CircRNA circ-ATAD1 suppresses the maturation of miR-168 to participate in colorectal cancer

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Research

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Abstract

Background: CircRNA circ-ATAD1 has been characterized as an oncogenic circRNA in gastric cancer, while its role in colorectal cancer is This study was carried out to explore the role of circ-ATAD1 in colorectal cancer (CRC).

Methods: Paired CRC and adjacent non-tumor tissue samples collected from 64 CRC patients were subjected to RNA extractions and RT-qPCRs to analyze the expression of circ-ATAD1, premature miR-168 and mature miR-168 in The effects of circ-ATAD1 overexpression on the maturation of miR-168 was analyzed by transfecting circ-ATAD1 expression vector into CRC cells, followed by determining the expression of premature miR-168 and mature miR-168 through RT-qPCR. The role of circ-ATAD1, premature miR-168 and mature miR-168 in regulating the proliferation of CRC cells was explored by CCK-8

Results: In this study we found that circ-ATAD1 was upregulated in CRC and predicted poor survival. In addition, circ-ATAD1 was inversely correlated with mature miR-168, but not premature miR-168. In CRC cells, circ-ATAD1 overexpression decreased the expression of mature miR-168, but not premature miR-168. Moreover, circ-ATAD1 overexpression reduced the inhibitory effects of miR-168 overexpression on cell proliferation.

Conclusions: Therefore, circ-ATAD1 is overexpressed in CRC and it may suppress the maturation of miR-168 to participate in CRC.

Introduction

Colorectal cancer (CRC), also known as rectal cancer, colon cancer or bowel cancer, is one of the most common types of malignancy in clinical practice (1, 2). It is estimated that one out of 21 males and one out of 23 females will develop CRC during the life time (3). In recent years, incidence of CRC among patients older than 50 years has been observed (1, 2). However, incidence in young population showed an increasing trend (1, 2). Despite the advances in the treatment and prevention of CRC, CRC is still a major cause of cancer deaths all over the world(4), mainly owing to the lack of cures for metastatic CRC and the low early diagnostic rate(5, 6). Therefore, novel therapeutic and diagnostic approaches are Smoking, alcohol abuse, poor dietary structure, and and obesity are the major risk factors for CRC(7). However, it has been well established that the development and progression also require the participation of molecular players (8) . Some molecular factors, such as HER2, have shown therapeutic potentials in the treatment of CRC through molecular target therapy, which can been performed to treat CRC by regulating gene expression (9, 10). However, molecular targeted therapy is still under research and effective targets remain lacking(11). Despite the lack of protein-coding capacity, circular RNAs (circRNAs, closed by covalent bonds) play critical roles in cancer biology mainly by regulating related gene expression (12, 13). Therefore, circRNAs are potential targets for molecular targeted therapy.

However, the function of most circRNAs in CRC remains CircRNA circ-ATAD1 has been characterized as an oncogenic circRNA in gastric cancer(14), while its role in CRC is unknown. We performed preliminary microarray analysis and observed the altered expression of circ-ATAD1 in CRC as well as the inverse correlation between circ-ATAD1 and miR-168, which is also a crucial player in cancer biology (15). We therefore carried out this study to explore the interactions between circ-ATAD1 and iR-168 in CRC.

Materials And Methods

Patients and follow-up

Ethics Committee of The General Hospital of People's Liberation Army approved this study before the admission of At this hospital, 64 CRC patients (38 males and 26 females) were enrolled from June 2013 to June Age range of the 64 patients was 47 to 67 years, with a mean of 1+/-6.7 years. CRC patients were all diagnosed by histopathological Patients with initiated therapy, recurrent CRC patients and patients with other severe clinical disorders were excluded from the Based on AJCC system, the 64 CRC patients were grouped into stage I (n=12), II (n=16), III (n=20) and IV (n=16). From the day of admission, this study performed a 5-year follow-up on the 64 patients through a monthly manner to record patients'

Patients died of causes unrelated to CRC were excluded from the 64 CRC patients included in this Informed consent was provided by all patients.

CRC biopsies and cell lines

All the 64 patients were subjected to fine needle aspirations to collect paired CRC and adjacent (within 3cm around tumors) non-tumor tissues. Following histopathological confirmation, tissue samples were kept in liquid nitrogen storage prior to the subsequent assays.

The cell models of CRC were WiDr and HT-29 CRC cell lines (ATCC, USA). Cells were cultivated in a medium composed of FBS (10%) and McCoy's 5a Medium (90%). Cell culture conditions were 37°C, 95% humidity and 5% CO2. Cells used in the subsequent assays were collected at about 85% confluence.

Overexpression of circ-ATAD1 and miR-168 in WiDr and HT-29 cells were achieved by transfecting circ-ATAD1 expression vector (1μg) or miR-168 (50 nM) mimic into 10^8 WiDr and HT-29 cells through transient transfections mediated by lipofectamine 2000 (Invitrogen). Expression vector was constructed using pcDNA3.1 vector (Invitrogen) as Mimic of miR-168 and negative control (NC) miRNA were provided by Sigma-Aldrich. Control (C, untransfected cells) and NC (cells transfected with empty vector or NC miRNA) cells were included in each Transfected cells were cultivated in fresh medium for 48h prior to the subsequent assays.

Preparation of RNA samples

Total RNA was extracted from tissue samples and in vitro cultivated cells using RNAzol (Sigma-Aldrich), followed by using DNase I (Invitrogen) to digest genomic DNA for 100 min at 37°C. Electrophoresis was

performed using 5% urea-PAGE gels to analyze RNA integrity. RNA purity was analyzed by determining the OD 260/280 ratios of all RNA samples.

RT-qPCRs

RNA samples with an OD260/280 ratio close to 2.0 (pure RNA) and satisfactory integrity were subjected to reverse transcriptions (RTs) to prepare cDNA samples. RTs were performed using SS-IV-RT system (Invitrogen). With 18S rRNA as an endogenous control, SYBR Green Master Mix (Bio-Rad) was used to perform qPCRs to determine the expression of circ-ATAD1.

Expression of premature miR-168 and mature miR-168 was analyzed using All-in-One™ miRNA qRT-PCR reagent kit (GeneCopoeia). Expression of mature miR-168 was added by addition poly (A), followed by using poly (T) to perform RTs and Expression of premature miR-168 was performed by using sequence-specific primers to perform RTs and Ct values of target genes were normalized to endogenous controls using the method of 2-ΔΔCT.

Cell Counting Kit-8 (CCK-8) assay

WiDr and HT-29 cells with transfections were subjected to cell proliferation analysis using a CCK-8 kit from Dojindo (Japan). In brief, cells were counted and were transferred to a 96-well cell culture plate with 3000 cells in 1 ml fresh medium per Three replicate wells were set for each experiment. Cells were cultivated under the aforementioned conditions, followed by determining OD values (450 nM) every 24h until At 2h prior to the determination of OD values, CCK-8 solution was added to reach the final concentration of 10%.

Statistical analysis

Gene expression data in paired CRC and non-tumor tissues was used to plot heatmaps using Heml 0 software to present the differential expression of genes in CRC and non-tumor tissues. Comparisons among multiple independent cell transfection groups were analyzed by ANOVA Tukey's Correlations between the expression of genes were analyzed by Pearson' correlation To analyze the prognostic value of circ-ATAD1 for CRC, the 64 CRC patients were grouped into high and low circ-ATAD1 level groups (n=32, cutoff value=the median level of circ-ATAD1 expression in CRC tissues). Survival curves were plotted based on the 5-year follow-up data and survival curves were compared by log-rank test. $P<0.05$ was deemed statistically significant.

Results

Circ-ATAD1 was overexpressed in CRC and predicted poor survival

Paired CRC and non-tumor tissues collected from CRC patients (n=64) were subjected to RNA extractions and RT-qPCRs to determine the expression of circ-ATAD1. Heatmap analysis showed that circ-ATAD1 expression was upregulated in CRC tissues compared to non-tumor tissues (Fig.1A). Survival analysis

showed that patients in high circ-ATAD1 level group showed significantly lower overall survival rate compared to patients in low circ-ATAD1 level group (Fig.1B).

CRC tissues exhibited downregulated mature miR-168, but not premature miR-168

Expression of mature miR-168 and premature miR-168 in the paired tissue samples from 64 CRC patients was also analyzed by RT-qPCR. Heatmap analysis showed that expression of miR-168 was upregulated in CRC tissues compared to non-tumor tissues (Fig.2A). In contrast, CRC and non-tumor exhibited no obvious differences in expression level of premature miR-168 (Fig.2B). Therefore, the maturation of miR-168 may participate in CRC.

Circ-ATAD1 was inversely correlated with mature miR-168 across CRC tissue samples

Pearson's correlation coefficient was performed to analyze the correlations between circ-ATAD1 and mature miR-168 or premature miR-168 across CRC tissue It was observed that circ-ATAD1 was significantly and inversely correlated with mature miR-168 across tumor tissues (Fig.3A). In contrast, no significant correlation between circ-ATAD1 and premature miR-168 was observed across non-tumor tissues (Fig.3B).

Therefore, circ-ATAD1 is likely related to the maturation of miR-168.

Circ-ATAD1 overexpression suppressed the maturation of miR-168 in WiDr and HT-29 cells

To analyze the effects of circ-ATAD1 on the maturation of miR-168, WiDr and HT-29 cells were transfected with either circ-ATAD1 expression vector or miR-168 mimic, followed by determining the expression of circ-ATAD1 and miR-168 every 24h until 144h through RT-qPCRs. It was observed that circ-ATAD1 and mature miR-168 were significantly overexpressed between 24h and 144h (Fig.4A, $p<0.05$). Interestingly, circ-ATAD1 overexpression decreased the expression of mature miR-168 (Fig.4B, $p<0.05$), but not premature miR-168 (Fig.4C). Moreover, overexpression of miR-168 failed to significantly affect the expression of circ-ATAD1 (Fig.4D).

Circ-ATAD1 expression increased the proliferation of WiDr and HT-29 cells through miR-168

The role of circ-ATAD1 and miR-168 in regulating the proliferation of WiDr and HT-29 cells was analyzed by CCK-8 It was observed that circ-ATAD1 overexpression increased cell proliferation, and miR-168 overexpression decreased cell Moreover, circ-ATAD1 overexpression reduced the inhibitory effects of miR-168 overexpression on cell proliferation (Fig.5, $p<0.05$).

Discussion

This study mainly investigated the involvement of circ-ATAD1 and miR-168 in CRC and explored the interaction between them. We found that circ-ATAD1 was significantly upregulated in CRC and it may downregulate the expression of mature miR-168 to promote CRC cell proliferation.

The role of circ-ATAD1 in cancer biology has only been explored in gastric cancer (14).

It has been reported that circ-ATAD1 is overexpressed in gastric cancer and it sponges miR-140-3p to upregulate YY1, thereby increasing cancer cell proliferation (14). Based on our knowledge, the role of circ-ATAD1 in other types of cancers is In this study we first reported the upregulation of circ-ATAD1 in CRC and its enhancing effects on CRC cell Therefore, circ-ATAD1 is likely an oncogenic lncRNA in CRC.

Treatment of CRC in clinical practice is challenged by the poor prognosis of metastatic cases (5, 6). Unfortunately, early diagnosis of CRC is unlikely to be significantly improved in near future, mainly owing to the lack of sensitive diagnostic biomarkers. In this study we showed that high expression levels of circ-ATAD1 were correlated to the poor survival of CRC Therefore, monitoring the expression of circ-ATAD1 in CRC patients may guide the determination of therapeutic approaches, which in turn improve patients' survival. However, the prognostic value of circ-ATAD1 remains to be further analyzed.

MiR-168 has been characterized as a tumor suppressor in different types of cancers (15, 16). For instance, miR-618 is downregulated in prostate cancer and suppresses cancer cell invasion and migration by targeting FOXP2 (15). Although the function of miR-618 in CRC is unknown, it is known that the susceptibility of CRC in a Han Chinese population is correlated with the polymorphism rs2682818 in miR-618 (15). In this study we showed that the decreased maturation of miR-168, but not the transcription of miR-168 gene is involved in In addition, the overexpression of circ-ATAD1 is likely responsible for the reduced production of mature miR-168 in CRC cells. We speculate that circ-ATAD1 may suppress the transportation of premature miR-168 from nucleus to cytoplasm, which is required for the maturation of miR-168.

Conclusions

In conclusion, circ-ATAD1 is upregulated in CRC and it may suppress the maturation of miR-168 gene to promote CRC cell proliferation.

Declarations

Ethics Approval and Consent to participate

For human experiments, the trial was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the human Ethics Committee of The General Hospital of People's Liberation Army and informed consent was taken from all individual participants.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of date and materials

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

The data used to support the finds of this study are available from the corresponding author upon request.

Competing interests

The author reports no conflicts of interest in this work.

Funding

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' contributions

(I) Conception and design: Guanglong Dong

(II) Administrative support: All authors

(III) Provision of study materials or patients: Guanglong Dong

(IV) Collection and assembly of data: All authors

(V) Data analysis and interpretation: All authors

(VI) Manuscript writing: Li Cao, Peng Chen

(VII) Final approval of manuscript: All authors

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Figures

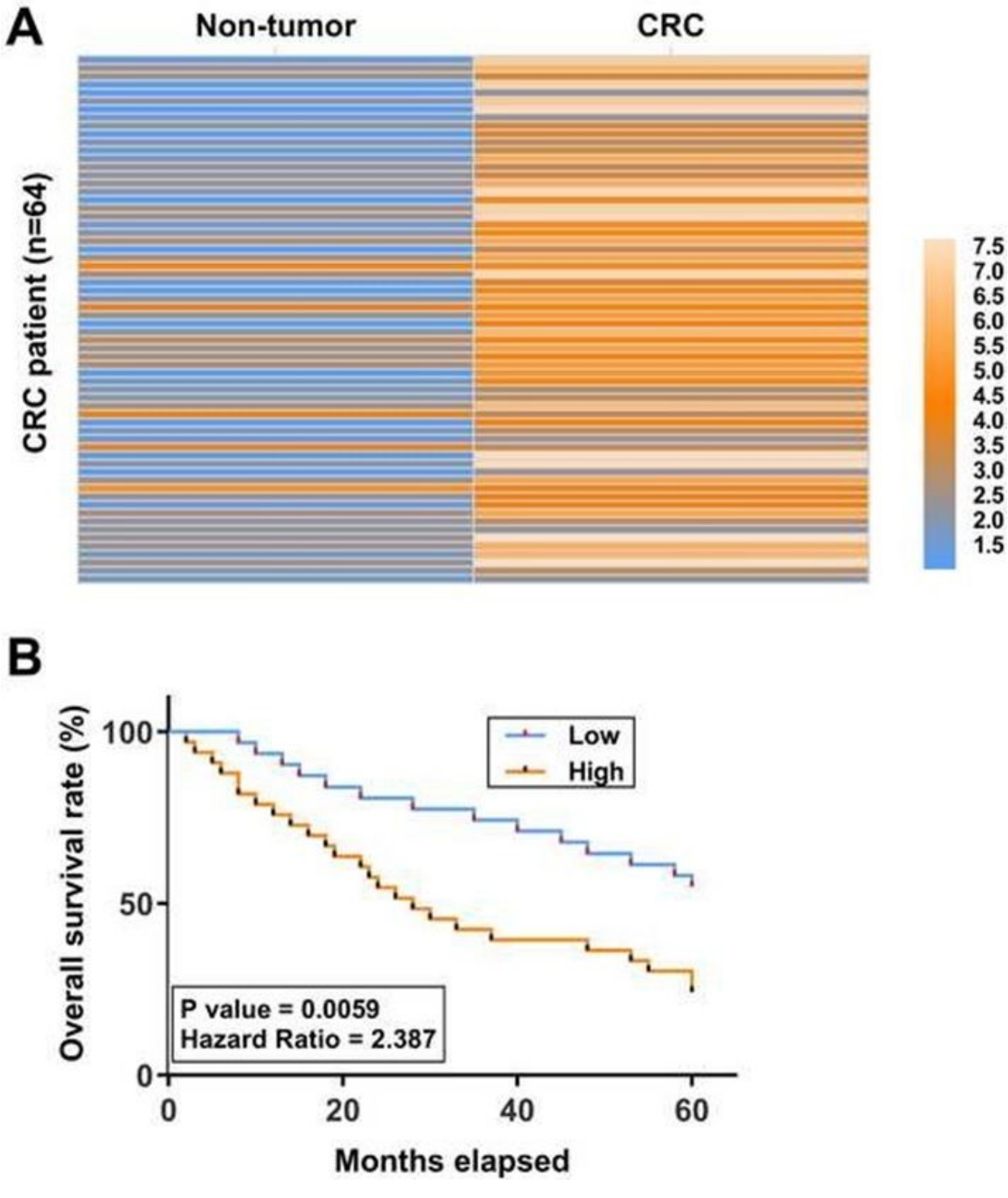

Figure 1

Circ-ATAD1 was overexpressed in CRC and predicted poor survival Paired CRC and non-tumor tissues collected from CRC patients (n=64) were subjected to RNA extractions and RT-qPCRs to determine the expression of circ-ATAD1. Gene expression data in paired CRC and non-tumor tissues was used to plot heatmaps using Heml 0 software to present the differential expression of circ-ATAD in CRC and non-tumor tissues (A). To analyze the prognostic value of circ-ATAD1 for CRC, the 64 CRC patients were grouped into high and low circ-ATAD1 level groups (n=32, cutoff value=the median level of circ-ATAD1 expression in CRC tissues). Survival curves were plotted based on the 5-year follow-up data and survival curves were compared by log-rank test (B).

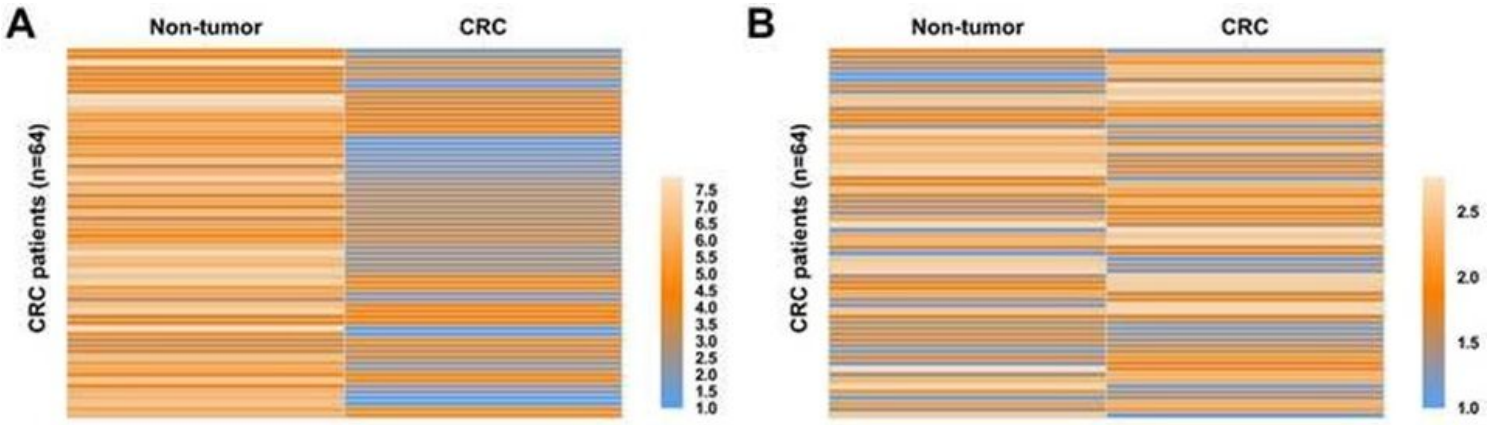

Figure 2

CRC tissues exhibited downregulated mature miR-168, but not premature miR-168 Expression of mature miR-168 (A) and premature miR-168 (B) in the paired tissue samples from 64 CRC patients was also analyzed by RT-qPCR. Differential expression of mature miR-168 and premature miR-168 was presented by plotting heatmaps.

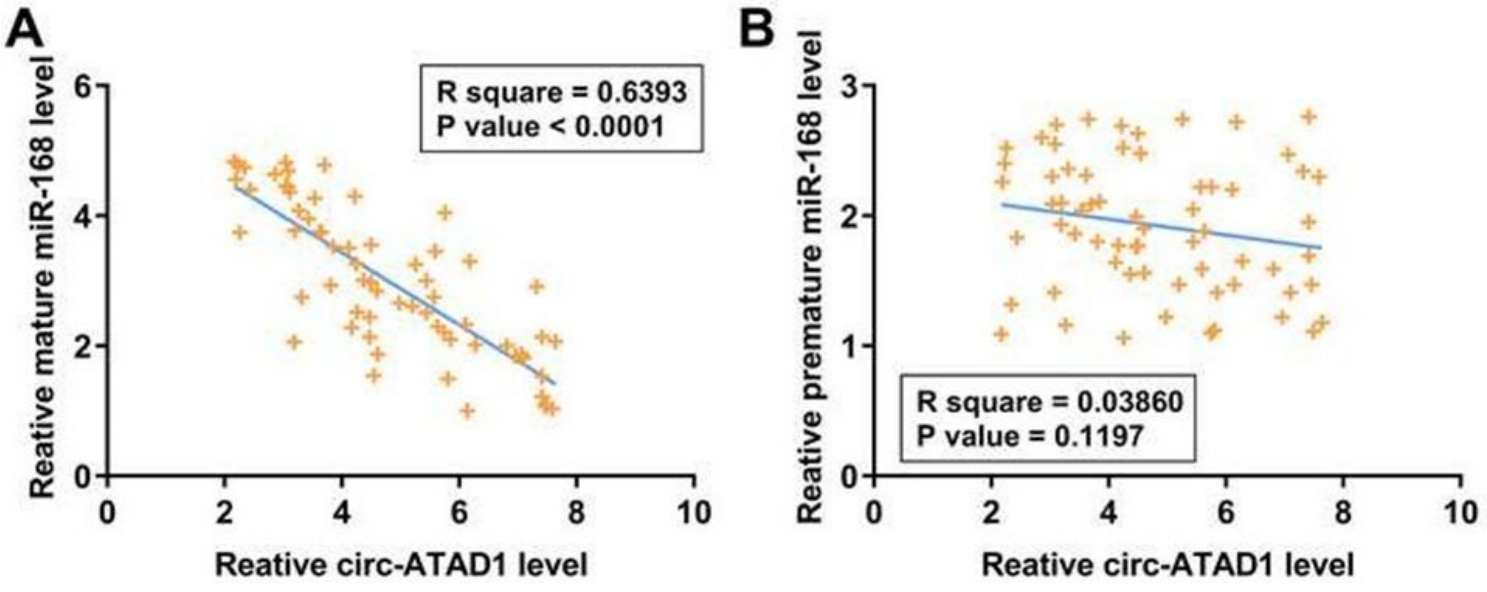

Figure 3

Circ-ATAD1 was inversely correlated with mature miR-168 across CRC tissue samples Pearson's correlation coefficient was performed to analyze the correlations between circ-ATAD1 and mature miR-168 or premature miR-168 across CRC tissue samples

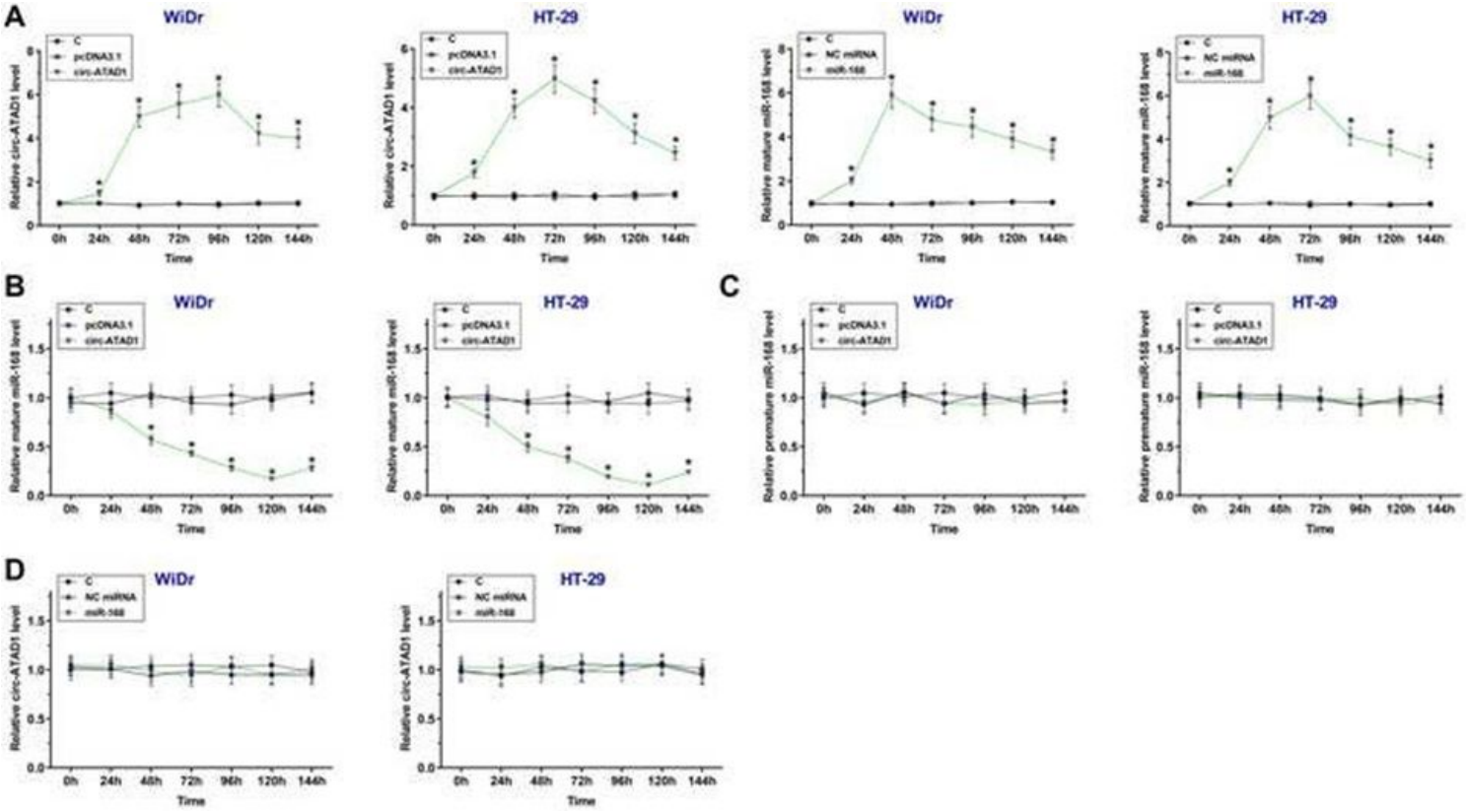

Figure 4

Circ-ATAD1 overexpression suppressed the maturation of miR-168 in WiDr and HT-29 cells To analyze the effects of circ-ATAD1 on the maturation of miR-168, WiDr and HT-29 cells were transfected with either circ-ATAD1 expression vector or miR-168 mimic, followed by determining the expression of circ-ATAD1 and miR-168 every 24 h until 144 h through RT-qPCRs (A). The effects of circ-ATAD1 overexpression on the expression of mature miR-168 (B) and premature miR-168 (C), and the effects of mature miR-168 overexpression on the expression of circ-ATAD1 (D) were analyzed by RT-qPCRs.*,$p<0.05$.

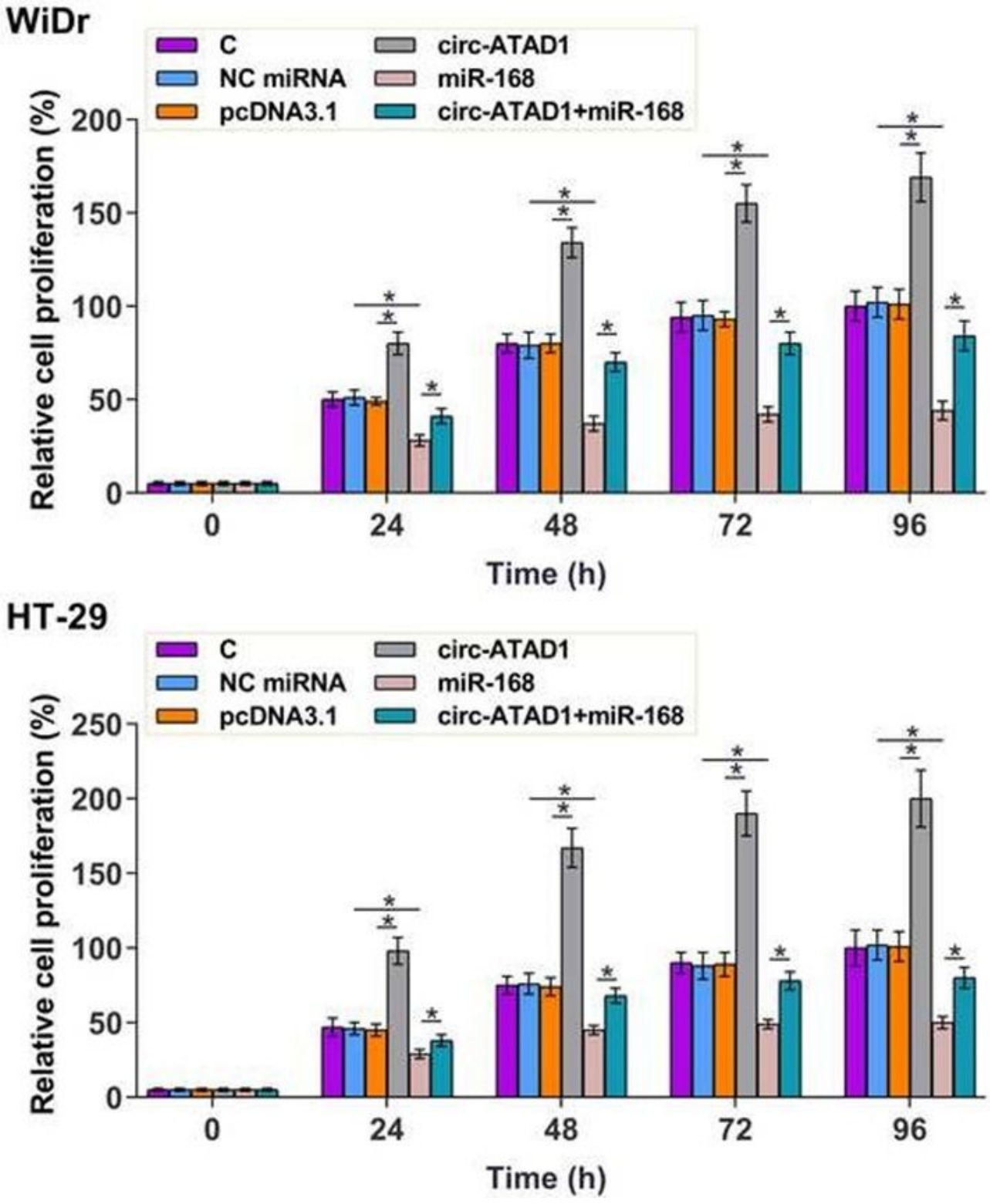

Figure 5

Circ-ATAD1 expression increased the proliferation of WiDr and HT-29 cells through miR-168 The role of circ-ATAD1 and miR-168 in regulating the proliferation of WiDr and HT-29 cells was analyzed by CCK-8 Cell proliferation was monitored by measuring OD values at 450n every 24 h until 96 h