CircRNA CircRIMS is Overexpressed in Esophageal Squamous Cell Carcinoma and Downregulate miR-613 through Methylation to Increase Cell Proliferation

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Research article

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Abstract

Background

Esophageal squamous cell carcinoma (ESCC) as the most common subtype of esophageal cancer accounts for about 95% of all cases. CircRNA CircRIMS has been characterized as an oncogenic circRNA in gastric cancer, while its role in other cancers is unknown. This study aimed to explore the role of CircRIMS in esophageal squamous cell carcinoma (ESCC).

Methods

Tissues collected from 60 ESCC patients were used in this study. The expression of CircRIMS and miR-613 were detected by RT-qPCRs. The 60 ESCC patients were followed up for 5 years to evaluate the prognostic value of CircRIMS for ESCC. The role of CircRIMS in regulating the expression of miR-613 and methylation was assess by overexpression experiments and RT-qPCRs. The role of CircRIMS and miR-613 in regulating cell proliferation was analyzed by BrdU assay.

Results

We found that CircRIMS was overexpressed in ESCC and predicted poor survival. In addition, miR-613 was downregulated in ESCC and inversely correlated with CircRIMS. In ESCC cells, overexpression of CircRIMS decreased the expression levels of miR-613 and increased the methylation of miR-613 gene. Cell proliferation assay showed that overexpression of CircRIMS reduced the inhibitory effects of overexpression of miR-613 on cell proliferation.

Conclusions

CircRIMS may downregulate miR-613 through methylation to increase cell proliferation in ESCC.

Background

Esophageal cancer is one of the most common types of cancer among males[1]. Esophageal squamous cell carcinoma (ESCC) is the most common subtype of esophageal cancer and accounts for about 95% of all cases[1,2]. With the development of anti-ESCC approaches, the survival of patients with resectable tumors such as localized tumors, has been significantly improved by neoadjuvant chemoradiotherapy in combination with surgery [3,4]. However, distant tumor metastasis to liver, lung, bone and brain is common in ESCC patients[5] It is estimated that more than half of ESCC patients are diagnosed with distant tumor metastasis, and more than 1/3 of patients received surgery and chemotherapies will develop distant tumor metastasis[5,6]. Unfortunately, there is no cure for metastasis ESCC.

With the growing understanding of cancer biology, molecularly targeted therapy, which can be applied to treat cancers by regulating the expression of cancer-related genes, has been developed[7,9]. In effect, certain molecular players, such as the NRF2 signaling, HER2 gene and FGFR2 gene, have been proven to

be potential targets for targeted ESCC therapy [10,11]. However, the molecularly targeted therapy for ESCC is still under research and more targets are needed. Circular RNAs (circRNAs) are covalently closed single-strand RNA transcripts that participate in cancer biology by regulating gene expression rather than coding proteins, suggesting their potential role as targets for cancer treatment [12,13]. However, the function of most circRAs in cancer biology remains to be elucidated. CircRNA CircRIMS has been characterized as an oncogenic circRNA in gastric cancer[14], while its role in other cancers is unknown. This study was performed to investigate the role of CircRIMS in ESCC.

Methods

Patients and follow-up

A total of 60 ESCC patients (37 males and 23 females, 62.4 +/- 5.6 years old) who were diagnosed as ESCC through histopathological exam between May 2015 and August 2015 were enrolled in this study. This study was approved by the Ethics Committee of General Hospital of Eastern Theater Command. All patients were diagnosed for the first time. This study excluded the factors that can potentially affect the expression of the target gene of the present study, such as initiated therapy within 3 months prior to admission, other clinical disorders and history of malignancies, from the 60 ESCC patients. All patients signed the informed consent. The 60 patients were grouped into stage I or II (n = 32), and III or IV (n = 28). All patients were followed up for 5 years from the day of admission to record survival. Follow-up was performed through a monthly manner and all patients completed the follow-up or died of ESCC during follow-up.

ESCC tissue acquisition and ESCC cells

ESCC and adjacent (within the area 3 cm around tumors) paired non-tumor tissue samples were collected from the 60 ESCC patients through fine needle aspiration. All tissue samples were confirmed by histopathological exam. Tissue samples were kept in liquid nitrogen prior to the subsequent assays. ESCC cell model used in this study was human ESCC cell line KYSE450 (Laboratory of Birth Defects and Reproductive Health, China). Cells were cultivated in medium composed of 10% FBS and 90% RPMI-1640 medium under the conditions of 5% CO_2, 37 °C and 95% humidity to reach about 80% confluence prior to the subsequent assays.

Cell transfections

CircRIMS and miR-613 were overexpressed in KYSE450 cells by transfecting KYSE450 cells (10^6) with either 1 μg CircRIMS expression vector or 50 nM miR-613 mimic. Mimic of miR-613 and negative control (NC) miRNA were obtained from Genecopoeia (Guangzhou, China). The expression vector of CircRIMS was constructed with pcDNA3.1 expression vector obtained from Invitrogen (Shanghai, China). NC experiments were performed by transfecting empty vector or NC miRNA into the same number of cells. In all transfections, untransfected cells were the control (C) cells. The transfected cells were kept for 48 h in fresh medium to perform the following experiments.

RNA preparations

Total RNAs were isolated from tissues and cells using Ribozol (VWR). RNA samples were digested with DNase I (Invitrogen) until to remove genomic DNA. RNA integrity analysis was performed through 5% urea-PAGE gel electrophoresis.

RT-qPCR assay

StaRT Reverse Transcription kit (AnyGenes) was used to prepare cDNA samples through reverse transcriptions (RTs) using RNA samples as template. PowerTrack SYBR Green Master Mix (Rhenium) was used to perform qPCRs to analyze the expression of CircRIMS. The endogenous control of CircRIMS was 18S RNA. The expression of mature miR-613 was analyzed using All-in-One™ miRNA qRT-PCR Detection Kit (Genecopoeia) by adding poly (A), followed by RTs and qPCRs with poly (T) as reverse primer. Ct values of target genes were normalized to endogenous controls using $2^{-\Delta\Delta CT}$ method.

Methylation-specific PCR (MSP)

Quick Genomic DNA Extraction Kit (Clinisciences) was used to isolate genomic DNA from KYSE450 cells with transfections. EZ DNA Methylation-Gold™ Kit (ZYMO RESEARCH) was used to convert genomic DNA samples. The methylation of miR-613 was analyze through routine PCRs and MSPs, which were all performed using 2x Taq mix (Invitrogen).

BrdU assay

Cells with transfections were cultivated in a 96-well plate with 4,000 cells in 0.1 ml medium per well. Cells were cultivated under the aforementioned conditions for 48 h, followed by incubation with BrdU (10 mM) for 48 h. After that, cells were fixed and incubated with peroxidase-coupled anti-BrdU-antibody (Sigma–Aldrich) for 48 h. After washing, peroxidase substrate was used to incubate the cells for 2 h. Cell proliferation was analyzed by measuring OD values at 450 nm.

Statistical analyses

Heml 1.0 software was used to plot heatmaps to present the differential expression of CircRIMS and miR-613 in paired tissue samples. ANOVA Tukey's test was used to compare differences among multiple cell transfection groups. Correlations were analyzed by Pearson's correlation coefficient. With the median expression levels of CircRIMS in ESCC tissues as the cutoff value, the 64 ESCC patients were grouped into high and low (n = 32) level groups. Survival curves of both groups were plotted and compared by log-rank test. $P < 0.05$ was considered as statistically significant.

Results

Poor prognosis of ESCC patients was correlated with the high expression levels of CircRIMS in ESCC tissues

The differential expression of CircRIMS between ESCC and paired non-tumor tissue samples were determined by RT-qPCR. Compared to non-tumor tissues, ESCC tissues exhibited higher expression levels of CircRIMS (Fig. 1A). Survival analysis showed that patients in high CircRIMS level group experienced worse overall survival compared to patients in low CircRIMS level group (Fig. 1B).

MiR-613 was downregulated in ESCC and inversely correlated with CircRIMS

The expression of miR-613 in paired ESCC and non-tumor tissues from the 64 ESCC patients was also analyzed by RT-qPCR. Heatmap analysis showed that the expression levels of miR-613 were lower in ESCC tissues compared to that in non-tumor tissues (Fig. 2A). Pearson's correlation coefficient analysis showed that the expression of CircRIMS and miR-613 were inversely and significantly correlated across ESCC tissue samples (Fig. 2B, $p < 0.05$), but not non-tumor tissues (Fig. 2C).

Overexpression of CircRIMS decreased the expression levels of miR-613 in KYSE450 cells through methylation

To investigate the crosstalk between CircRIMS and miR-613, KYSE450 cells were transfected with either CircRIMS expression vector or miR-613 mimic, followed by the confirmation of the overexpression of CircRIMS and miR-613 by RT-qPCR. It was observed that CircRIMS and miR-613 were significantly overexpressed between 24 h and 96 h post-transfection (Fig. 3A, $p < 0.05$). In addition, Overexpression of CircRIMS significantly decreased the expression levels of miR-613 (Fig. 3B), while overexpression of miR-613 did not affect the expression of CircRIMS (Fig. 3C). MSP was performed to analyze the role of CircRIMS in regulating the methylation of miR-613 gene. Compared to cells transfected with empty pcDNA3.1 vector, cells transfected with CircRIMS expression vector exhibited increased methylation of miR-613 (Fig. 3D).

Overexpression of CircRIMS reduced the inhibitory effects of overexpression of miR-613 on cell proliferation

The role of CircRIMS and miR-613 in regulating the proliferation of KYSE450 cells was analyzed by BrdU assay. Overexpression of CircRIMS significantly increased cell proliferation rate, while overexpression of miR-613 significantly decreased cell proliferation rate. Moreover, overexpression of CircRIMS reduced the inhibitory effects of overexpression of miR-613 on cell proliferation (Fig. 4, $p < 0.05$).

Discussion

The present study explored the role of CircRIMS in ESCC and analyzed its crosstalk with miR-613, which is a critical player in ESCC [15]. We found that CircRIMS was upregulated in ESCC and it could downregulate miR-613 through methylation to increase cell proliferation.

A previous study reported that CircRIMS played an oncogenic role in gastric cancer [14]. CircRIMS is overexpressed in gastric cancer and overexpression of CircRIMS promotes the *in vitro* metastasis of gastric tumors by sponging hsa-miR-148a-5p and hsa-miR-218-5p[14]. Based on our understanding, the

role of CircRIMS in other types of cancer is unknown. In this study we reported the overexpression of CircRIMS in ESCC. Moreover, overexpression of CircRIMS significantly increased the proliferation of ESCC cells. Therefore, CircRIMS may play oncogenic roles by increasing cell proliferation.

Clinical treatment of ESCC is challenged by the poor prognosis, which is mainly caused by the low early diagnostic rate[16]. Due to the lack of sensitive biomarkers, the early diagnosis of ESCC is unlikely to be significantly improved in the near future. In this study we showed that the expression levels of CircRIMS were closely correlated with the poor survival of ESCC patients, suggesting that CircRIMS may serve as a potential prognostic biomarker for ESCC. Therefore, monitoring the expression of CircRIMS may guide the determination of treatment approaches for ESCC, thereby improving the survival of patients. However, clinical trials are needed to further test our hypothesis.

It has been reported that miR-613 is downregulated in ESCC and overexpression of miR-613 targets G6PD to suppress the invasion and migration of ESCC cells[15] Consistently, our study confirmed the overexpression of miR-613. Moreover, our study showed that overexpression of miR-613 decreased cell proliferation. Interestingly, our data showed that CircRIMS may downregulate miR-613 through methylation in ESCC cells. However, the regulation of miR-613 is likely mediated by certain ESCC-related factors due to the fact that the expression of CircRIMS and miR-613 were closely correlated only across ESCC tissues but not non-tumor tissues.

Conclusions

In conclusion, CircRIMS is overexpressed in ESCC and it may downregulate miR-613 through methylation to promote cancer cell proliferation.

Abbreviations

Not applicable

Declarations

Acknowledgments

The authors would like to thank the participants in this study.

Authors' contributions

FW designed the study. HW carried out experiments and wrote the manuscipt, FW revised the paper, BY, KJ, JW, XF and BS collected patient specimens and related information. HW, BT, KJ, JW, XF and BS contributed to analysing the data. All authors reviewed the results and approved the final version of the manuscript.

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Availability of data and materials

All data are included in this published article. Any additional information related to this study is available from the author for correspondence upon reasonable request.

Declarations

Not applicable.

Ethics approval and consent to participate

The study was granted ethical approval by the Ethical Committee of General Hospital of Eastern Theater Command, Nanjing City, and all the patients or parents/ guardians of patients provided written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

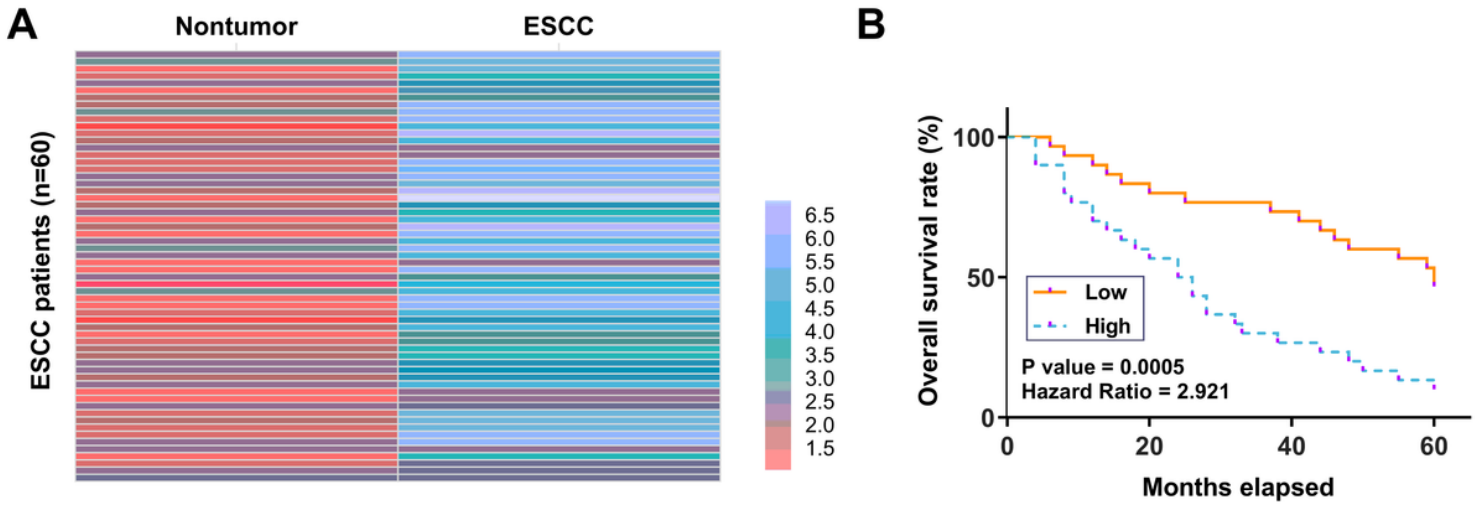

Figure 1

Poor prognosis of ESCC patients was correlated with the high expression levels of CircRIMS in ESCC tissues ESCC and paired non-tumor tissue samples were subjected to RNA isolation and RT-qPCR to determine the differential expression of CircRIMS in ESCC, which is expressed as a heatmap plotted using Heml 1.0 software (A). With the median expression level of CircRIMS in ESCC tissues as a cutoff value, the 64 ESCC patients were grouped into high and low (n = 32) level groups (B).

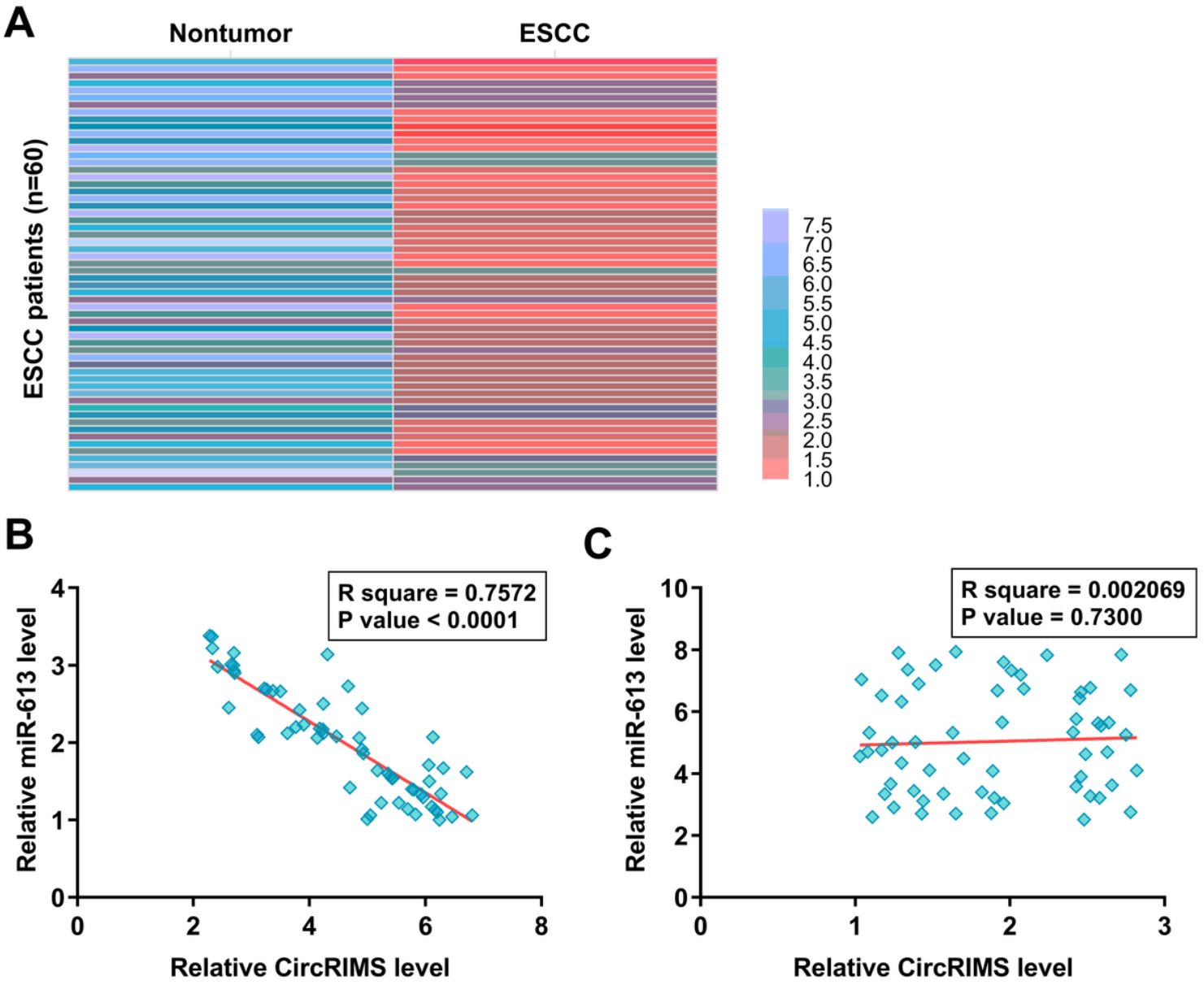

Figure 2

MiR-613 was downregulated in ESCC and inversely correlated with CircRIMS ESCC and paired non-tumor tissue samples were subjected to RNA isolation and RT-qPCR to determine the differential expression of miR-613 in ESCC, which is expressed as a heatmap plotted using Heml 1.0 software (A). Pearson's correlation coefficient analysis was performed to analyze the correlations between CircRIMS and miR-613 ESCC tissue samples (B) and non-tumor tissues (C).

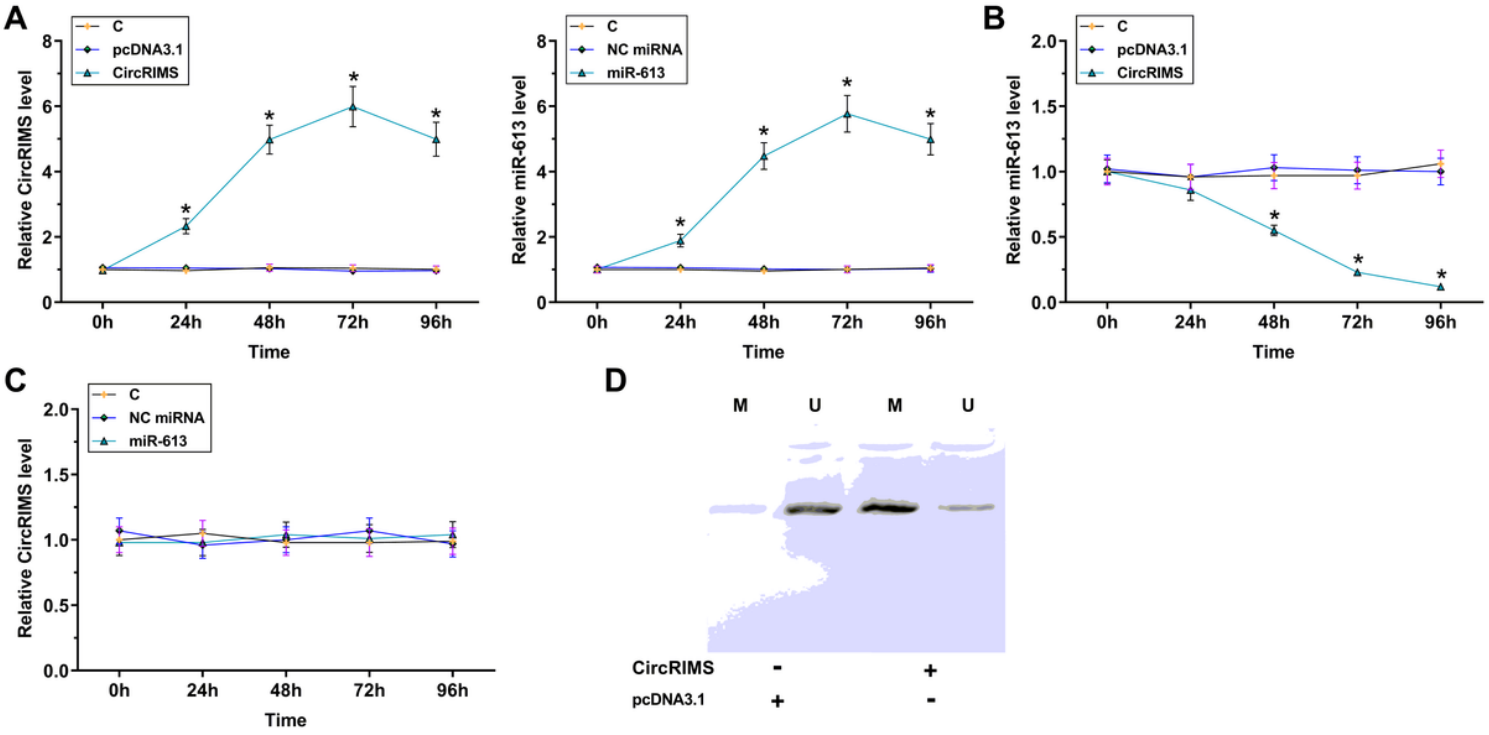

Figure 3

Overexpression of CircRIMS decreased the expression levels of miR-613 in KYSE450 cells through methylation To study the crosstalk between CircRIMS and miR-613, KYSE450 cells were transfected with either CircRIMS expression vector or miR-613 mimic, followed by the confirmation of the overexpression of CircRIMS and miR-613 by RT-qPCR every 24 h until 96 h (A). The effects of CircRIMS overexpression on the expression of miR-613 (B), and the effects of overexpression of miR-613 on the expression of CircRIMS (C) were analyze by RT-qPCR. MSP was performed to analyze the role of CircRIMS in regulating the methylation of miR-613 gene (D). M, methylated PCR products; U, un-methylated PCR products. *, $p < 0.05$.

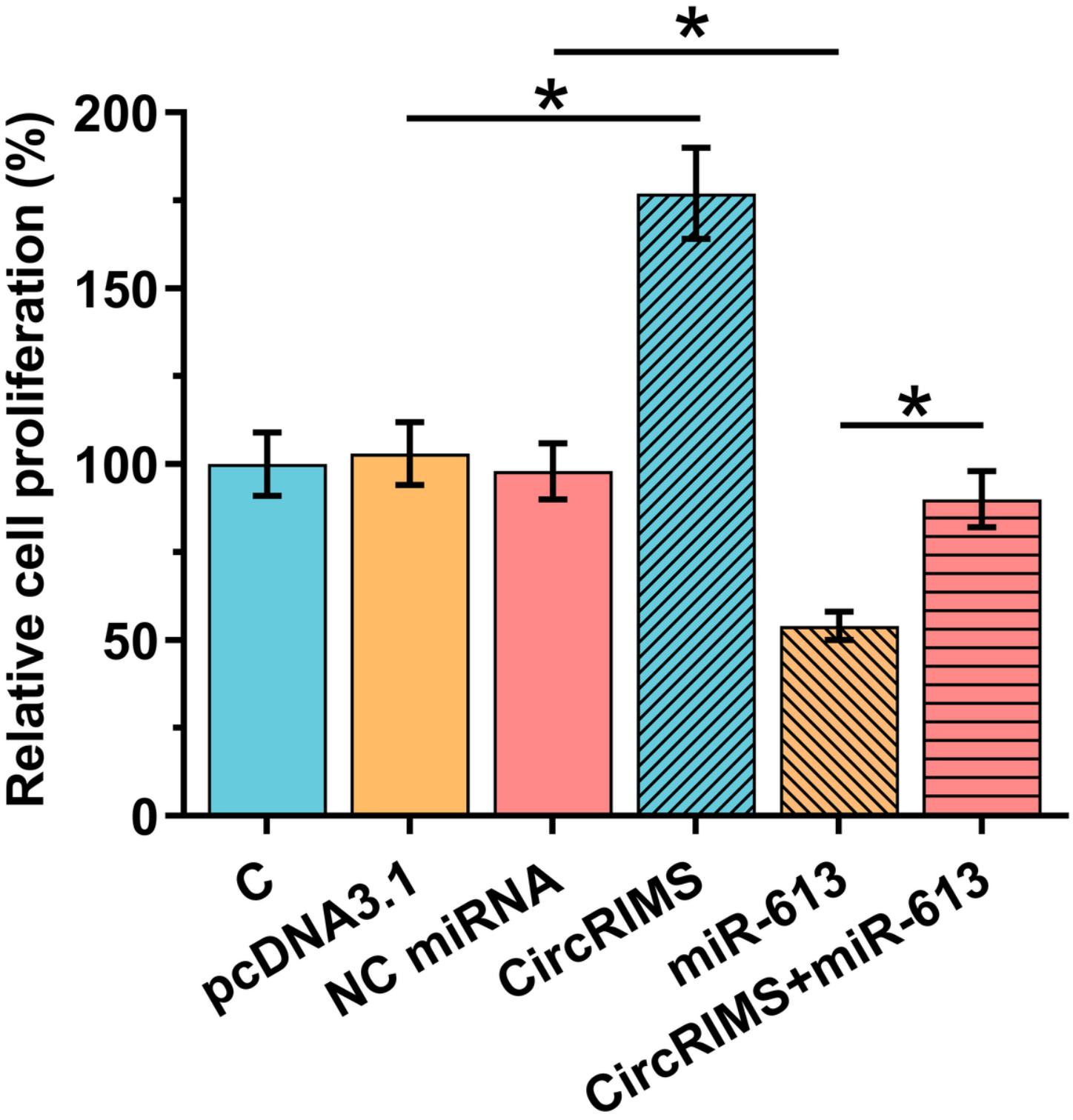

Figure 4

Overexpression of CircRIMS reduced the inhibitory effects of overexpression of miR-613 on cell proliferation The role of CircRIMS and miR-613 in regulating the proliferation of KYSE450 cells was analyzed by BrdU assay. Experiments were repeated 3 times and mean+/-values of three biological replicates were present an compared. *, $p < 0.05$.

Supplementary Files

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