt/β-catenin pathway activity, CRC cell proliferation, apoptosis as well as drug sensitivity of Adriamycin (ADM).

MATERIALS AND METHODS: Dual luciferase gene reporter assay demonstrated the regulatory relationship between miR-224 and GSK-3β. Expression of miR-224, GSK-3β, β-catenin, and Survivin was measured in normal colon epithelium NCM460, CRC cell line SW480, and drug-resistant SW480/ADM cell line. Flow cytometry measured apoptosis under ADM with an IC50 concentration of SW480 cells, followed by CCK-8 analysis of cell proliferation. SW480/ADM cells were treated with miR-224 inhibitor and/or pSicoR-GSK-3β, followed by analysis of the expressions of GSK-3β, β-catenin and Survivin, cell apoptosis, and cell proliferation by EdU staining.

RESULTS: MiR-224 targeted and inhibited GSK-3β expression. In SW480/ADM cells, GSK-3β expression and cell apoptosis rate were lower than those in SW480 cells, whilst miR-224, β-catenin, and Survivin expression or proliferation were higher than those in SW480 cells. Transfection of miR-224 inhibitor and/or pSicoR-GSK-3β significantly increased GSK-3β expression in SW480/ADM cells, and decreased β-catenin and Survivin expression, leading to reduced proliferation potency, enhanced cell apoptosis and suppressed ADM resistance.

CONCLUSIONS: MiR-224 up-regulation is associated with ADM resistance of CRC cells. Suppression of miR-224 expression up-regulated GSK-3β expression, inhibited Wnt/β-catenin signal pathway activity and Survivin expression, as well as reduced ADM resistance of CRC SW480 cells.

Key Words: MiR-224, GSK-3β, Wnt/β-catenin, Adriamycin, Colorectal 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. 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%.

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

C.-Q. LIANG1, Y.-M. FU1, Z.-Y. LIU1, B.-R. XING1, Y. JIN2, J.-L. HUANG3

1Department of Emergency Medicine, the Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China
2Department of Pathology, the Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China
3Department of Gastrointestinal Surgery, the third Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China

Caiqian Liang and Yongmei Fu contributed equally to this work

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 miR-224 and GSK-3β. This study investigated if miR-224 plays a role in mediating GSK-3β expression, Wnt/β-catenin pathway activity, CRC cell proliferation, apoptosis as well as drug sensitivity of Adriamycin (ADM).

MATERIALS AND METHODS: Dual luciferase gene reporter assay demonstrated the regulatory relationship between miR-224 and GSK-3β. Expression of miR-224, GSK-3β, β-catenin, and Survivin was measured in normal colon epithelium NCM460, CRC cell line SW480, and drug-resistant SW480/ADM cell line. Flow cytometry measured apoptosis under ADM with an IC50 concentration of SW480 cells, followed by CCK-8 analysis of cell proliferation. SW480/ADM cells were treated with miR-224 inhibitor and/or pSicoR-GSK-3β, followed by analysis of the expressions of GSK-3β, β-catenin and Survivin, cell apoptosis, and cell proliferation by EdU staining.

RESULTS: MiR-224 targeted and inhibited GSK-3β expression. In SW480/ADM cells, GSK-3β expression and cell apoptosis rate were lower than those in SW480 cells, whilst miR-224, β-catenin, and Survivin expression or proliferation were higher than those in SW480 cells. Transfection of miR-224 inhibitor and/or pSicoR-GSK-3β significantly increased GSK-3β expression in SW480/ADM cells, and decreased β-catenin and Survivin expression, leading to reduced proliferation potency, enhanced cell apoptosis and suppressed ADM resistance.

CONCLUSIONS: MiR-224 up-regulation is associated with ADM resistance of CRC cells. Suppression of miR-224 expression up-regulated GSK-3β expression, inhibited Wnt/β-catenin signal pathway activity and Survivin expression, as well as reduced ADM resistance of CRC SW480 cells.

Key Words: MiR-224, GSK-3β, Wnt/β-catenin, Adriamycin, Colorectal 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. 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%.
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. Various studies 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. Recent reports 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 inhibiting its translation. Abnormal expression or function of miRNA plays an important role in tumor pathogenesis. Multiple investigations showed significantly elevated miR-224 expression in CRC tumor tissues, and its expression level was correlated with treatment sensitivity and patient prognosis. Bioinformatics analysis showed complementary binding sites between miR-224 and 3'-UTR of GSK-3β mRNA. This study investigated if miR-224 plays a role in modulating GSK-3β expression, Wnt/β-catenin pathway activity, CRC cell proliferation, apoptosis as well as drug sensitivity against Adriamycin (ADM).

**Materials and Methods**

**Major Reagents and Materials**

Normal colon epithelial cell line NCM460 was purchased from Scien cell (Carlsbad, CA, USA). Human CRC cell line SW480 was purchased from ATCC (Manassas, VA, USA). Roswell Park Memorial Institute-1640 (RPMI 1640) culture medium was purchased from Gibco (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Gemini Bio Product (Woodland, Northern California, USA). RNA extraction kit GenElute™, Total RNA Purification Kit was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluorescent quantitative PCR reagent TransScrip Green One-Step qRT-PCR SuperMix was purchased from Quanshijin Bio (Beijing, China). MiR-224 mimic, miR-214 inhibitor, miR-NC and transfection reagent riboFECT™ CP was purchased from Ribobio (Changzhou, China). Rabbit anti-human GSK-3β and β-catenin was purchased from Active Motif (Carlsbad, CA, USA). Mouse anti-human Survivin and β-actin were purchased from R&D System (Minneapolis, MN, USA). HRP conjugated secondary antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). RIPA lysis buffer and BCA protein quantification kit, Annexin/PI cell apoptosis test kit, BeyoECL Plus hyper-sensitive ECL development kit were all purchased from Beyotime Bio (Nantong, China). pSicoR plasmid was purchased from BioVector (Beijing, China). Luciferase activity kit (Dual-Glo Luciferase Assay System) and pMIR luciferase reporter plasmid were purchased from Promega (Madison, WI, USA). CCK-8 cell viability assay kit was purchased from Dojindo (Kumamoto, Japan). Adriamycin (ADM) was purchased from Meiji Pharma (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 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-
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 IC50 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, CuSO4, Alexa Fluor 488, buffer additive for 30 min incubation under dark at room temperature. 3 mL Click-iT saponin-based permeabilization and wash reagent were used for centrifugation and washing. Cells were then re-suspended in 500 μL Click-iT saponin-based permeabilization and wash reagent. Next, they were tested for cell proliferation using FC 500 MCL/MPL flow cytometry (Beckman Coulter, Fullerton, CA, USA).

**Dual Luciferase Activity Assay**

Full-length fragment of wild-type or mutant forms of 3’-UTR of GSK-3β gene was amplified and was dual digested and ligated into a pMIR plasmid for transforming competent bacteria. Positive clones with correct sequences were screened by sequencing and were named as pMIR-GSK-3β-wt and pMIR-GSK-3β-mut. RiboFECT™ was used to co-transfect pMIR-GSK-3β-wt (or pMIR-GSK-3β-mut) and miR-224 mimic (or miR-224 inhibitor or miR-NC) into SW480 cells. After 48 h incubation, the dual luciferase activity was measured by Dual-Glo Luciferase Assay System.

**Construction of GSK-3β Over-Expression Plasmid**

CDS fragment of GSK-3β gene was amplified and determined for the length by gel electrophoresis. After dual enzymatic digestion, the fragment was ligated into a pSicoR plasmid for transforming bacteria. Positive clones were amplified to extract recombinant plasmids 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 SW480/ADM cells were assigned into five transfection groups: miR-NC transfection group, miR-224 inhibitor transfection group, pSicoR-blank transfection group, pSicoR-GSK-3β group, and miR-224 inhibitor + pSicoR-GSK-3β group. During transfection, 100 μL 1 X riboFECT™ CP buffer was used to dilute 3 μL pSicoR-blank, pSicoR-GSK-3β, miR-NC, and miR-224 inhibitor. After room temperature incubation for 5 min, 10 μL riboFECT™ CP reagent was added for a gentle mixture for 0-15 min at room temperature. riboFECT™ CP reagent mixture was added into the culture medium for 72 h continuous incubation. Cells were collected for assays.

**qRT-PCR for Gene Expression**

GenElute™ Total RNA Purification Kit was used to extract cell RNA following the manual instruction. TransScript Green One-Step qRT-PCR SuperMix 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 ViiATM7 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, USA) was used for data analysis. All measurement data were presented as mean ± standard deviation (SD). Student t-test was used to compare measurement data between groups. p < 0.05 was considered statistically significant.

**Results**

**MiR-224 Targeted and Inhibited GSK-3β Expression**

Online prediction of microRNA.org showed complementary binding sites between miR-224 and 3'-UTR of GSK-3β mRNA (Figure 1A). Dual luciferase gene reporter assay showed that transfection of miR-224 mimic or inhibitor significantly reduced or increased relative luciferase activity.

![Figure 1](image.png)

*Figure 1. A-miR-224 targeted and inhibited GSK-3β expression. A, Binding sites between miR-224 and 3'-UTR of GSK-3β mRNA. 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.*
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 IC₅₀ value of 2.51 μg/mL for ADM, whilst SW480/ADM cells had IC₅₀ 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 also 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 resistance of ADM.

**Discussion**

Wnt/β-catenin signal pathway is a Wnt pathway using β-catenin as the core molecule. Canonical Wnt/β-catenin signal pathway is highly conserved in evolution and is widely involved in regulating various biological effects including cell proliferation, cycle, and apoptosis. β-catenin is the core regulatory protein in canonical Wnt/β-catenin signal pathway. The abnormally elevated β-catenin expression can over-activate Wnt/β-catenin signal pathway and is closely associated with the occurrence, progression, and

---

**Figure 3.** MiR-224 down-regulation induced SW480/ADM cell apoptosis and enhanced ADM sensitivity. **A**, Western blot for protein expression. **B**, Flow cytometry for cell apoptosis. **C**, EdU staining for cell proliferation. a, p < 0.05 comparing between miR-224 inhibitor and miR-NC groups; b, p < 0.05 comparing between pSicoR-GSK-3β and pSicoR-blank groups; c, p < 0.05 comparing 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.
metastasis of various tumors such as prostate cancer, breast carcinoma, and pancreatic cancer. 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, pancreatic cancer, lung cancer, and CRC. 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). 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 occurrence of multiple tumors such as breast cancer, oral cavity cancer, and non-small cell lung cancer.

Various researches showed that decreased expression or function of GSK-3β was associated with the drug resistance of various tumor cells such as lung cancer and breast cancer. Recent investigations revealed the important regulatory role of GSK-3β abnormal expression in the occurrence, progression and metastasis of CRC. Multiple studies showed significantly elevated miR-224 expression in CRC tumor tissues, and the correlation between its expression level and treatment responsiveness or patient prognosis. Bioinformatics analysis showed complementary binding sites between miR-224 and 3'-UTR of GSK-3β mRNA. This work investigated if miR-224 expression to decrease lung cancer cell apoptosis and proliferation activity and apoptosis resistance, which can target and suppress p21 expression, which can target and suppress p21 expression to decrease lung cancer cell apoptosis and proliferation activity and apoptosis resistance.
MiR-224 up-regulation is associated with ADM resistance of CRC cells. Inhibition of miR-224 can up-regulate GSK-3β expression, reduce Wnt/β-catenin signal transduction and target gene Survivin expression, thus suppressing ADM drug resistance of CRC cell line SW480.

Conclusions

MiR-224 up-regulation is associated with ADM resistance of CRC cells. Inhibition of miR-224 can up-regulate GSK-3β expression, reduce Wnt/β-catenin signal transduction and target gene Survivin expression, thus suppressing ADM drug resistance of CRC cell line SW480.
miRNA-224 promotes cell progression in NSCLC. Oncotarget 2014; 5: 5736-5749.


