MiR-150 alleviates EMT and cell invasion of colorectal cancer through targeting Gli1


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Abstract. – OBJECTIVE: Epithelial-mesenchymal transition (EMT) is related to colorectal cancer invasion and metastasis. Glioma-associated oncogene homolog 1 (Gli1) abnormal expression is associated with EMT, invasion, and metastasis in various cancers. MiR-150 is found downregulated in colorectal cancer pathogenesis. Bioinformatics analysis shows the complementary targeted relationship between miR-150 and the 3’-UTR of Gli1 mRNA. This study explores the role of miR-150 in regulating Gli1 expression, colorectal cancer cell EMT, and invasion.

MATERIALS AND METHODS: Dual luciferase assay confirmed the targeted relationship between miR-150 and Gli1 predicted by bioinformatics analysis. MiR-150 and Gli1 expressions were compared in NCM460, SW480, and SW620 cells. Cell colony formation and invasion were tested in SW480 and SW620 cells. Anip973 and AGYZ83-a cells were treated by 10 ng/mL TGF-β1 to detect miR-150 and Gli1 expressions. SW620 cells were cultured in vitro and divided into five groups, including miR-NC, miR-150 mimic, si-NC, si-Gli1, and miR-150 mimic + si-Gli1 groups.

RESULTS: MiR-150 specifically inhibited Gli1 expression. The level of miR-150 was significantly downregulated, while Gli1 was elevated in SW480 and SW620 cells compared with that in NCM460 cells. SW620 exhibited markedly stronger invasive and colony formation abilities than SW480. The level of miR-150 was apparently reduced, whereas Gli1 was increased in SW620 than that in SW480 cells after the treatment of TGFβ1. MiR-150 mimic and/or si-Gli1 transfection markedly reduced Gli1 and Snail levels, upregulated E-cadherin expression, and attenuated cell colony formation and invasion.

CONCLUSIONS: Downregulation of miR-150 and elevation of Gli1 promote the development and invasion of colorectal cancer cell EMT. MiR-150 attenuated the progression of colorectal cancer cell EMT via inhibiting Gli1.

Key Words: miR-150, Gli1, EMT, Colorectal cancer, Invasion.

Introduction

Colorectal cancer (CRC) is the most common malignant tumor of digestive tract worldwide. It mainly occurs in the junction of the rectum and sigmoid colon. The age range of 40-50 years old represents the higher risk of CRC, and the incidence of male is 2-3 times higher than that of female. CRC is characterized as invasive and metastatic, which is related to poor curative effect, high recurrence rate, and poor prognosis.

Epithelial-mesenchymal transition (EMT) refers to the biological process of epithelial cell transiting into mesenchymal cells. Downregulation of E-cadherin mediated tight junction between cells and extracellular matrix is an important indicator of EMT. Cancer cell EMT is closely related to tumor progression, metastasis, recurrence, and poor prognosis. Hedgehog signaling pathway widely expresses in multiple tissues and cells, and it participates in regulating embryonic development and damage repair. Physically, Hedgehog signaling pathway is in the resting status. It is involved in various tumor occurrence, development, metastasis, and recurrence when activated by gene mutation, overexpression, or other mechanisms.

Glioma-associated oncogene protein 1 (Gli1) is an important transcription factor in Hedgehog signaling pathway. It can regulate various intranuclear target genes transcription and expression, thus affecting cell proliferation, apoptosis, migration, and invasion. Gli1 overexpression can activate Hedgehog signaling pathway, which is associated with cancer occurrence, progression, and metastasis. Gli1 was found abnormally increased in CRC tissue. MiRNA is a type of endogenous single-stranded noncoding RNA with the length of 22-25 nt discovered in eukaryote. It plays a degradation
or transcriptional inhibition role on mRNA via the binding between miRNA and 3'-UTR of mRNA, thus participating in cell proliferation, differentiation, and migration. MiRNA plays an oncogene or tumor suppressor gene in tumorigenesis. Recent researches showed that miR-150 expression was reduced in CRC tissue. In silico study by bioinformatics analysis shows the complementary targeted relationship between miR-150 and the 3'-UTR of Gli1 mRNA. In our work, we aimed to investigate the effect and possible mechanism of miR-150 on colorectal cancer cell EMT.

**Materials and Methods**

**Main Reagents and Materials**

Human high metastatic CRC cell line SW620, low metastatic CRC cell line SW480, and normal colon epithelial cell line NCM460 were purchased from Jining Cell Culture Center (Shanghai, China). RPMI-1640, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Thermo Fisher Scientific (Waltham, MA, USA). EasyPure RNA Kit and Real-time PCR reagent TransScript Green One-Step qRT-PCR SuperMix were obtained from TransGen Biotech (Beijing, China). miR-NC, miR-150 mimic, miR-150 inhibitor, and riboFECT™ CP were bought from RiboBio (Guangzhou, China). Rabbit anti-human Snail and E-cadherin primary antibodies were obtained from CST (Cell Signaling Technology, Danvers, MA, USA). Mouse anti Gli1 and β-actin primary antibodies were got from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horse radish peroxidase (HRP) conjugated secondary antibody was derived from Bio-Rad (Hercules, CA, USA). Transwell chamber was bought from Greiner Bio-One (Frickenhausen, Germany). Matrigel was derived from BD Biosciences (Franklin Lakes, NJ, USA). Dual luciferase activity detection kit and pGL3 luciferase gene reporter plasmid were purchased from Promega (Madison, WI, USA). TGF-β1 was obtained from Sino Biological (Beijing, China).

**Cell Culture and EMT Induction**

SW620, SW480, and NCM460 cells were cultured in vitro and divided into five groups, including miR-NC, miR-150 mimic, si-NC, si-Gli1, and miR-150 mimic + si-Gli1 groups. Nucleotide fragments and riboFECT™ CP Reagent were added to 1×riboFECT™ CP Buffer and incubated at room temperature for 0-15 min, respectively. Then, they were added to the cells, which were used for detection after 72 h.

**qRT-PCR**

Total RNA was extracted using EasyPure RNA Kit and adopted for PCR reaction by TransScript Green One-Step qRT-PCR SuperMix. The reaction system contained 1 μg RNA template, 0.3 μM primers, 10 μL 2×TransStart Tip Green qPCR SuperMix, 0.4 μL RT Enzyme Mix, 0.4 μL Dye II, and ddH2O. The PCR reaction was composed of 45°C reverse transcription for 5 min, 94°C pre-denaturation for 30 s, followed by 40 cycles of 94°C for 5 s and 60°C for 30 s. Real-time PCR was performed on Bio-Rad (Hercules, CA, USA) CFX96 connect to test the relative expression.

**Western Blot**

Total protein was extracted by SDS buffer from cells. A total of 40 μg protein was separated by 8-10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membrane. Next, the membrane was blocked and incubated in primary antibody at 4°C overnight (E-cadherin, Gli1, Snail, and β-actin at 1:3000, 1:2000, 1:2000, and 1:10000, respectively). Then the membrane was incubated in secondary antibody (1:25000) for 60 min after

For EMT induction, SW620 and SW480 cells were seeded in six-well plate at 3×10³/well for 24 h. Then, the cells were treated by 10 ng/ml TGF-β1 for 48 h to induce EMT.

**Dual-Luciferase Reporter Gene Assay**

The PCR products containing the full-length of Gli1 gene 3'-UTR segment from SW480 cells were cloned to pGL3. Next, it was transformed to DH5α competent cells and sequenced to select the plasmid with correct sequence. Then, pGL3-Gli1-3'-UTR-wt (or pGL3-Gli1-3'-UTR-mut) was co-transfected to HEK293T cells using riboFECT™ CP together with miR-150 mimic (or miR-NC). The luciferase activity was detected according to the Dual-Glo Luciferase Assay manual after cultured for 48 h.
washed by PBST for three times. At last, the protein expression was detected by electrochemiluminescence (ECL).

**Colony Formation Assay**

The cells were seeded in 10 cm dish at 100/well and cultured at 37°C and 5% CO₂ for 14-21 days. The dish was washed by phosphate buffered saline (PBS) for twice and fixed by 4% paraformaldehyde when the macroscopic clone appeared. Next, the dish was stained by Giemsa for 20 min to count the clone with more than 10 cells. At last, the colony formation rate was calculated by (clone number/seeded cell number) ×100%.

**Transwell Assay**

A total of 500 μl RPMI-1640 medium containing 10% FBS were added to the 24-well plate. Then, the Transwell chamber paved 100 μl Matrigel was put onto the plate and added with SW620 cells resuspended in 200 μl serum-free RPMI-1640 medium (1×10⁶/mL). After 48 h, the membrane was fixed in methanol and stained by crystal violet. At last, the membrane was observed under the microscope.

**Statistical Analysis**

All data analyses were performed with SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The measurement data were depicted as mean ± standard deviation and compared by t-test. p < 0.05 was considered as statistical significance.

**Results**

**MiR-150 Inhibited Gli1 Expression**

Online target gene prediction by microRNA.org showed the targeted binding site between miR-150 and 3’-UTR of Gli1 mRNA (Figure 1A). Dual luciferase assay revealed that miR-150 mimics or inhibitor transfection significantly declined or elevated the relative luciferase activity of HEK293 cells (Figure 1B), indicating the regulatory relationship between miR-150 and Gli1 mRNA.

**MiR-150 was Downregulated, while Gli1 Was Overexpressed in CRC Cells**

The detection of qRT-PCR showed that Gli1 mRNA expression was highest in SW620 cells, followed by that in SW480 and NCM460 cells (Figure 2A). Also, miR-150 level was lowest in SW620 cells, compared with that in SW480 and NCM460 cells (Figure 2B).

**Gli1 Was Increased, While miR-150 Was Reduced During the EMT Process**

Colony formation assay demonstrated that clone formation capability in SW620 cells was markedly stronger than that in SW480 cells (Figure 3A, B). qRT-PCR showed that the level of miR-150 was declined, and Gli1 mRNA was markedly enhanced in SW620 and SW480 cells treated by TGF-β1, among which stronger metastatic ability was found in SW620 cells (Figure 3C, D).

**The Overexpression of miR-150 Downregulated Gli1 Level, Suppressed EMT Process, and Attenuated Cell Invasion in CRC Cells**

After the transfection of miR-150 mimic and/or si-Gli1, Gli1, and Snail levels were markedly reduced, E-cadherin expression was upregulated (Figure 4A), and cell colony formation (Figure 4B) and invasion were attenuated (Figure 4C).

**Discussion**

Hedgehog (Hh) gene is originally found in drosophila genetic mutation. Hedgehog signaling pathway is activated by Hedgehog ligand²⁰. Hedgehog signaling pathway is widely expressed in a variety of tissues and cells, and
participates in regulating pathological physiological processes, such as organ development and damage repair\textsuperscript{21-24}. Hedgehog signaling pathways are composed of secretory signal protein Hedgehog, transmembrane protein receptor Patched (Ptch) and Smoothened (Smo), middle molecule, transcription factor protein Gli, and downstream target genes, etc. When Hedgehog signaling pathways is inactive, Ptch combines with Smo to suppress its activity, leading to Gli phosphorylation and ubiquitin, thus impeding its entry into the nucleus in full-length to regulate the transcription and expression of downstream target genes. Once the Hedgehog signaling pathway is activated, Hh ligands bind with transmembrane protein receptor Ptch to abrogate the inhibitory effect on Smo, thus invaliding the degradation of Gli. Gli1 enters the nucleus in the form of a full-length to promote target gene transcription and expression, so as to facilitate cell proliferation, migration, and malignant transformation\textsuperscript{25}. Several studies revealed that the aberrant activation of Hedgehog signaling pathway was closely associated with tumorigenesis, progression, metastasis, and recurrence, such as breast cancer\textsuperscript{9}, pancreatic cancer\textsuperscript{10}, cervical cancer\textsuperscript{7}, and gallbladder carcinoma\textsuperscript{8}. As a member of zinc finger protein family, Gli1 gene locates in human chromosome 12 q13.2-13.3\textsuperscript{26}. Gli1 is a member of human Gli protein family (Gli1, Gli2, and Gli3). The abnormal upregulation of Gli1 is an important symbol of Hedgehog signaling pathway excessive activation\textsuperscript{27}. It was found that Gli1 elevation is related to multiple tumorigenesis, progression, and metastasis, including breast cancer\textsuperscript{11}, esophageal cancer\textsuperscript{12}, and lung cancer\textsuperscript{11}. It was showed that Gli1 expression abnormally increased in CRC tissue and affected cancer metastasis and progression\textsuperscript{14,15}. MiR-150 poses as a downstream effector of myocardial infarction associated transcript (MIAT) in the development of cardiac hypertrophy\textsuperscript{28}. Recent research also revealed that miR-150 downregulated in CRC tissue and was associated with cancer progression, treatment efficacy, and prognosis\textsuperscript{18,19}.

After the regulatory relationship between miR-150 and Gli1 mRNA was demonstrated by dual luciferase assay, we further compared miR-150, Gli1 levels among human normal colon epithelial cells and CRC cells. Our data suggested that miR-150 reduction may play a role in elevating Gli1 and promoting tumorigenesis. Ma et al\textsuperscript{18} found that miR-150 was significantly declined in CRC tumor tissue compared with that in adjacent normal control by fluorescence in situ hybridization and qRT-PCR. Moreover, the survival rate and chemotherapy effect of patients with lower miR-150 expression were worse than that with higher miR-150 level. Sarlinova et al\textsuperscript{19} reported that miR-150 expression was reduced in the peripheral blood from CRC patients compared with healthy control. Aheme et al\textsuperscript{29} demonstrated that miR-150 was markedly declined in peripheral blood from CRC patients and was correlated with disease progression. In this study, miR-150 level was decreased in CRC cells, revealing that miR-150 may be a tumor suppressor gene of CRC, which was similar with Ma et al\textsuperscript{18}, Sarlinova et al\textsuperscript{19}, and Aheme et al\textsuperscript{29}. Hong et al\textsuperscript{30} exhibited that Gli1 expression was upregulated in CRC tissue compared with that in normal control. Zhang et al\textsuperscript{30} discovered...
Figure 3. Gli1 increased, while miR-150 reduced in the EMT process of CRC cells. A, Colony formation assay detection of clone formation. B, Representative images of colony-forming assay by Giemsa staining (20×). C, qRT-PCR detection of Gli1 mRNA expression. D, qRT-PCR detection of miR-150 expression. *p < 0.05, compared with SW480 cells; #p < 0.05, compared with TGF-β1 (0 ng/ml).

Figure 4. MiR-150 overexpression downregulated Gli1 level, suppressed EMT process, and attenuated cell invasion in CRC cells. A, Western blot detection of protein expression. B, Colony formation assay detection of clone formation. C, Transwell assay detection of cell invasion. *p < 0.05, compared with miR-NC; a*p < 0.05, compared with si-NC; b*p < 0.05, compared with miR-150 mimic; c*p < 0.05, compared with si-Gli1 group.
that Gli1 level was increased in CRC tissue compared with that in adjacent normal control, while its level was significantly higher in CRC cells than that in normal colon epithelial cell NCM460. This research found that Gli1 expression was higher in CRC cells than that in normal colon epithelial cells, indicating that Gli1 was a stimulus of colon cancer tumorigenesis, which was in accordance with Hong et al\textsuperscript{14} and Zhang et al\textsuperscript{15}. Furthermore, Gli1 was upregulated, while miR-150 was reduced in SW620 cells compared with that in SW480 cells. This became more significant in EMT process induced by TGF-β1, suggesting that miR-150 mediated the upregulation of Gli1 and represented a motivator of CRC EMT and invasion. It was proposed that the increase of Snail expression was closely associated with Gli1 in promoting EMT and cancer cell invasion\textsuperscript{16}. This study further observed that miR-150 mimic and/or si-Gli1 transfection markedly reduced Gli1 and Snail levels, upregulated E-cadherin expression, and attenuated cell colony formation and invasion, indicating that Gli1 may facilitate SW620 cell EMT and invasion via upregulating Snail. Feng et al\textsuperscript{13} reported that miR-150 played a tumor suppressor role in CRC. MiR-150 elevation suppressed CRC cell proliferation, blocked cell cycle, restrained migration and invasion, and attenuated cell growth \textit{in vivo} by targeting c-Myb expression. Inhibition of miR-150 obtained the opposite phenomenon. Wang et al\textsuperscript{32} found that miR-150 weakened the migration and invasion of CRC cell and HCT-116 through inhibiting MUC4 expression. This study showed that the upregulation of miR-150 alleviated CRC cell EMT and invasion, which was in accordance with Feng et al\textsuperscript{13} and Wang et al\textsuperscript{32}. Cheng et al\textsuperscript{33} demonstrated that the downregulation of Gli1 markedly suppressed the proliferation and invasion of CRC cell line Caco-2 cell, induced cell apoptosis, and attenuated drug resistance. Zhang et al\textsuperscript{15} observed that the overexpression of Gli1 significantly facilitated CRC cell EMT, and enhanced cell migration, invasion, and metastasis \textit{in vivo}. Inhibition of Gli1 alleviated CRC cell metastasis \textit{in vivo}, which was similar to our results. This study revealed that the reduction of miR-150 and upregulation of Gli1 promoted the EMT and invasion of CRC cell. However, our investigation failed to test miR-150 and Gli1 dynamic expressions in CRC tumor tissues in different stages and metastatic status, which requires further validation to accurately reflect the condition in human body.

**Conclusions**

Downregulation of miR-150 and elevation of Gli1 facilitate the EMT and invasion of CRC cells. MiR-150 inhibited the EMT and invasion of CRC cells via inhibiting Gli1.

**Acknowledgements**

This work was supported by Fujian natural fund project (No. 2015J01391).

**Conflict of Interest**

The Authors declare that they have no conflict of interests.

**References**

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