LncRNA MCM3AP-AS1 sponges miR-148a to cell invasion and migration in small cell lung cancer

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Abstract

Background: MCM3AP-AS1 is a recently characterized lncRNA in hepatocellular carcinoma (HCC) and (GBM). We found that miR-148a may be able to bind MCM3AP-AS1. We explored the functions of lncRNA MCM3AP-AS1 in small cell lung cancer (SCLC).

Methods: Gene expression in SCLC patients was analyzed by qPCR. Transient transfections were performed to analyze gene interactions. Cell invasion and migration were analyzed by Transwell assays.

Results: We found that MCM3AP-AS1 was upregulated in SCLC and high expression levels were accompanied by low survival rate. Bioinfromatics analysis showed that MCM3AP-AS1 may bind miR-148a, which can target ROCK1. In SCLC tissues, MCM3AP-AS1 was positively correlated with ROCK1. In SCLC cells, MCM3AP-AS1 overexpression mediated the upregulated ROCK1 expression and increased cell invasion and migration rates. However, MCM3AP-AS1 and miR-148a could not regulate each other. However, miR-148a overexpressed led to downregulated ROCK1 expression, decreased cell invasion and migration rates, and reduced effects of MCM3AP-AS1 overexpression.

Conclusions: Therefore, MCM3AP-AS1 may sponge miR-148a to cell invasion and migration in SCLC through the upregulation of ROCK1.

Background

In 2018, lung cancer accounts for 11.6% (2,093,876 cases) of all new cases of cancer and caused 18.4% of cancer-related deaths [1]. It is predicted that lung cancer will be the most common cancer in a long-term [2]. Small-cell lung cancer (SCLC) accounts for about 15% of all lung cancer patients [3]. Although the development of novel therapeutic approaches, survival of SCLC patients has been improved significantly during the past century [3]. However, no significant treatment advances were achieved in recent years [3]. Smoking is the most common risk factor of SCLC [4]. However, SCLC also affects never-smokers, indicating the critical roles of genetic factors [5].

Studies on the pathogenesis of SCLC have revealed genetic alterations involved in the tumorigenesis of SCLC [6, 7]. As a consequence of these studies targeted therapies have been proposed, designed, tested and applied [6, 7]. Rho-associated, coiled-coil-containing protein kinase 1 (ROCK1), also known as ROKβ, is a downstream effector GTPase RhoA and participates in cancer biology by mainly by regulating cancer angiogenesis and metastasis [8, 9]. Some tumor suppressive miRNAs, such as miR-148a, inhibit cancer metastasis by targeting ROCK1 [10]. MCM3AP-AS1 is a recently characterized long non-coding RNA (lncRNA) in hepatocellular carcinoma (HCC) and (GBM) [11, 12]. Although lncRNAs encode no proteins, they can participate in diverse physiological and pathological processes, such as the development and progression of cancer, by interaction with protein-coding genes and other non-coding RNAs, such as miRNAs [13]. Analysis of the interactions between lncRNAs and other molecular players may provide novel insights to the development of anti-cancer therapies[13]. Our bioinformatics analysis showed that

that miR-148a may be able to bind MCM3AP-AS1. The present study aimed to investigate the interaction between MCM3AP-AS1 and miR-148a in SCLC.

Methods

Patients, treatment and follow-up

A total of 105 cases of SCLC were admitted to Yongchuan Hospital of Chongqing Medical University between April 2010 and April 2014. Form these patients the present study enrolled 60 cases (42 males and 18 females; 40 to 69 years, 54.1 ± 6.6 years). This study was approved by Yongchuan Hospital of Chongqing Medical University committee before patient admission, and that was conducted in accordance with the Declaration of Helsinki. The written informed consent was obtained from all patients. Inclusion criteria: 1) new SCLC cases; 2) no therapies were initiated; 3) confirmed by histopathological exam. Exclusion criteria: 1) patients transferred from other hospital; 2) patients with recurrent SCLC; 3) therapies were initiated; 4) multiple clinical disorders were diagnosed.

The 60 patients included 15, 20 and 25 cases at AJCC clinical stage II-IV, respectively. According to patients' conditions, surgery only, or surgery in combination with chemotherapy or radiotherapy, or chemotherapy or radiotherapy only, were performed. A 5-year follow-up was performed since admission in a monthly manner through telephone or outpatient visit. Patients died of causes unrelated to SCLC were excluded.

Specimen collection and SCLC cell model

MRI-guided biopsy was performed to collect both SCLC (tumor) and adjacent non-tumor lung tissues from each patient. Histopathological exams were performed to confirm all tissue samples.

Human SCLC cell line SHP-77 (ATCC, USA) was used. 90% RPMI-1640 medium was mixed with 10% FBS to cultivate cells at 37 °C with 5% CO_2.

Transient transfections

Negative control (NC) miRNA and miR-148a mimic were from Sigma-Aldrich (USA). Vectors expressing MCM3AP-AS1 and ROCK1 were constructed using pcDNA3 vector by Sangon (Shanghai, China). SHP-77 cells collated at 80% confluence were transfected with 45 nM miRNAor 10 nM vector using lipofectamine 2000 (Sigma-Aldrich). Control (C) cells were untransfected cells. Empty vector- or NC miRNA-transfected cells were NC cells. At 4h after transfections, cells were harvested and used in following experiments.

RNA extractions and digestion

To detect gene expression, total RNAs in tissue sample (0.03g per sample) or SHP-77 cells (3×10^5 cells per transfection group) were extracted using RNAzol (Sigma-Aldrich). All steps were completed following

the instructions from Sigma-Aldrich. It is worth noting that RNA precipitation was performed using 75% ethanol. The purpose is to harvest miRNA. DNase I digestion was performed on all RNA samples.

QPCR

Tetro Reverse Transcriptase (Bioline) was used to perform all reverse transcriptions and qPCR reactions were carried out using QuantiTect SYBR Green PCR Kit (Qiagen). With GAPDH as endougenous control, MCM3AP-AS1 and ROCK1 mRNA expression levels were normalized.

All-in-One™ miRNA qRT-PCR Detection Kit (Genecopoeia) was used to measure the expression levels of mature miR-148a. After the addition of poly (A), miRNA reverse transcription and qPCR assays were sequentially performed. Expression of miR-148a was normalized to U6.

Primer sequences were MCM3AP-AS1: Forward-CTGCTAATGGCAACACTGA and Reverse-AGGTGCTGTCTGGTGGAGA; GAPDH : Forward-CAGGAGGCATTGCTGATGAT and Reverse-GAAGGCTGGGGCTCATTT; ROCK1: Forward-CCTGTAACCCAAGGAGATGT and Reverse-CACAATTGGCAGGAAAGTG; miR-148a: Forward-AAAGTTCTGAGACACTCCG. U6 primers and miR-148a reverse primer were included in the kit. All Ct values were processed using $2^{-\Delta\Delta CT}$ method and PCR reactions were repeated 3 times.

Cell invasion and migration analysis

SHP-77 cells were harvested at 48 h post-transfection and cell invasion as well as migration abilities were analyzed by performing Transwell assays. To prepare single cell suspensions, non-serum RPMI-1640 Medium was mixed with SHP-77 cells in a ratio of 4×10^4 cells per 1 ml cell culture medium. After that, cell suspensions were injected into the upper Transwell chamber (0.1ml per well). In contrast, the lower chamber was filled with 80% RPMI-1640 medium and 20% FBS. Before invasion assays, Matrigel (200ug/ml, Millipore, USA) was used to coat the membranes for 8h at 37 °C to mimic cell invasion. The chambers were cultivated at 37 °C for 15 h. After that, membranes were collected at stained for 15min at room temperature with 0.1% crystal violet (Sigma-Aldrich, USA). Invasion and migrating cells were observed under a light microscope.

Western blot

SHP-77 cells were harvested at 48h post-transfection and the expression of ROCK1 was detected by performing western blot. RIPA solution (Sigma-Aldrich) was mixed with SHP-77 cells in a ratio of 10^5 cells per 1 ml to extract total proteins. BCA assay (Sigma-Aldrich) was then performed to quantify protein samples. Electrophoresis (12 % SDS-PAGE gel) was performed after the protein samples were denatured in boiling water for 15 min, followed by transferring the proteins to PVDF membranes. Blocking was performed in 5% fat-free milk at 24°C for 1h, following by incubation with primary antibodies including rabbit anti-GAPDH (1: 1600, ab22555, Abcam) and anti-ROCK1 (1: 1600, ab97592, Abcam), and the incubation was performed at 4°C for 12h. Following that, the membranes were further incubated with

secondary antibody of goat HRP (IgG) (1:2000; ab6721; Abcam) at 24°C for 1h. Following that, membranes were incubated with ECL Western Blotting Substrate (ab65623, Abcam) to develop signals, which were processed using Image J v1.46 software.

Statistical analysis

Mean values of 3 replicates were calculated and used to perform following statistical analyses. Pearson's correlation coefficient was for correlation analysis. The 60 patients were grouped into low and high MCM3AP-AS1 level groups with the median expression level of MCM3AP-AS1 in SCLC as cutoff value. Survival curves were plotted and compared by log-rank test. To explore differences between tissue types (non-tumor and SCLC) and among multiple cell groups, paired t test and ANOVA (on-way) and Tukey test were used. $p<0.05$ was statistically significant.

Results

Upregulated MCM3AP-AS1 in SCLC predicted poor survival

The differential expression of MCM3AP-AS1 in SCLC and non-tumor tissues was analyzed by performing qPCR and paired t test. The results revealed significantly higher expression levels of MCM3AP-AS1 in SCLC tissues comparing to non-tumor tissues (Fig.1A, $p<0.05$). Survival curve analysis showed that, compared to low level MCM3AP-AS1 group, patients in high level MCM3AP-AS1 group had significantly lower overall survival rate (Fig.1B).

MCM3AP-AS1 may bind miR-148a but MCM3AP-AS1 and miR-148a failed to affect the expression of each other

The interaction between MCM3AP-AS1 and miR-148a was predicted using IntaRNA (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp). It was observed that miR-148a can form base pairing with MCM3AP-AS1 (Fig.2A). SHP-77 cells were transfected with MCM3AP-AS1 expression vector or miR-148a mimic and the overexpression of MCM3AP-AS1 and miR-148a was confirmed by performing qPCR. Expression levels of MCM3AP-AS1 and miR-148a were significantly increased after transfection (Fig.2B, $p<0.05$). However, overexpression of MCM3AP-AS1 and miR-148a failed to affect the expression of each other (Fig.2C, $p<0.05$).

MCM3AP-AS1 was positively correlated with ROCK1 in SCLC

ROCK1 is a direct target of miR-148a. The differential expression of ROCK1 mRNA in SCLC was analyzed by performing qPCR and paired t test. The results revealed significantly higher expression levels of ROCK1 mRNA in SCLC tissues comparing to non-tumor tissues (Fig.3A, $p<0.05$). Correlations between MCM3AP-AS1 and ROCK1 mRNA across SCLC (Fig.3B) and non-tumor (Fig.3C) tissues were analyzed. Expression levels of MCM3AP-AS1 and ROCK1 mRNA were significantly and positively correlated in both SCLC (Fig.3B) and non-tumor (Fig.3C) tissues.

MCM3AP-AS1 upregulated ROCK1 through miR-148a

Comparing to C and NC (NC miRNA or empty pcDNA3 vector) groups, MCM3AP-AS1 overexpression mediated the upregulated ROCK1 mRNA (Fig.4A) and protein (Fig.4B) expression ($p<0.05$). However, miR-148a overexpressed led to downregulated ROCK1 expression ($p<0.05$). In addition, co-transfection (transfection of miR-148a mimic and MCM3AP-AS1 expression vector at the same time) experiment showed that miR-148a overexpressed led to reduced effects of MCM3AP-AS1 overexpression.

MCM3AP-AS1 promoted SCLC cell invasion and migration through ROCK1 and miR-148a

The effects of MCM3AP-AS1, miR-148a and ROCK1 overexpression on the invasion (Fig.5A) and migration (Fig.5B) of SHP-77 cells were analyzed by performing Transwell invasion and migration assays, respectively. Overexpression of ROCK1 was confirmed by western blot (Supplemental Fig.1, $p<0.05$). Comparing to C and NC (NC miRNA or empty pcDNA3 vector) groups, MCM3AP-AS1 and ROCK1 overexpression increased cell invasion and migration rates ($p<0.05$). However, co-transfection (transfection of miR-148a mimic and MCM3AP-AS1 expression vector at the same time) experiment showed that miR-148a overexpressed led to decreased cell invasion and migration rates, and reduced effects of MCM3AP-AS1 overexpression ($p<0.05$).

Discussion

The functions and expression pattern of MCM3AP-AS1 in SCLC were analyzed in this study. We found that MCM3AP-AS1 was upregulated in SCLC and predict the poor survival of SCLC patients. We also found that MCM3AP-AS1 may upregulate ROCK1 possibly by sponging miR-148a.

The functions of MCM3AP-AS1 have only been characterized in HCC and GBM. In HCC, MCM3AP-AS1 is upregulated and inhibits cancer growth by regulating miR-194-5p/FOXA1 axis [11]. In GBM, MCM3AP-AS1 is also upregulated and interacts with miR-211/KLF5/AGGF1 Axis to promote tumor angiogenesis [12]. In this study we first reported the upregulation of MCM3AP-AS1 in SCLC. Our in vitro cell experiments showed the increased invasion and migration after the overexpression of MCM3AP-AS1. Therefore, we proved MCM3AP-AS1 as an oncogenic lncRNA in SCLC. Our 5-year follow-up study is the first analysis of the prognostic values of MCM3AP-AS1 for cancer. We found that the high expression level of MCM3AP-AS1 was significantly correlated with the poor survival of SCLC patients. However, more clinical trials are needed to further verify the prognostic values, such as the reliability and accuracy.

The tumor suppressive roles of miR-148a have been investigated in many types of cancers, such as non-SCLC (NSCLC), which is another form of lung cancer [14, 15]. In NSCLC, miR-148a inhibits cancer metastasis and downregulated miR-148a in serum is considered as a screening marker of NSCLC [16]. In this study we proved the tumor suppressive role of miR-148a in the regulating of SCLC cell invasion and migration. It is known that miR-148a can directly target ROCK1 in gastric cancer [10]. In this study we observed the downregulated ROCK1 after miR-148a overexpression. Therefore, miR-148a may also target ROCK1 in SCLC. ROCK1 as an effector of the small GTPase RhoA is usually overexpressed in different

types of cancers and can increase cancer cell motility to promote tumor invasion and metastasis[17]. Consistently, we also showed the enhancing effects of ROCK1 on SCLC cell invasion and migration. Therefore, overexpression of miR-148a may serve as a potential target to inhibit tumor metastasis in SCLC.

MiRNAs mainly functions as gene expression regulators by cleaving mRNAs or inhibiting translation [18]. In this study we found miR-148a may bind MCM3AP-AS1, while overexpression experiments revealed no regulatory role of miR-148a on the expression of MCM3AP-AS1. It is known that lncRNAs can sponge miRNAs to attenuate their effects on downstream genes [19]. Combined with the observation of upregulated ROCK1 after MCM3AP-AS1 overexpression, we may speculate that MCM3AP-AS1 can sponge miR-148a to upregulate ROCK1.

Conclusions

In conclusion, MCM3AP-AS1 is upregulated in SCLC and may sponge miR-148a to upregulate ROCK1, thereby promoting cancer cell invasion and migration.

Abbreviations

HCC: hepatocellular carcinoma; SCLC: small cell lung cancer; lncRNA: long non-coding RNA; HCC: hepatocellular carcinoma; NC: Negative control.

Declarations

Authors' contributions

H.L, L.L.M and Y.K.Z designed the research; H.L and B.J collected the data; H.L and G.M.Q performed experiments and statistical modelling, H.L and Y.K.Z wrote the paper. All authors contributed to data interpretation and critically revised and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the ethics committee of the Yongchuan Hospital of Chongqing Medical University and informed consent was obtained from all participants of this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

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Figures

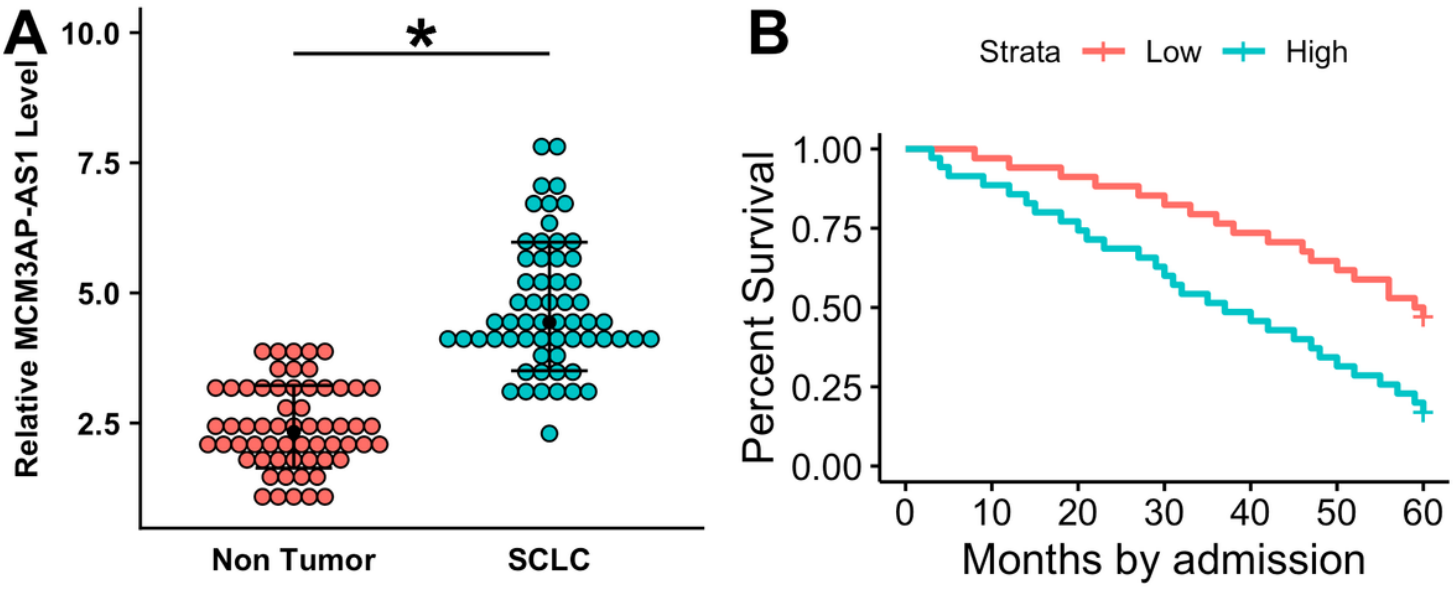

Figure 1

Upregulated MCM3AP-AS1 in SCLC predicted poor survival. The differential expression of MCM3AP-AS1 in SCLC and non-tumor tissues was analyzed by performing qPCR and paired t test (A). To perform survival analysis, the 60 patients were grouped into low and high MCM3AP-AS1 level groups with the median expression level of MCM3AP-AS1 in SCLC as cutoff value. K-M plotter and log-rank test were used to plot and compare survival curves (B). Mean values of 3 biological replicates were presented, *, p<0.05.

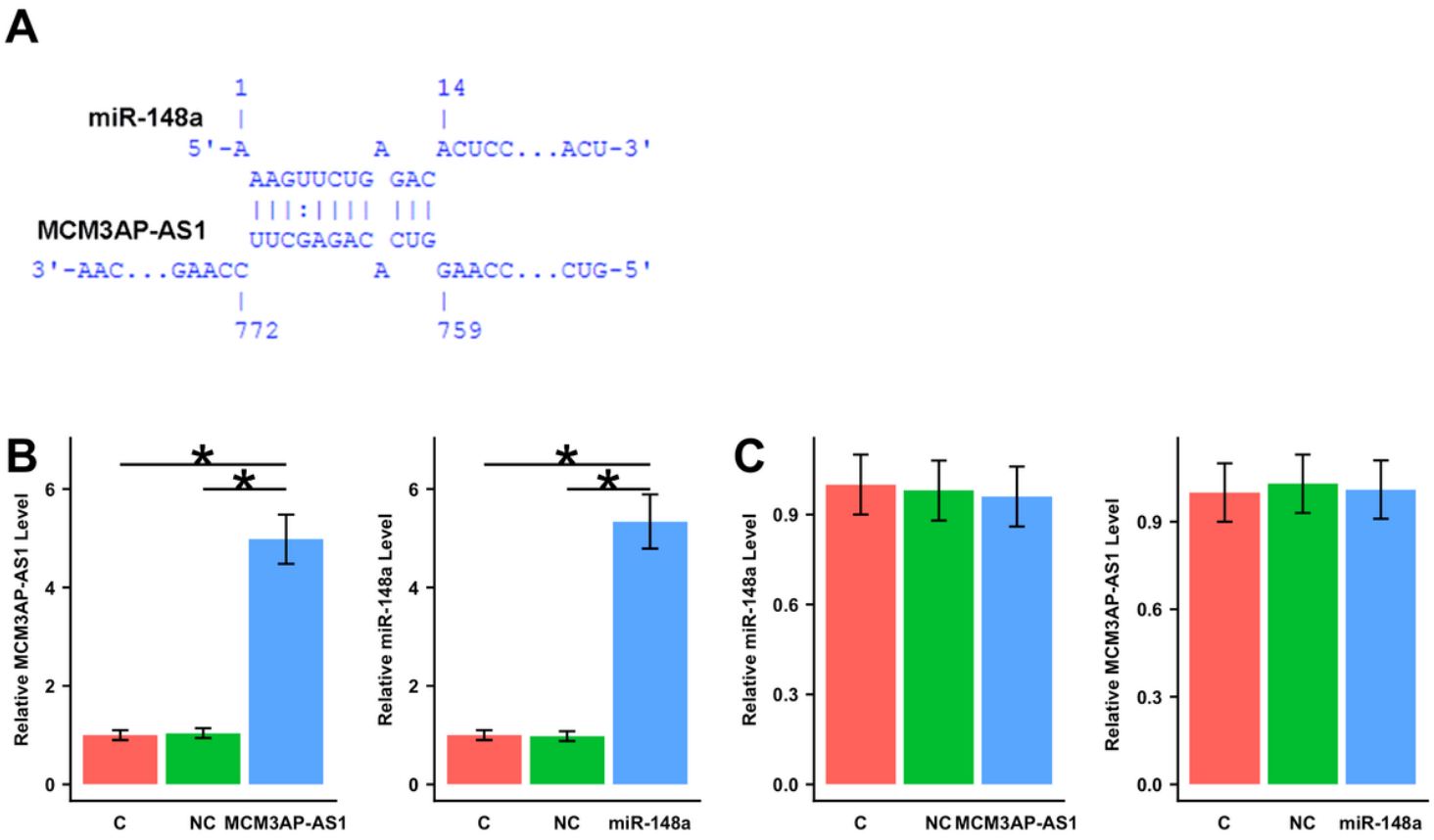

Figure 2

MCM3AP-AS1 may bind miR-148a but MCM3AP-AS1 and miR-148a failed to affect the expression of each other. The interaction between MCM3AP-AS1 and miR-148a was predicted using IntaRNA (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp). It was observed that miR-148a can form base pairing with MCM3AP-AS1 (A). To further analyze the interaction between MCM3AP-AS1 and miR-148a. SHP-77 cells were transfected with MCM3AP-AS1 expression vector or miR-148a mimic and the overexpression of MCM3AP-AS1 and miR-148a was confirmed by performing qPCR (B). The interaction between MCM3AP-AS1 and miR-148a was also analyzed by qPCR. Mean values of 3 biological replicates were presented, *, p<0.05.

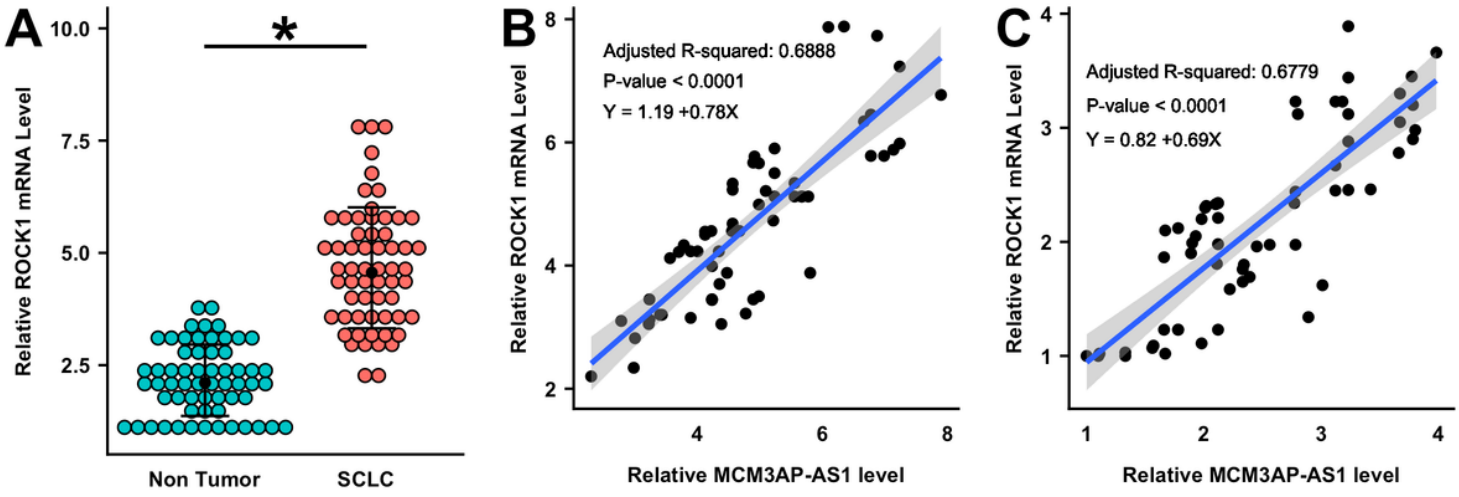

Figure 3

MCM3AP-AS1 was positively correlated with ROCK1 in SCLC. ROCK1 is a direct target of miR-148a. The differential expression of ROCK1 mRNA in SCLC and non-tumor tissues was analyzed by performing qPCR and paired t test (A). Correlations between MCM3AP-AS1 and ROCK1 mRNA across SCLC (B) and non-tumor (C) tissues were analyzed by Pearson correlation coefficient. Mean values of 3 biological replicates were presented, *, $p<0.05$. Full-length blots/gels are presented in Supplementary Figure 2.

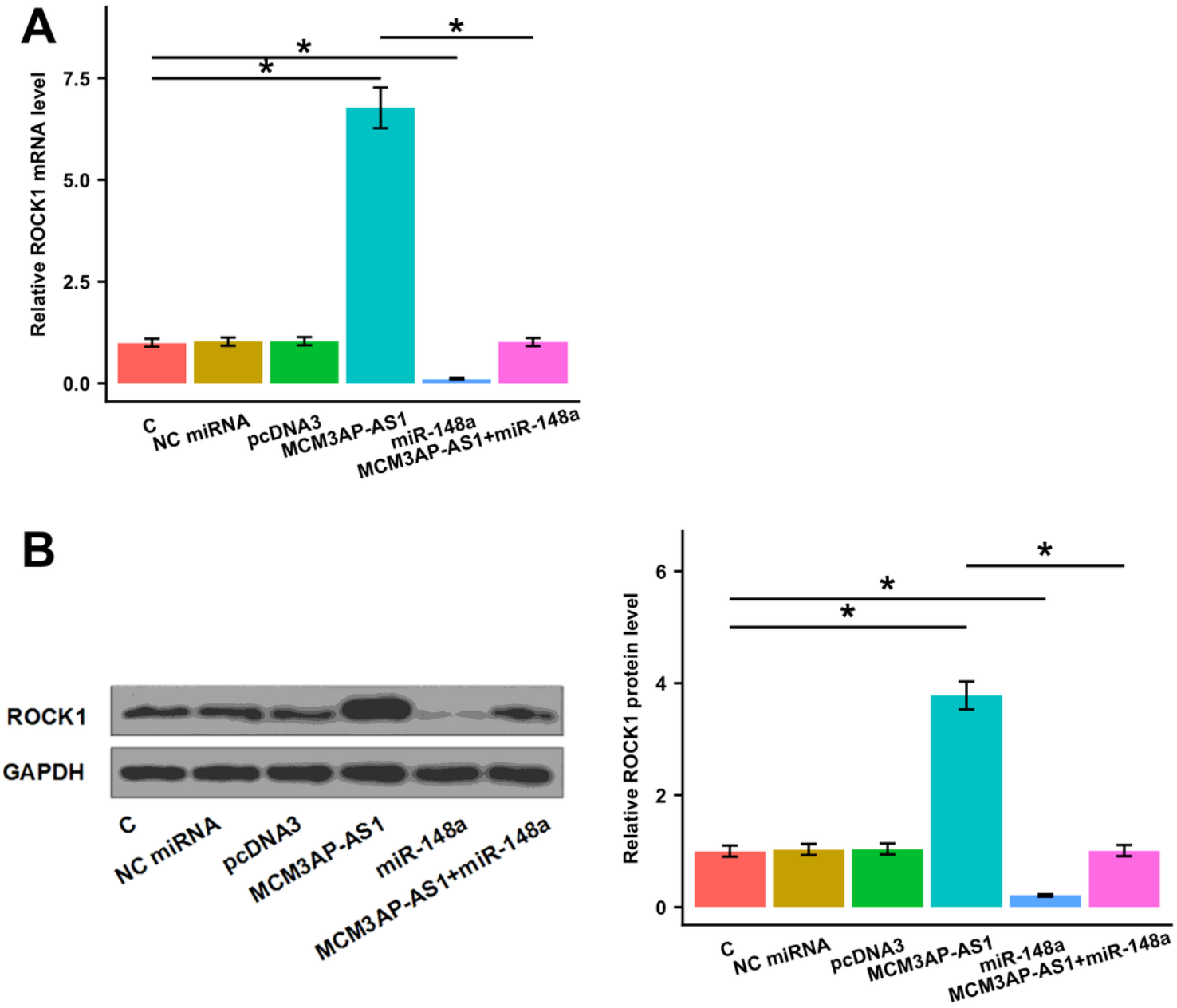

Figure 4

MCM3AP-AS1 upregulated ROCK1 through miR-148a. The effects of MCM3AP-AS1 and miR-148a overexpression on the expression of ROCK1 at mRNA (A) and protein (B) levels were analyzed by performing qPCR and western-blot, respectively. Mean values of 3 biological replicates were presented, *, $p<0.05$. Full of

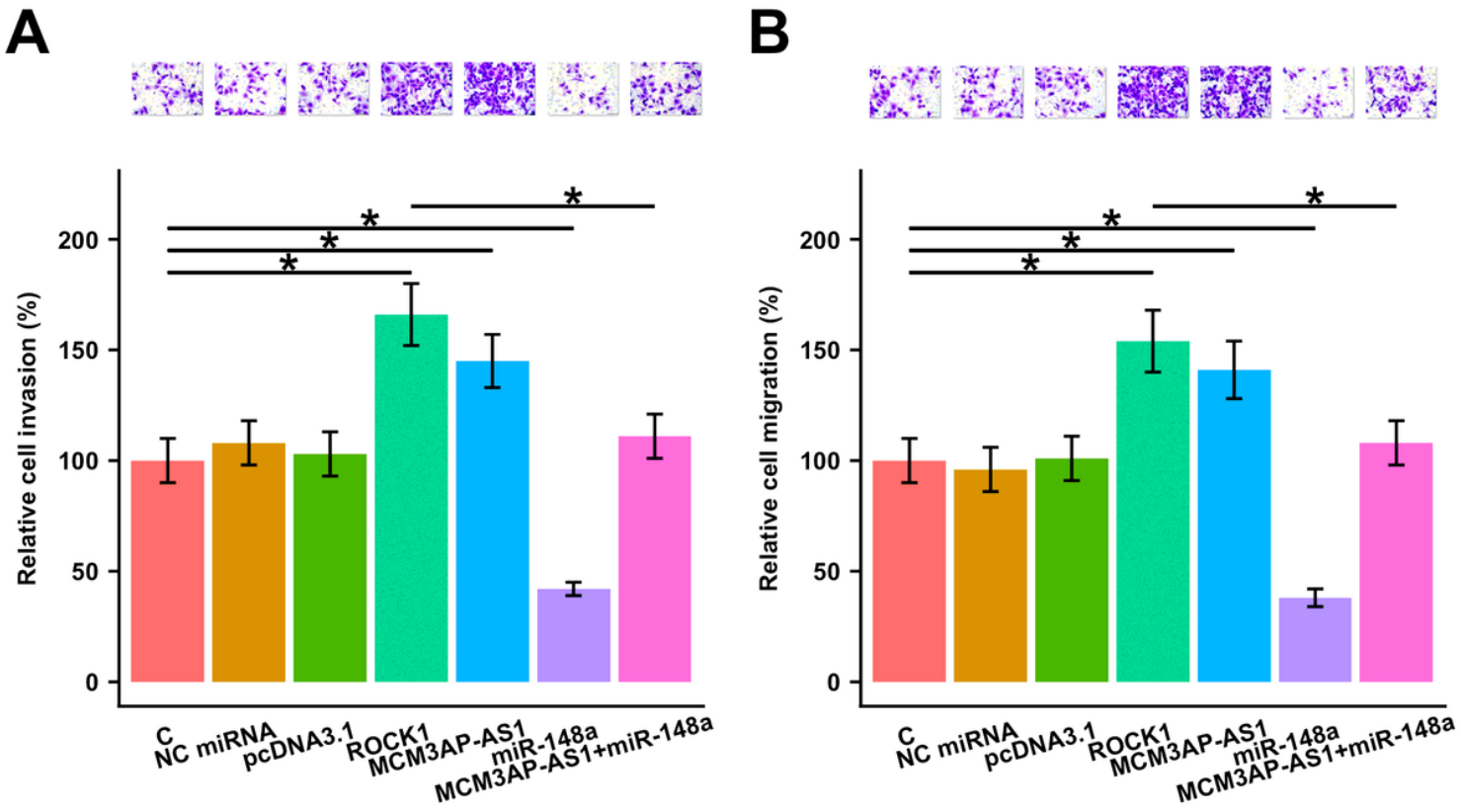

Figure 5

MCM3AP-AS1 promoted SCLC cell invasion and migration through ROCK1 and miR-148a. The effects of MCM3AP-AS1, miR-148a and ROCK1 overexpression on the invasion (A) and migration (B) of SHP-77 cells were analyzed by performing Transwell invasion and migration assays, respectively. Mean values of 3 biological replicates were presented, *, $p<0.05$.

Supplementary Files

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