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Circular RNA ITCH suppresses proliferation and promotes apoptosis in human epithelial ovarian cancer cells by sponging miR-10a-α

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Abstract. – OBJECTIVE: To explore the effect of Circular RNA Itchy E3 ubiquitin protein ligase (Circ-ITCH) on regulating epithelial ovarian cancer (EOC) cells proliferation and apoptosis, as well as its potential target microRNAs (miR) *in vitro*.

MATERIALS AND METHODS: Circ-ITCH mimic, blank mimic, Circ-ITCH inhibitor and blank inhibitor plasmids were transfected into SKOV3 cells. Rescue experiments were carried out by transfecting blank mimic, miR-10a mimic, Circ-ITCH mimic and miR-10a mimic/Circ-ITCH mimic plasmids into SKOV3 cells. Quantitative polymerase chain reaction (qPCR) was performed to detect RNA expression. Cells proliferation was determined by Cell Counting Kit-8 (CCK-8) assay, and cells apoptosis rate was detected using Annexin V (AV) / propidium iodide (PI) assay.

RESULTS: Circ-ITCH expression was decreased in Human EOC cell lines SKOV3, A-2780, OVCAR-3 and HO-8910 compared with human normal ovarian epithelial cell line IOSE80, and the most significant reduction of Circ-ITCH expression was presented in SKOV3 cells. Cells proliferation was suppressed by Circ-ITCH mimic transfection and promoted by Circ-ITCH inhibitor transfection in SKOV3 cells. Cells apoptosis was enhanced by Circ-ITCH mimic transfection and inhibited by Circ-ITCH inhibitor transfection in SKOV3 cells. In addition, Circ-ITCH adversely regulated miR-10a expression in SKOV3 cells but not miR-4251 or miR-6505. Rescue experiments were subsequently performed, which exhibited that Circ-ITCH adversely regulated miR-10a expression, whereas miR-10a did not affect Circ-ITCH expression. And most importantly, miR-10a mimic attenuated the effect of Circ-ITCH on reducing proliferation and promoting apoptosis in SKOV3 cells.

CONCLUSIONS: Circ-ITCH suppresses cells proliferation and promotes cells apoptosis via sponging miR-10a in EOC cells.

Key Words:
Apoptosis, Circ-ITCH, Epithelial ovarian cancer, miR-10a, Proliferation.

Introduction

Epithelial ovarian cancer (EOC), a pathologic subtype accounting for over 90% cases of ovarian cancer, is one of the most commonly diagnosed and death-leading malignancies in women worldwide[1]. The incidence of ovarian cancer exceeds 8 out of 100,000 populations in developed areas, and it also presents an increasing trend in China partially accounting for the change of lifestyle and environment in recent decades[2]. Although modern therapeutic approaches such as tumorectomy, radiotherapies and chemotherapies have improved survival of EOC patients, the mortality rate still ranges from 40% to 50%[3]. Therefore, it is essential to explore more potential molecular mechanisms of EOC pathogenesis and investigate corresponding therapeutic targets to improve the treatment outcomes. Circular RNA (circRNA), defined as RNA whose 3' and 5' ends are covalently joined together forming a closed and continuous loop, is resistant to RNA degradation and have limited protein coding ability[4]. Circular RNA Itchy E3 ubiquitin protein ligase (Circ-ITCH), located on human chromosome 20, is a novelty identi-

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fied circRNA that is reported to be involved in development and progression of tumors[5]. *In vitro* studies reveal that Circ-ITCH suppresses cancer cells proliferation and invasion, meanwhile promotes cells apoptosis through regulating multiple cancer-related microRNAs (miRNAs) in various carcinomas such as breast cancer, bladder cancer and colorectal cancer, while its underlying mechanism in EOC is still unclear[5-8]. Based on the data retrieved from Tissue-Specific CircRNA Database on gb.whu.edu.cn/TSCD/, Circ-ITCH expresses in ovary with entry No. chr20: 34424479|34457474. Considering the published studies about the effect of Circ-ITCH on cancer cells activities, we hypothesized that Circ-ITCH might play an important role in EOC pathogenesis. Therefore, the aim of our study was to explore the effect of Circ-ITCH on regulating EOC cells proliferation and apoptosis, as well as its potential target miRNAs *in vitro*.

Materials and Methods

Cells Culture

Human EOC cell lines including SKOV3, A-2780, OVCAR-3 and HO-8910 and human normal ovarian epithelial cell line IOSE80 were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SKOV3 cells were cultured in 90% McCOY's 5A medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Gran Island, NY, USA); A-2780 and HO-8910 cells were cultured in 90% RPMI-1640 (Roswell Park Memorial Institute-1640) medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Gran Island, NY, USA); OVCAR-3 cells were cultured in 80% RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% FBS (Gibco, Gran Island, NY, USA) and 0.01 mg/ml bovine insulin; IOSE80 cells were cultured in 89% RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% FBS (Gibco, Gran Island, NY, USA) and 1% penicillin and streptomycin. All the cells were incubated in a humidified incubator under 95% air and 5% CO_2 at 37°C.

Circ-ITCH Expression in EOC Cell Lines And Normal Ovarian Epithelial Cell Line

Total RNA was extracted from each cell line and quantitative polymerase chain reaction (qPCR) assay was performed to determine the expression of Circ-ITCH in SKOV3, A-2780, OVCAR-3, HO-8910 and IOSE80 cell lines. The process of qPCR assay was listed in "qPCR assay" subsection.

Transfection of Circ-ITCH Mimic and Inhibitor Plasmids Into SKOV3 Cells

Blank mimic, Circ-ITCH mimic, blank inhibitor and Circ-ITCH inhibitor plasmids were constructed by Shanghai Qeejen Bio-tech Institution (Shanghai China) and then transfected into SKOV3 cells as negative control NC (+), Circ-ITCH (+), NC (-), Circ-ITCH (-) groups, respectively. And the following assays were performed: (1) Circ-ITCH expression at 24 h by qPCR assay. (2) Cells proliferation ability by Cells Counting Kit-8 (CCK-8) assay at 0 h, 24 h, 48 h and 72 h. (3) Cells apoptosis rate by Annexin V (AV) / propidium iodide (PI) assay at 72 h. The process of each assay was listed in subsequent subsections "qPCR assay", "CCK8 assay" and "AV/PI assay".

Evaluation of Predicting Target miRNAs of Circ-ITCH in SKOV3 Cells

Target miRNAs of Circ-ITCH was predicted using Potential Binding MicroRNAs analysis in Tissue-Specific CircRNA Database on gb.whu.edu.cn/TSCD/, which disclosed that 30 potential target miRNAs existed including 3 miRNAs with 8 matched bases, and 27 miRNAs with 7 matched bases. Subsequently, expressions of these 3 miRNAs with 8 matched bases which consist of miRNA (miR)-10a, miR-4251 and miR-6505 were detected at 24 h after Circ-ITCH mimic and inhibitor plasmids transfection in SKOV3 cells by qPCR assay. The process of qPCR assay was listed in "qPCR assay" subsection.

Rescue Experiment

Blank mimic, miR-10a mimic, Circ-ITCH mimic, Circ-ITCH/miR-10a mimic plasmids were constructed by Shanghai Qeejen Bio-tech Institution (Shanghai China) and then transfected into SKOV3 cells as NC (+), miR-10a (+), Circ-ITCH (+) and Circ-ITCH (+)/miR-10a (+) groups, respectively. And the following assays were performed: (1) Circ-ITCH and miR-10a expressions by qPCR assay at 24 h. (2) Cells proliferation ability by CK-8 assay at 0 h, 24 h, 48 h and 72 h. (3) Cells apoptosis rate by AV/PI assay at 72 h. The process of each assay was listed in subsequent subsections "qPCR assay", "CCK8 assay" and "AV/PI assay".

qPCR Assay

TRIzol reagent (Invitrogen, Carlsbad USA) was used to extract the total RNA extracted from cells; then, 1 μg total RNA from each sample was applied for the synthesis of cDNA with PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). Subsequently, cDNA product was subjected to qPCR with SYBR Green kit (TaKaRa, Otsu, Shiga, Japan). The amplification of PCR was conducted in the following conditions: 95°C for 3 min, 40 cycles of 95°C for 5 s, 61°C for 10 s, and then 72°C for 30 s. qPCR results were calculated by the method of $2^{-\Delta\Delta Ct}$ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or U6 was served as the internal reference. The sequence of Circ-ITCH in ovarian was retrieved from Tissue-Specific CircRNA Database (TSCD) on gb.whu.edu.cn/TSCD/ with entry No. chr20:34424479|34457474. The primers of Circ-ITCH and GAPDH were as follows: Circ-ITCH forward primer 5'-CCTTGAGCAAGAAGACTATGCCAAT-3', Circ-ITCH reverse primer 5'-CCGCATTCTGTGGTAAGCAATCA-3', GAPDH forward primer 5'-TGACCACAGTCCATGCCATCAC-3', GAPDH reverse primer 5'- GCCTGCTTCACCACCTTCTTGA-3'. The primers of miR-10a and U6 were as follows: miR-10a forward primer 5'-ACACTCCAGCTGGGTACCCTGTAGATCCGAAT-3',

miR-10a reverse primer 5'-TGTCGTGGAGTCGGCAATTC-3', U6 forward primer 5'-CTCGCTTCGGCAGCACATATACTA-3';

U6 reverse primer 5'-ACGAATTTGCGTGTCATCCTTGC-3'. The primers for miR-4251 and miR-6505 were as follows: miR-4251 forward primer 5'-ACACTCCAGCTGGGCCTGAGAAAAGGGCCAA-3', miR-4251 reverse primer 5'-TGTCGTGGAGTCGGCAATTC-3', miR-6505 forward primer 5'-ACACTCCAGCTGGGTTGGAATAGGGGATATCT-3', miR-6505 reverse primer 5'-TGTCGTGGAGTCGGCAATTC-3'.

CCK8 Assay

10 ul CCK-8 (Abcam, Cambridge, MA, USA) and 90 ul medium were added to each group of SKOV3 cells, then the cells were incubated under 95% air and 5% CO_2 at 37°C. Optical density (OD) value was calculated by microplate reader (BioTek, Winooski, VT, USA) to represent the cells proliferation ability.

AV/PI Assay

SKOV3 cells in each group were digested by pancreatin and washed with phosphate-buffered solution (PBS) and then suspended in 100 ul blinding buffer. 2 ul AV (Invitrogen, Carlsbad, CA, USA) were added to each group, then they were placed on the ice in the darkness for 15 min. Subsequently, 1 ul PI (Invitrogen, Carlsbad, CA, USA) was added and the apoptosis rate was analyzed by using flow cytometry (FCM) (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical Analysis

Statistical analysis was performed by SPSS 21.0 software (IBM, Armonk, NY, USA) and Graphpad Prism 5.01 software (GraphPad Software Inc., La Jolla, CA, USA). Data were presented as mean ± standard error. Comparison between two sets of data was carried out using *t*-test. $p<0.05$ was considered significant.

Results

Circ-ITCH Expression in EOC Cell Lines and Normal Ovarian Epithelial Cell Line

Circ-ITCH expression was declined in human EOC cell lines SKOV3 ($p<0.001$), A-2780 ($p<0.01$), OVCAR-3 ($p<0.05$) and HO-8910 ($p<0.05$) compared with human normal ovarian epithelial cell line IOSE80 (Figure 1). Among these four candidate EOC cell lines, the most significant reduction in Circ-ITCH expression was observed in SKOV3 cells compared with IOSE80 cells. Thus, SKOV3 cells were chosen for transfection in the following experiments.

Effect of Circ-ITCH Transfection on Proliferation and Apoptosis in SKOV3 Cells

CircRNA-ITCH expression was upregulated in Circ-ITCH (+) group compared with NC (+) group ($p<0.001$) and downregulated in Circ-ITCH (-) group compared with NC (-) group ($p<0.001$) at 24 h after transfection (Figure 2A). Cells proliferation was reduced in Circ-ITCH (+) group compared to NC (+) groups at 48 h ($p<0.05$) and 72 h ($p<0.01$), while it was increased in Circ-ITCH (-) group compared with NC (-) group at 72 h ($p<0.05$) after transfection (Figure 2B). Cells apoptosis rate was elevated in Circ-ITCH (+) group compared to NC (+) group ($p<0.001$) while lowered in Circ-ITCH (-) group compared with NC (-) group ($p<0.05$) at 72 h after transfection (Figure 2C, 2D). These results implied that Circ-ITCH suppressed proliferation and promoted apoptosis in SKOV3 cells.

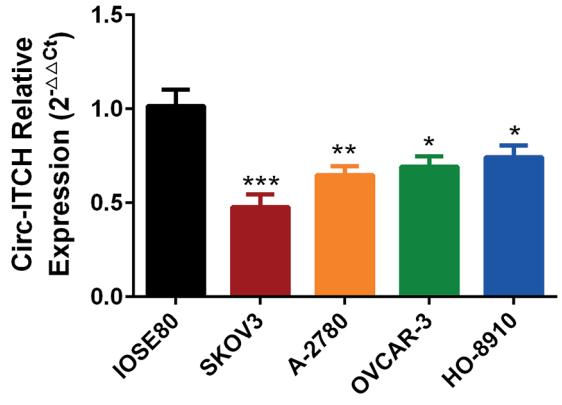

Figure 1. Circ-ITCH expression in EOC cell lines and normal ovarian epithelial cells. Circ-ITCH expression was lowered in SKOV3, A-2780, OVCAR-3 and HO-8910 compared to IOSE80, and the most significant reduction was in SKOV3 cells. *t*-test was used to compare the Circ-ITCH expression level between two cell lines. *p<0.05, **p<0.01, ***p<0.001. EOC, epithelial ovarian cancer; Circ-ITCH, Circular RNA Itchy E3 ubiquitin protein ligase.

Effect of Circ-ITCH on Regulating miR-10a, miR-4251 and miR-6505 in SKOV3 Cells

Using potential binding MicroRNAs analysis in tissue-specific CircRNA Database on gb.whu.edu.cn/TSCD/, 3 miRNAs with 8 matched bases to Circ-ITCH in human ovary were selected as candidate target miRNAs. MiR-10a expression was downregulated in Circ-ITCH (+) group compared with NC (+) group (p<0.01) and upregulated in Circ-ITCH (-) group compared with NC (-) group (p<0.05) (Figure 3A). Decreased miR-4251 expression was observed in Circ-ITCH (+) group compared with NC (+) group (p<0.05), while there was no difference between Circ-ITCH (-) group and NC (-) group (Figure 3B). As to miR-6505 expression, no change was observed in Circ-ITCH (+) group or Circ-ITCH (-) group compared to their corresponding NC groups (Figure 3C). This implied that Circ-ITCH negatively regulated miR-10a but not miR-4251 or miR-6505 expression in SKOV3 cells.

Circ-ITCH and miR-10a Expressions After Transfection in Rescue Experiments

For the purpose of further investigating the interaction between Circ-ITCH and miR-10a in SKOV3 cells, rescue experiment was performed. No difference in Circ-ITCH expression was found between miR-10a (+) group and NC (+) group, nor between miR-10a (+)/Circ-ITCH (+) group and Circ-ITCH (+) group (Both p>0.05) (Figure 4A). In addition, miR-10a expression was lower in Circ-ITCH (+) group compared with NC (+) group (p<0.01), while its expression was elevated in miR-10a (+)/Circ-ITCH (+) group than that in Circ-ITCH (+) group (p<0.01) (Figure 4B). Hence, these data suggested that Circ-ITCH adversely regulated miR-10a expression, whereas miR-10a did not affect Circ-ITCH expression in SKOV3 cells.

Cells Proliferation and Apoptosis After Transfection In Rescue Experiments

At 72 h after transfection, cells proliferation was increased in miR-10a (+) group compared with NC (+) group (p<0.05), as well as in miR-10a (+)/ Circ-ITCH (+) group than that in Circ-ITCH (+) group (p<0.05) (Figure 5A). Cells apoptosis rate was reduced in miR-10a (+) group compared with NC (+) group (p<0.05), as well as in miR-10a (+)/ Circ-ITCH (+) group compared with Circ-ITCH (+) group (p<0.01) at 72 h after transfection (Figure 5B, 5C). Thus, these results suggested that Circ-ITCH reduced cells proliferation and promoted cells apoptosis by targeting miR-10a in SKOV3 cells.

Discussion

In the present study, we observed that: (1) Circ-ITCH was downregulated in EOC cells lines compared to human normal ovarian epithelial cell line, and it inhibited cells proliferation and promoted cells apoptosis in SKOV3 cells. (2) Rescue experiment revealed that Circ-ITCH suppressed proliferation and enhanced apoptosis of SKOV3 cells via targeting miR-10a. CircRNAs, a diversified class of greatly abundant and evolutionally conserved RNAs in human genome, have been demonstrated to be involved in the development of various malignancies by functioning as miRNAs sponges or regulating genes transcription via binding to RNA-associated proteins and forming RNA-protein complexes[9,10]. A recent experiment reveals that Circ-FOXO3 represses cells proliferation, migration and invasion in non-small cell lung cancer by targeting miR-155 and increasing FOXO3 expression[11]. Liu et al[12] reveal that Circ-ZFR restrains cells proliferation and promote cells apoptosis in gastric cancer by sponging miR-103a/miR-107 and regulating PTEN. In addition, Circ-C3P1 suppresses cells growth and metastasis in hepatocellular carcinoma through miR-4641/PCK1 pathway[13]. Thus, these previous

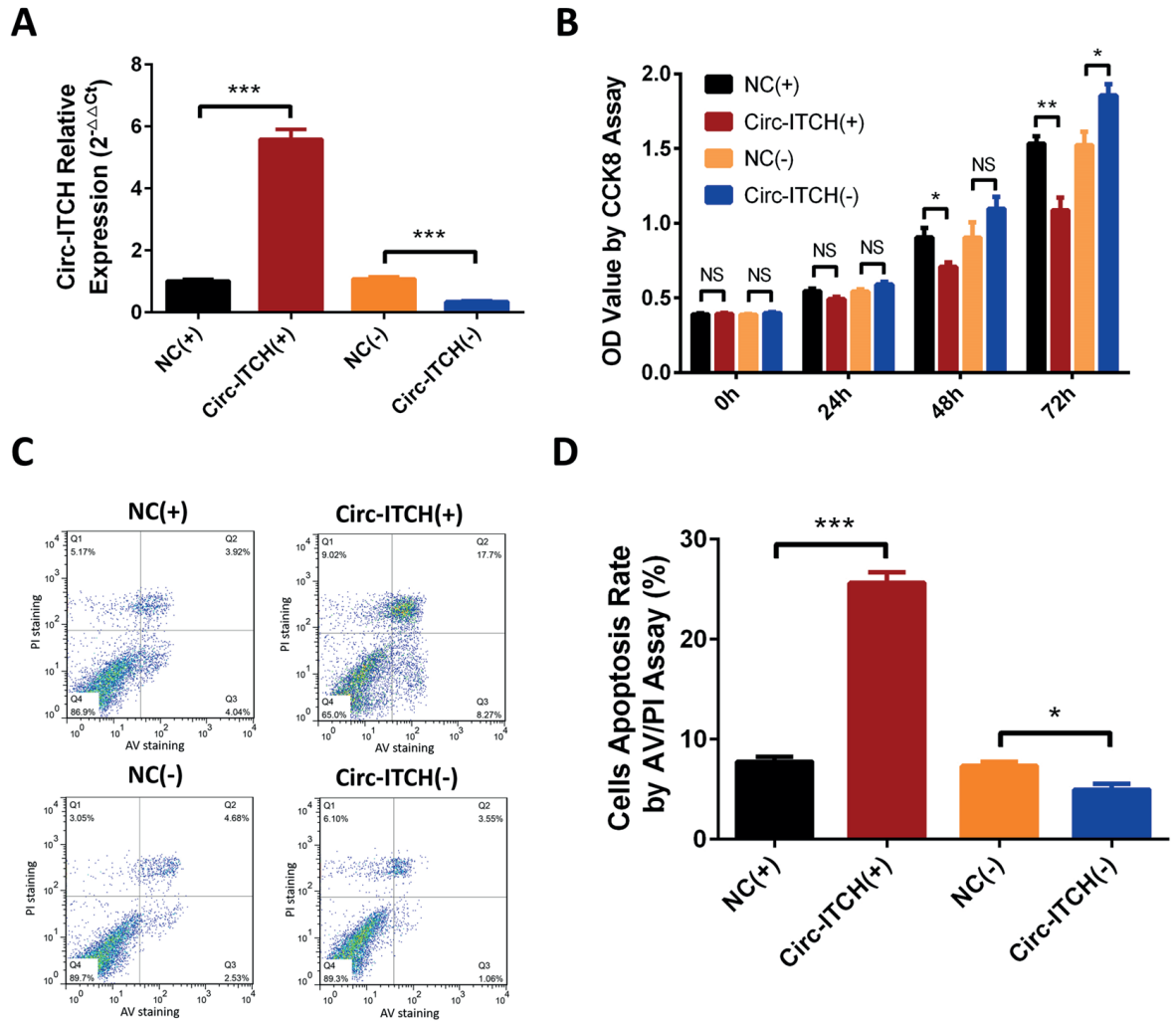

Figure 2. Effect of Circ-ITCH on proliferation and apoptosis in SKOV3 cells. (A) Circ-ITCH expression was upregulated by Circ-ITCH mimic transfection and downregulated by Circ-ITCH inhibitor transfection. (B) Cells proliferation was decreased in Circ-ITCH (+) group compared to NC (+) group at 48 h and 72 h after transfection, while elevated in Circ-ITCH (-) group compared to NC (-) group at 72 h after transfection. (C, D) Cells apoptosis rate was higher in Circ-ITCH (+) group compared to NC (+) group and lower in Circ-ITCH (-) group compared to NC (-) group at 72 h after transfection. *t*-test was used to determine the differences between two groups. *p<0.05, **p<0.01, ***p<0.001, NS non-significant. Circ-ITCH, Circular RNA Itchy E3 ubiquitin protein ligase; NC, negative control.

works show that circRNAs may play critical roles in pathogenesis of various carcinomas via multiple pathways. Circ-ITCH is a member of circRNA family that has been discovered to serve as a tumorigenesis suppressor in quite a lot of distinct types of carcinomas. For instance, enforced expression of Circ-ITCH inhibits cells proliferation and induces cells apoptosis in breast cancer cells by sponging miR-17 and miR-224[7]. Wan et al[14] disclose that Circ-ITCH suppresses lung cancer cells proliferation via functioning as a sponge of miR-7 and miR-214. Also, in esophageal cancer, Circ-ITCH targets *c-Myc* gene to limit cancer cells growth through altering miR-7, miR-17 and miR-214 and the down-stream Wnt/β-Catenin pathway[8]. Despite that these previous works reveal that Circ-ITCH reduces cells proliferation and induces cells apoptosis in numerous carcinomas via sponging miRNAs, the role of Circ-ITCH in EOC remains unclear. In the present study, we investigated the mechanism of Circ-ITCH in pathogenesis of EOC and discovered that Circ-ITCH suppressed proliferation and promoted apoptosis of SKOV3 cells, suggesting that Circ-ITCH participated in EOC pathology by impacting cells proliferation and apoptosis, providing a potential

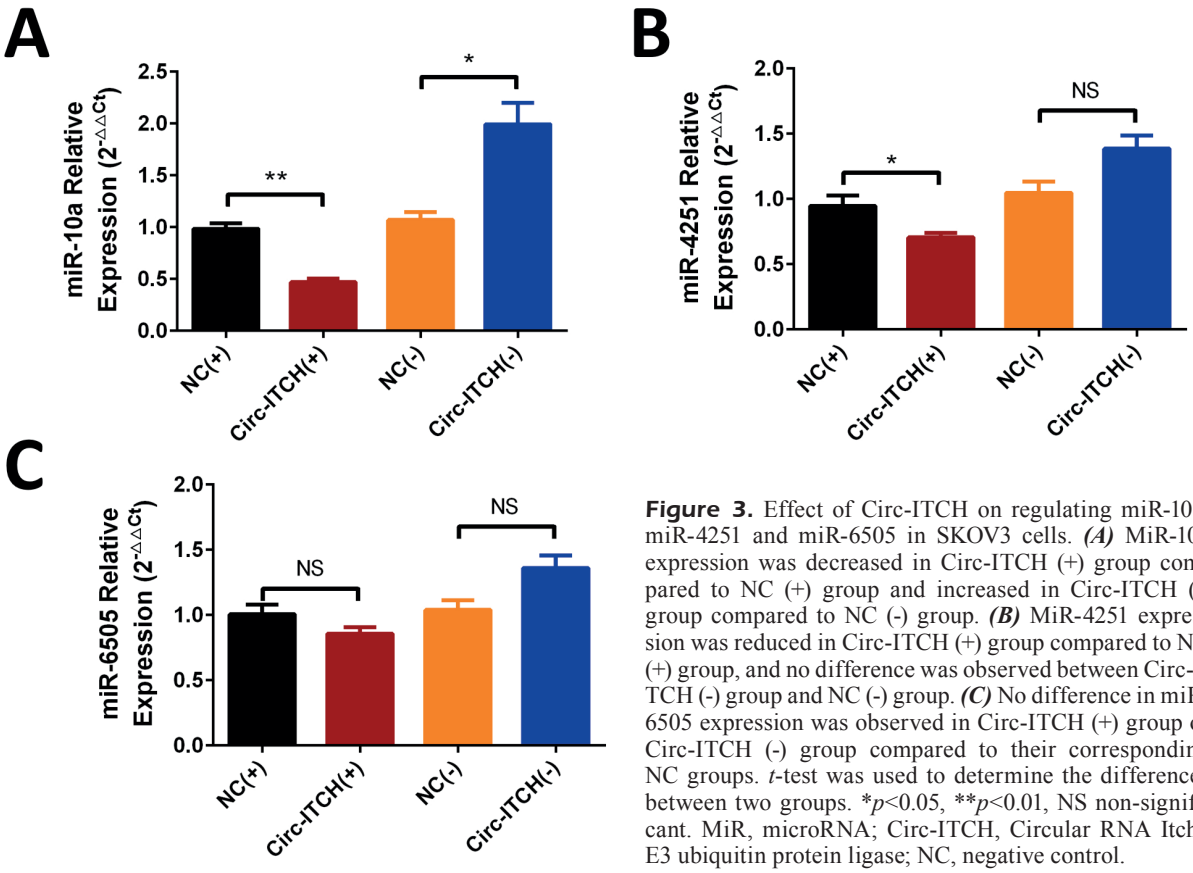

Figure 3. Effect of Circ-ITCH on regulating miR-10a, miR-4251 and miR-6505 in SKOV3 cells. ***(A)*** MiR-10a expression was decreased in Circ-ITCH (+) group compared to NC (+) group and increased in Circ-ITCH (-) group compared to NC (-) group. ***(B)*** MiR-4251 expression was reduced in Circ-ITCH (+) group compared to NC (+) group, and no difference was observed between Circ-ITCH (-) group and NC (-) group. ***(C)*** No difference in miR-6505 expression was observed in Circ-ITCH (+) group or Circ-ITCH (-) group compared to their corresponding NC groups. *t*-test was used to determine the differences between two groups. **p*<0.05, ***p*<0.01, NS non-significant. MiR, microRNA; Circ-ITCH, Circular RNA Itchy E3 ubiquitin protein ligase; NC, negative control.

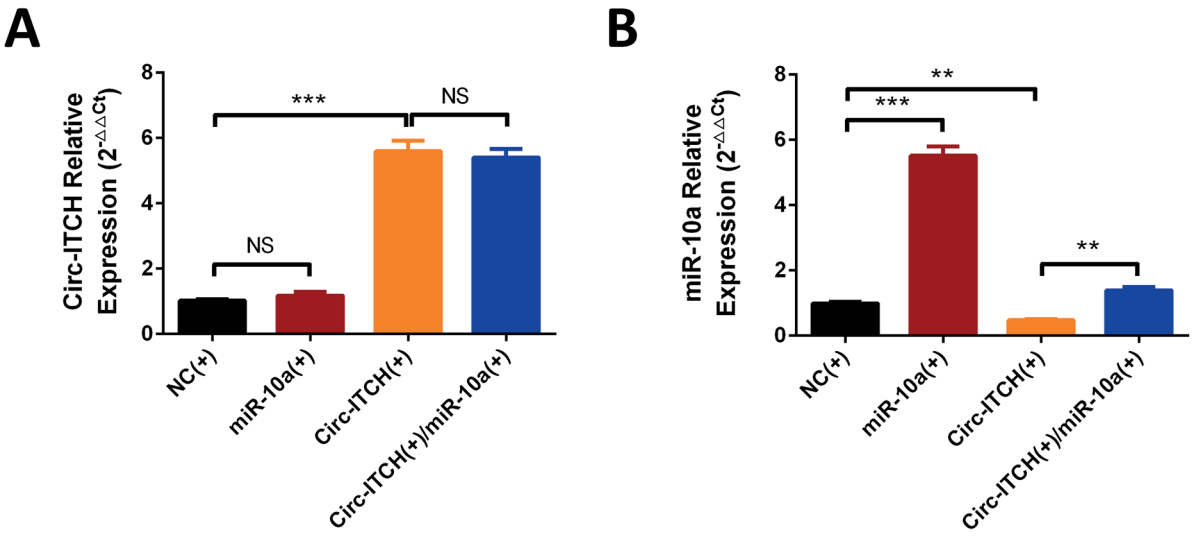

Figure 4. CircRNA-ITCH and miR-10a expressions after transfection in rescue experiments. ***(A)*** There was no difference in Circ-ITCH expression between miR-10a (+) group and NC (+) group, as well as between miR-10a (+)/Circ-ITCH (+) group and Circ-ITCH (+) group. ***(B)*** MiR-10a expression was lower in Circ-ITCH (+) group compared to NC (+) group but higher in miR-10a (+)/Circ-ITCH (+) group compared to Circ-ITCH (+) group. Differences between two groups were determined by *t*-test. ***p*<0.01, ****p*<0.001, NS non-significant. MiR, microRNA; Circ-ITCH, Circular RNA Itchy E3 ubiquitin protein ligase; NC, negative control.

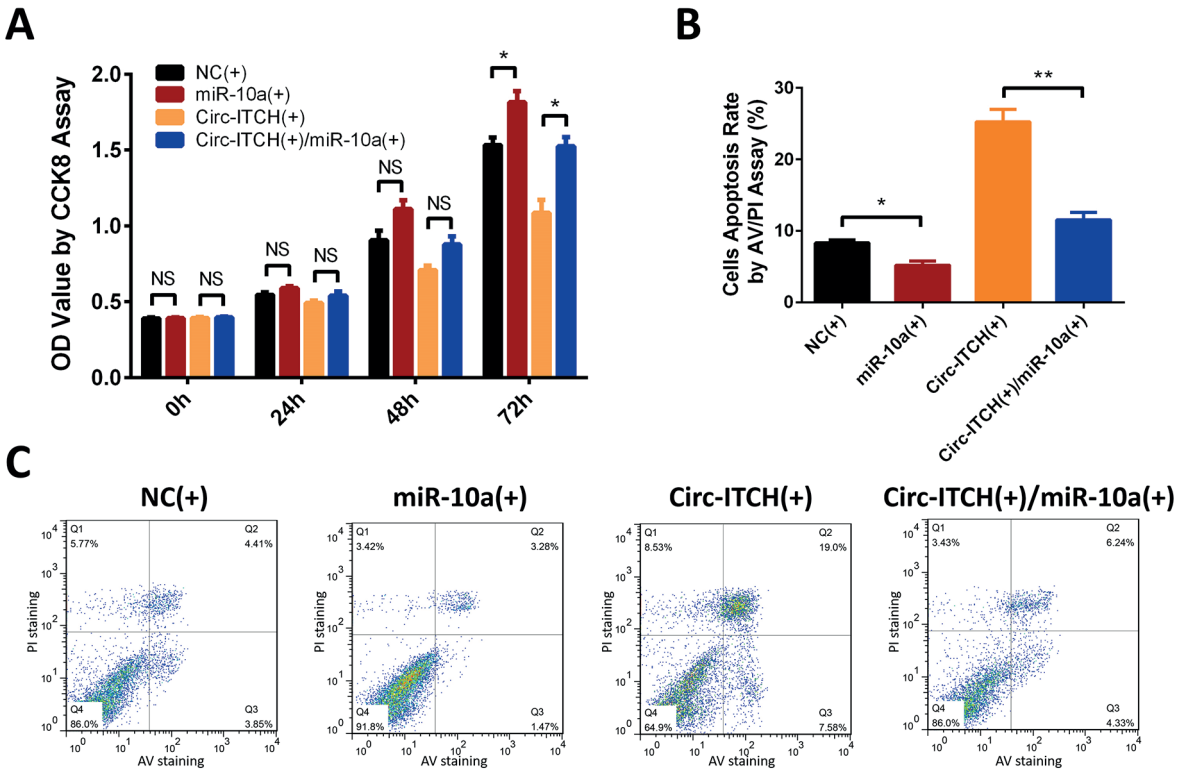

Figure 5. Cells proliferation and apoptosis after transfection in rescue experiments. ***(A)*** At 72 h after transfection, cells proliferation was increased in miR-10a (+) group compared to NC (+) group and in miR-10a (+)/ Circ-ITCH (+) group compared to Circ-ITCH (+) group. ***(B, C)*** At 72 h after transfection, cells apoptosis rate was reduced in miR-10a (+) group compared to NC (+) group and in miR-10a (+)/ Circ-ITCH (+) group compared to Circ-ITCH (+) group. Differences between two groups were determined by *t*-test. **p*<0.05, ***p*<0.01, NS non-significant. MiR, microRNA; Circ-ITCH, Circular RNA Itchy E3 ubiquitin protein ligase; NC, negative control.

therapeutic target for EOC. MiRNAs are defined as non-coding RNAs whose lengths range from 19 to 25 nucleotides, and their dysregulation has been disclosed to be associated with development and progression of various carcinomas including ovarian cancer[15-19]. MiR-10a, a non-coding miRNA located on chromosome 17, is predicted to be a potential downstream target of Circ-ITCH by using potential binding microRNAs analysis in tissue-specific CircRNA database. Long et al[20] reveal that miR-10a promotes cells growth and migration via targeting CHL1 in human cervical cancer cells. Also, increased expression of miR-10a is reported to facilitate migration and invasion of hepatocellular cancer cells[21]. Lu et al[22] show that miR-10a targets MAPK8IP1 in gastric cancer (GC) cells and enhances GC metastasis. These previous studies imply that miR-10a acts as an oncogene in several carcinomas. In this study, we discovered that Circ-ITCH suppressed proliferation and promoted apoptosis in SKOV3 cells by downregulating miR-10a. In order to testify this suggestion, we performed rescue experiments subsequently and found that Circ-ITCH mediated cells proliferation and increased cells apoptosis via sponging miR-10a, which further illustrated the underlying mechanism of Circ-ITCH in EOC.

Conclusions

We found that circ-ITCH suppressed cells proliferation and promoted cells apoptosis via sponging miR-10a in EOC cells.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Acknowledgments

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