



hsa-miR-195-5p inhibits cell proliferation of human thyroid carcinoma cells via modulation of p21/cyclin D1 axis

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Background: Based on existing evidence, microRNAs (miRs) are gene regulators that undertake key functions in the oncogenesis and tumor progression of every single human malignant disease, such as thyroid carcinoma (TC). Previous clinical findings showed that expression of miR-195 is down-regulated in TC, which implies that miR-195 may be practically involved in TC pathogenesis. Nevertheless, the function of hsa-miR-195-5p in TC is still largely unclear. Herein, we detected the conceivable involvement of hsa-miR-195-5p in TC cell proliferation.

Methods: Real time PCR examination was performed to assess the expression level of hsa-miR-195-5p in TC cell lines TPC-1 and B-CPAP. TPC-1 cells were transfected with either hsa-miR-195-5p mimics or hsa-miR-195-5p inhibitor. After confirmation of transfection efficiency, the effect of hsa-miR-195-5p on proliferation and cell cycle of TPC-1 cells was assessed. The expression of cyclin D1 and p21 was simultaneously detected by western blotting. Moreover, targetScan 6.2 was used to predict hsa-miR-195-5p target genes. Subsequently, luciferase reporter was performed to examine whether there is a possible binding of hsa-miR-195-5p to 3'-UTR of cyclin D1 mRNA. Furthermore, cyclin D1 mRNA and protein levels were measured to check whether hsa-miR-195-5p exerts its function at the post-transcriptional level. In addition, to explore the function of cyclin D1 in TPC-1 cells overexpressing hsa-miR-195-5p, cyclin D1 siRNA was used to silence the expression of cyclin D1 in TPC-1 cells overexpressing hsa-miR-195-5p.

Results: We quantified the expression of hsa-miR-195-5p in TC cells and normal thyroid cells and found a remarkable decrease in hsa-miR-195-5p expression in TC cells. Over-expression of hsa-miR-195-5p obviously resulted in downgraded proliferation of TC cells. Moreover, hsa-miR-195-5p caused cell arrest at the G0/G1 phase. Further *in silico* analyses and the dual-luciferase reporter assay confirmed that 3'-UTR of cyclin D1 is a direct target of hsa-miR-195-5p. Western blot analysis uncovered that hsa-miR-195-5p over-expression led to decreased levels of cyclin D1 and p21. In mechanistic analyses, we found that silencing of cyclin D1 reversed the inhibitory effect of hsa-miR-195-5p on the proliferation of TC cells, which indicates that hsa-miR-195-5p suppresses TC cell proliferation by adversely regulating cyclin D1.

Conclusions: We concluded that hsa-miR-195-5p is a candidate tumor-suppressor miRNA in TC and that the hsa-miR-195-5p/p21/cyclin D1 pathway could be a potential therapeutic target for TC.

Keywords: Cyclin D1; cell cycle; cell proliferation; hsa-miR-195-5p; thyroid carcinoma (TC)

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Introduction

Thyroid cancer (TC), one of the sporadic human carcinomas and representing about 5% of thyroid nodules, is the most widespread endocrine cancer around the world (1). The incidence rate of TC in developed countries and developing countries such as China has been undergoing a marked global upsurge in recent decades (2-5), making TC an emerging public health concern. Recent advances in TC treatment such as chemotherapy or surgery have improved the prognosis of TC (6). Notwithstanding, due to the lack of in-depth understanding of the molecular mechanism on the occurrence and development of the disease, patients with advanced TC still have few treatment alternatives. Therefore, it is of far-reaching clinical significance to explore novel molecular targets for TC therapy.

A growing body of evidence suggested that microRNAs (miRNAs) are able to control the expression of genes related to proliferation, invasion and migration of cancer cells by targeting mRNA 3'UTRs, so as to prevent or accelerate the progress of tumor (7-15). Previous medical and basic research data suggested that miR-195 plays an important role in cancer (16-26). The expression of miR-195-5p was previously reported to be elevated in several diseases, such as deep vein thrombosis (27), while in other diseases, including collapse of steroid-induced osteonecrosis of the femoral head, non-small cell lung cancer and cervical carcinoma, decreased expression of miR-195-5p was confirmed (28-30). In particular, it has been recently reported that miR-195 expression in TC tissue is decreased, and its ectopic high expression has been proved to significantly inhibit the expression of raf1, thus inhibiting the proliferation of TC cells (31). More recent studies showed that miR-195 acts as a tumor suppressor in TC by inhibiting tumor growth and metastasis, which is probably related to regulation of Wnt/ β -catenin signaling pathway, VEGF-A and p53 in mechanism (32,33). Moreover, when the function of miR-195 is interfered by other factors, the malignancy of TC cells is remarkably enhanced (34). All things considered, the link between TC and the expression of hsa-miR-195-5p is still largely obscure and poorly understood, especially in the aspect of what concerns its inhibitory effect on TC cell proliferation and the underlying molecular mechanism at cell cycle levels.

Along these lines, the aim of this study was to investigate the function of hsa-miR-195-5p in regulation of TC cell proliferation and the underlying molecular mechanism.

Methods

Cell culture

The human TC cell lines TPC-1 (RRID:CVCL_6298) as well as normal thyroid cell line HT-ori3 (RRID:CVCL_4W02) used in the present study were provided by American Type Culture Collection (Manassas, VA, USA). B-CPAP (DSMZ Cat# ACC-273, RRID:CVCL_0153) were purchased from University of Colorado Cancer Center Cell Bank. Cells were cultured at 37 °C in a 5% CO₂ and 95% humidity incubator using DMEM medium (Gibco, USA) complemented with 10% FBS (Sigma, USA).

Transient transfection

hsa-miR-195-5p mimic, hsa-miR-195-5p inhibitor and the respective negative control oligonucleotides were purchased from GeneCopoeia (Guangzhou, China). The cyclin D1 siRNA (h): sc-29286 was obtained from Santa Cruz Biotechnology, Inc and used following the vendor's recommended protocol. Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) was employed in line with the manufacturer's instructions.

RNA extraction and real-time quantitative PCR

The extraction of total RNA from cells was performed with mirVana miRNA Isolation Kit (Ambion) following the protocol described in the manufacturer provided manual. To determine the hsa-miR-195-5p expression levels, real time qRT-PCR experiments were carried out using the gene-specific TaqMan miRNA Assay Probes (Applied Biosystems, Foster City, CA, USA). The real-time PCR reaction was performed in triplicate. The relative hsa-miR-195-5p expression was assessed by normalization to the small nuclear RNA U6 using the 2^{- $\Delta\Delta C_t$} method.

Western blotting

Cells were lysed using a lysis buffer (Beyotime, Jiangsu, China) and proteins were separated by SDS-PAGE. Next, purified proteins were transferred onto nitrocellulose membranes followed by blocking in TBST containing 5% non-fat milk for 2 hours. After an overnight incubation with primary antibodies against cyclin D1 (Abcam Cat# AB190194, RRID:AB_2728784), p21 (Abcam Cat# ab188224, RRID:AB_2734729) or GAPDH (Abcam

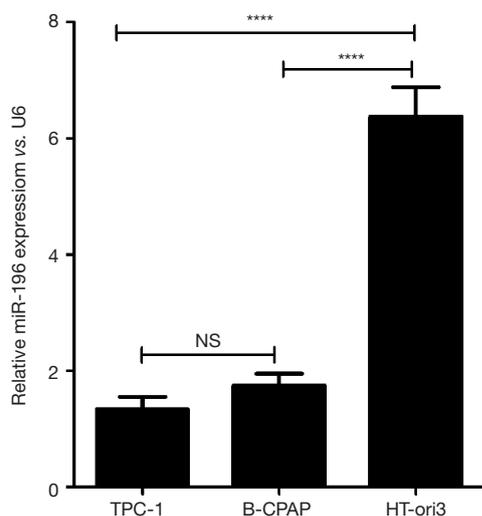


Figure 1 Expression of hsa-miR-195-5p in human thyroid carcinoma cell lines. Relative hsa-miR-195-5p mRNA expression levels in TC cell lines TPC-1 and B-CPAP and normal thyroid cell line HT-ori3 were determined by real time qRT-PCR. Experiments were repeated at least three times. Each bar represents the mean of three independent experiments. ****, $P < 0.0001$; NS, non-significant in intergroup comparisons.

Cat# ab9485, RRID:AB_307275), membranes were incubated with anti-rabbit HRP-conjugated secondary antibody (Millipore Cat# 12-348, RRID:AB_390191). Next, membranes were washed with TBST and proteins were revealed by chemiluminescence. Image J was used for densitometry quantification analysis.

MTT assay

The MTT cell proliferation assay was performed using the TPC-1 cells. Briefly, TPC-1 cells (2,000 cells/well) were seeded into 96-well microplates in medium containing 10% FBS for 72 hours. Afterward, cells were stained with 100 μ L MTT reagent (0.5 mg/mL, Sigma, St Louis, MO, USA) and incubated for another 4 h at 37 °C. After discarding the culture medium and replacing 150 μ L DMSO as its substitution, the absorbance at 490 nm was assessed in a Thermo Scientific Multiskan (Thermo Fisher Scientific, USA).

Luciferase assays

Approximately 5×10^4 cells/well were cultured in 24-well microplates for 24 hours and then cells were co-transfected

with hsa-miR-195-5p mimic or miR-NC and luciferase reporter vector containing the mutated cyclin D1 3'-UTR (cyclin D1-3'-UTR-mut) or the wild type cyclin D1 3'-UTR (WT 3'-UTR) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The luciferase activity was measured after additional 48 hours incubation using the dual luciferase assay kit in line with the manufacturer's protocol (Promega, Wisconsin, WI, USA).

Statistical analysis

The statistical analysis was done using One-way or two-way ANOVA with bonferroni post test in GraphPad Prism Software for Windows, version 6.00, San Diego California USA (www.graphpad.com). $P < 0.05$ was the statistically significant cutoff P value.

Results

hsa-miR-195-5p expression was down-regulated in TC cell lines

The expression level of hsa-miR-195-5p in TC cell lines TPC-1 and B-CPAP was assessed by real time PCR examination. The results (*Figure 1*) revealed that the relative expression of hsa-miR-195-5p was significantly down-regulated in both TPC-1 (1.34 ± 0.21) and B-CPAP (1.75 ± 0.20) cells compared with the normal thyroid cell line HT-ori3 (6.38 ± 0.50). These results reveal that hsa-miR-195-5p is abnormally down-regulated in human TC cell lines, indicating it could be involved in TC pathogenesis.

hsa-miR-195-5p inhibited TC cell proliferation

To evaluate whether hsa-miR-195-5p is involved in the proliferation phenotype of TC cells, TPC-1 cells were transfected with either hsa-miR-195-5p mimics or hsa-miR-195-5p inhibitor. The relative expression of hsa-miR-195-5p in the transfected cells was detected by qRT-PCR. The results displayed that transfection of hsa-miR-195-5p mimic reestablished its expression, while hsa-miR-195-5p inhibitor efficiently suppressed this expression (*Figure 2A*). After having confirmed the transfection efficiency, we assessed the effects of hsa-miR-195-5p on the proliferation of TPC-1 cells. Transfection with hsa-miR-195-5p mimics resulted in obvious inhibition of cell proliferation (*Figure 2B*). On the contrary, inhibition of hsa-miR-195-5p expression in TPC-1 cells could significantly increase cell proliferation

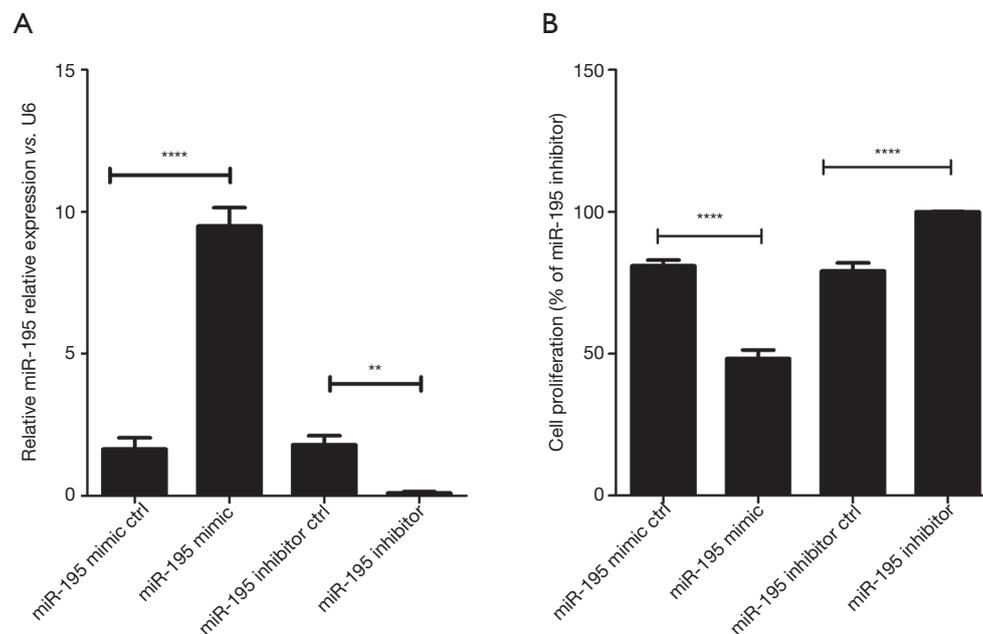


Figure 2 Exogenous over-expression of hsa-miR-195-5p inhibited TC cell proliferation. (A) Verification of hsa-miR-195-5p expression levels by PCR analysis after transfection. (B) MTT assays revealed that over-expression of hsa-miR-195-5p inhibited growth of TPC-1 cells while inhibition of hsa-miR-195-5p promoted growth of TPC-1 cells. Each bar represents the mean of three independent experiments. **, $P < 0.05$, ****, $P < 0.0001$ in intergroup comparisons.

(Figure 2B). These results indicate an inhibitory effect of hsa-miR-195-5p on TC cell proliferation.

hsa-miR-195-5p over-expression induced cell cycle arrest at G0/G1 phase

To deeply explore the mechanism underlying the anti-proliferative effect of hsa-miR-195-5p on TC cell, flow cytometry for cell cycle analysis was performed. The results (Figure 3A) showed that over-expression of hsa-miR-195-5p significantly increased the frequency of TPC-1 cells at the G0/G1 phase, which implied that hsa-miR-195-5p over-expression induced cell cycle arrest at the G0/G1 phase.

To confirm whether hsa-miR-195-5p could regulate the expression of cell cycle related markers at the molecular level, the levels of cyclin D1, a cell cycle related regulatory proteins and p21, a cell cycle inhibitor were simultaneously detected by western blotting. As expected, results indicated that over-expression of hsa-miR-195-5p markedly hindered the expression of cyclin D1 and up-regulated p21 while its inhibition triggered the opposite effects (Figure 3B). This observation supported the inhibitory effect of hsa-miR-195-5p on TC cell proliferation and suggested that hsa-miR-

195-5p may induce cell cycle arrest at the G0/G1 phase.

Cyclin D1 is a direct target of hsa-miR-195-5p

In order to understand the molecular mechanism on cell cycle and cell proliferation regulation by hsa-miR-195-5p, we used TargetScan 6.2 to predict target genes of hsa-miR-195-5p. The results revealed that cyclin D1 was a potential target of hsa-miR-195-5p (Figure 4A). Subsequently, luciferase reporter assay was performed to examine whether there is a possible binding of hsa-miR-195-5p to 3'-UTR of cyclin D1 mRNA. As shown in Figure 4B, co-transfection of the reporter plasmid harboring the wild type 3'-UTR of cyclin D1 mRNA with hsa-miR-195-5p mimics resulted in a remarkable inhibition of luciferase activity contrary to the activity measured in cells co-transfected with cyclin D1 3'-UTR-Mut and hsa-miR-195-5p mimics. These findings imply that hsa-miR-195-5p negatively regulates cyclin D1 by directly binding to its 3'-UTR in TC cells.

In addition, to check whether hsa-miR-195-5p exerts its function at the post-transcriptional level, cyclin D1 mRNA and protein levels were measured. The results showed that over-expression of hsa-miR-195-5p did not affect the cyclin

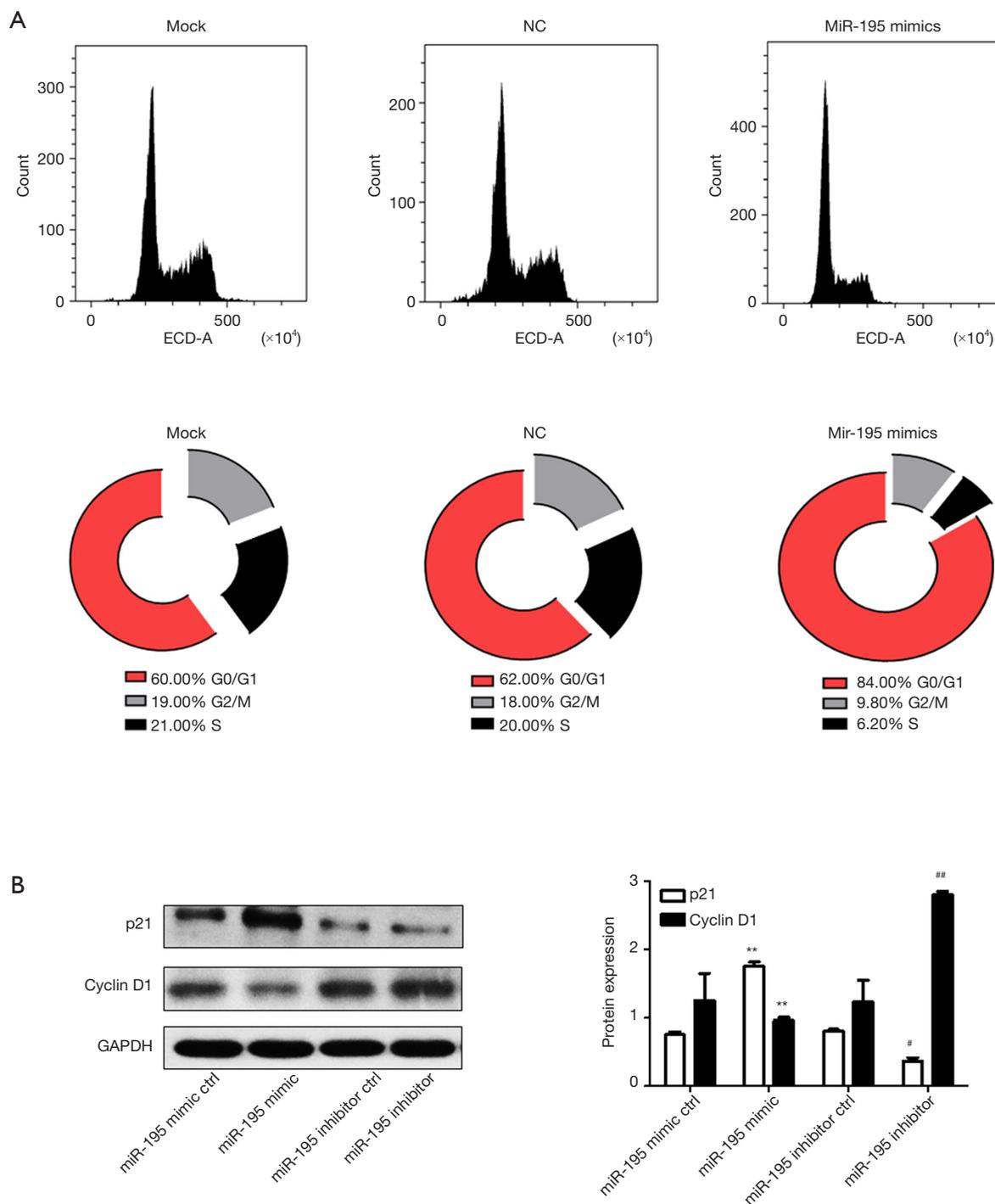


Figure 3 Hsa-miR-195-5p induces cell cycle arrest at G0/G1 phase by inhibition of cyclin D1 expression and upregulation of p21 expression in TPC-1 cells. (A) Flow cytometry analysis of cell cycle and quantitative representation. (B) Effects of hsa-miR-195-5p mimics on the endogenous cyclin D1 and p21 protein levels. TPC-1 cells were co-transfected with hsa-miR-195-5p mimics or negative control oligonucleotides. The expression of cyclin D1 and p21 was in cells was evaluated by Western blotting. GAPDH was used as loading control. The data were subjected to two-way ANOVA analysis. **, $P < 0.01$ compared with mimic control, #, $P < 0.05$, ##, $P < 0.01$ compared with inhibitor control; NC, negative control oligonucleotides.

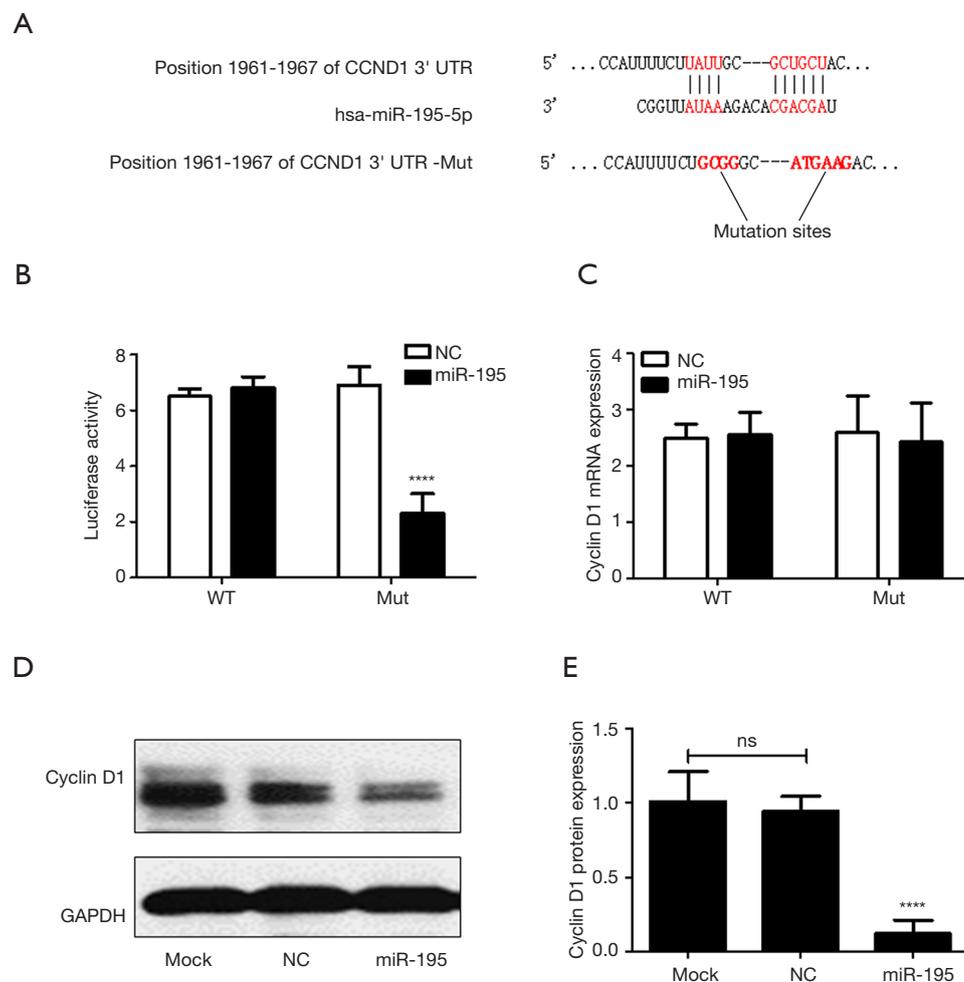


Figure 4 hsa-miR-195-5p suppresses cyclin D1 expression by directly targeting the cyclin D1 3'-UTR in TPC-1 cells. (A) TargetScan prediction of hsa-miR-195-5p targeting of cyclin D1 and Mutagenesis of cyclin D1 3'-UTR. (B) Luciferase reporter assay. (C) Effects of hsa-miR-195-5p mimics on the endogenous cyclin D1 mRNA levels. TPC-1 cells were cotransfected with hsa-miR-195-5p mimics or negative control oligonucleotides. Forty-eight hours after transfection, cells were isolated, the expression of cyclin D1 was analyzed by qRT-PCR. (D,E) Effects of hsa-miR-195-5p on the endogenous cyclin D1 protein levels. Forty-eight hours after transfection with the hsa-miR-195-5p mimics or negative control oligonucleotides, proteins were isolated from TPC-1 cells and analyzed by Western blotting, with GAPDH as an internal control. ****, $P < 0.0001$ compared with respective controls. NC, negative control oligonucleotides.

D1 mRNA levels (Figure 4C). Nevertheless, the protein level of cyclin D1 was significantly decreased in cells over-expressing hsa-miR-195-5p (Figure 4D,E). We inferred that hsa-miR-195-5p regulates cyclin D1 expression post-transcriptionally.

Silencing of cyclin D1 reversed the inhibitory effects of hsa-miR-195-5p on TC cell proliferation

In order to investigate the role of cyclin D1 in the TPC-

1 cells over-expressing hsa-miR-195-5p, we transfected the TPC-1 cells harboring hsa-miR-195-5p mimics with cyclin D1 siRNA to silence the expression of cyclin D1. Western blot analysis confirmed the efficiency of silencing (Figure 5A). In MTT assay, we found that silencing of cyclin D1 in hsa-miR-195-5p mimics-transfected TPC-1 cells reversed the anti-proliferative effect of hsa-miR-195-5p (Figure 5B). Further analysis confirmed that after silencing of cyclin D1, the cell cycle arrest caused by hsa-miR-195-5p was equally reversed (Figure 5C). These data support the

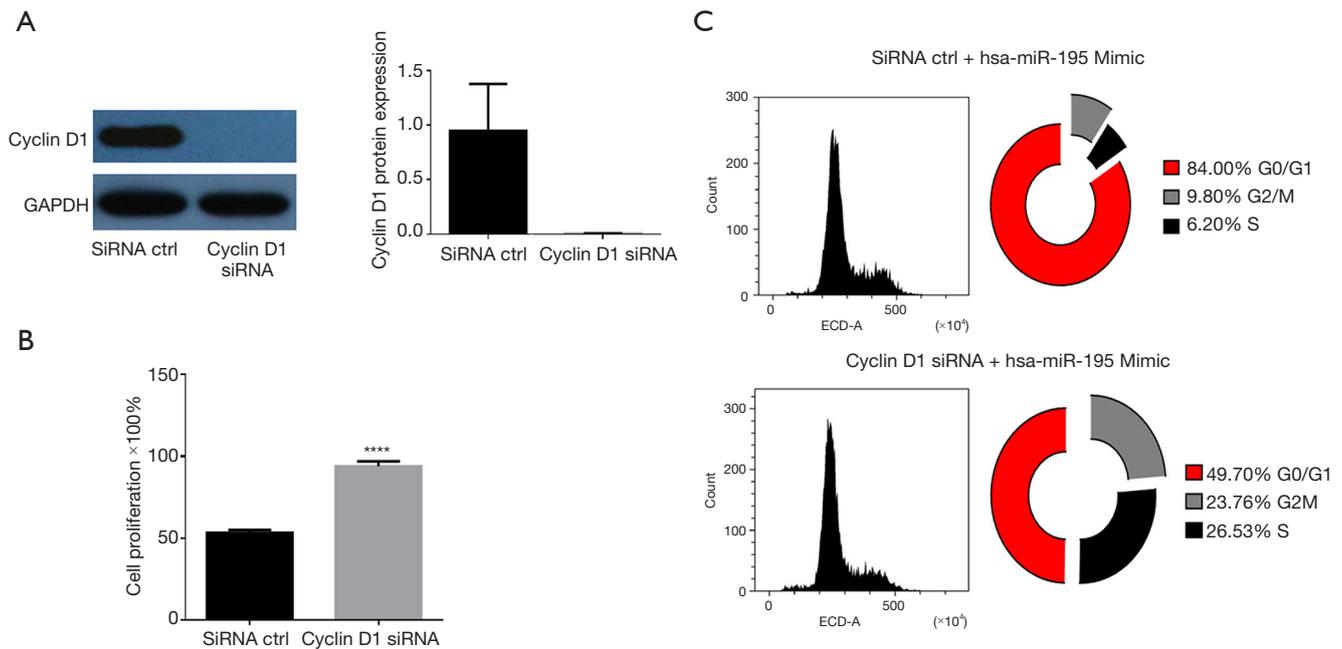


Figure 5 Cyclin D1 up-regulation is required for hsa-miR-195-5p inhibitor induced proliferation of TC cells. (A) Western blot analysis verified that silencing cyclin D1 effectively inhibited the expression of cyclin D1 TPC-1 cells. (B) Increased cell proliferation in hsa-miR-195-5p mimics-transfected TPC-1 cells. (C) Hsa-miR-195-5p induced cell cycle arrest is mediated through down-regulation of cyclin D1.

idea that hsa-miR-195-5p inhibited TC cell proliferation by prompting cell cycle arrest at the G0/G1 phase through interference of cyclin D1 expression.

Discussion

MicroRNAs (miRNAs) are a group of novel gene regulatory factors, which play an important role in the process of tumor cells growth. Clinical evidence has shown that miRNAs are abnormally expressed in a variety of human tumors (35).

Latest studies indicated that miR-195 expression is considerably decreased in breast cancer and miR-195 acts as a tumor suppressor (36). It was also reported that the expression of miR-195 in adrenocortical carcinoma is significantly down-regulated, and the down-regulated level is positively correlated with poor prognosis (37). Comparable reports linked the down-regulation of miR-195 in colorectal cancer with lymph node metastasis (38). Moreover, studies have demonstrated the involvement of miR-195 in acquired temozolomide resistance in glioblastoma multiform cells (39). Nonetheless, the significance of hsa-miR-195-5p in TC pathogenesis and its plausible application as a therapeutic target for this disorder

has yet to be assessed.

In the present study, we measured the expression level of hsa-miR-195-5p in TC cell lines and evaluated its regulatory role on cell proliferation. Our findings showed that hsa-miR-195-5p expression was markedly decreased in TC cell lines compared with normal cells. This result also confirms the previous research results (36-39). Moreover, we discovered that over-expression of hsa-miR-195-5p markedly blocked TC cell proliferation by promoting cell cycle arrest at the G0/G1 phase, which was further confirmed by its regulatory effects on cyclin D1 and p21 protein. This result is consistent with that of Furuta *et al.* (40) who demonstrated that the molecular pathways regulating cell cycle progression are characteristically altered by decreased expression of miR-195, thus instigating abnormal cell proliferation in hepatocarcinogenesis.

In further experiments, we found that hsa-miR-195-5p inhibited TC cell proliferation via negative regulation of cyclin D1 in post-translational level. We also found that hsa-miR-195-5p over-expression was accompanied with increased expression of p21. Therefore, we speculated that hsa-miR-195-5p might inhibit TC cell proliferation by up-regulating p21 and down-regulating cyclin D1. The down-regulation of cyclin D1 was probably mediated by

direct binding of hsa-miR-195-5p to its 3'-UTR or by the up-regulated p21 caused by hsa-miR-195-5p through an unknown mechanism. Accordingly, we found that hsa-miR-195-5p might regulate TC cell proliferation via modulation of the p21/cyclin D1-pathway. The present observation was consistent with the views of Luo *et al.* (41) indicating the implications of cyclin D1/p21 pathway in the induction of cell cycle arrest and subsequent inhibition of cell proliferation. The current findings were also similar to those of former works considering that cyclins was targets for miR-195, which trigger cell cycle arrest at the G1/S phase (42-44).

Cyclin D1, a critical protein involved in cell cycle regulation, is up-regulated in most human tumors including TC (45,46). It has also been reported that cyclin D1 plays a key role in the formation of activated CDK4/CDK6 complex, which in turn, initiates phosphorylation of pRb (47,48). Consistent with these reports, our data show that cyclin D1 was down-regulated in TPC-1 cells over-expressing hsa-miR-195-5p mimic. Furthermore, silencing of cyclin D1 in TPC-1 cells transfected with hsa-miR-195-5p mimics reversed the negative effect of hsa-miR-195-5p on cell proliferation, which implies that hsa-miR-195-5p inhibited TC cell proliferation, partly at least, by direct targeting of cyclin D1.

Conclusions

Conclusively, our present study indicated that hsa-miR-195-5p is a candidate tumor-suppressor miRNA in TC and that the hsa-miR-195-5p/p21/cyclin D1 pathway could be a potential therapeutic target for TC.

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Footnote

Data Sharing Statement: Available at <http://dx.doi.org/10.21037/tcr-20-1083>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr-20-1083>). DL reports grants from

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Ethical Statement: 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.

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