MicroRNA-150 suppresses p27^{Kip1} expression and promotes cell proliferation in HeLa human cervical cancer cells

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Abstract. MicroRNAs (miRNAs) exert critical roles in the majority of biological and pathological processes. Recent studies have associated miR-150 with a number of different cancer types. However, little is known about miR-150 targets in cervical cancer. In the present study, the HeLa human cervical cancer cell line was transfected with hsa-miR-150-5p mimics, hsa-miR-150-5p inhibitors or miRNA controls. miR-150 was predicted to bind the 3'untranslated region (3'UTR) of the *CDKN1B* gene, which encodes the cyclin-dependent kinase inhibitor 1B (p27^{Kip1}). The direct binding between miR-150 and the 3'UTR of *CDKN1B* was confirmed using dual-luciferase reporter assays. The effects of miR-150 on *CDKN1B* mRNA expression, p27^{Kip1} protein expression, cell cycle and cell proliferation were determined using reverse-transcription quantitative PCR, western blot analysis, flow cytometry and WST-8 assays, respectively. miR-150 was demonstrated to directly target the 3'UTR of *CDKN1B* in transfected HeLa cells. The expression of *CDKN1B* mRNA and p27^{Kip1} protein was reduced by miR-150 mimics, and increased by miR-150 inhibitors. Moreover, the overexpression of miR-150 promoted cell cycle progression from the G0/G1 to the S phase and led to a significant increase in HeLa cell proliferation. The results of the present study indicated that miR-150 promotes HeLa cell cycle progression and proliferation via the suppression of p27^{Kip1} expression.

Introduction

Cervical cancer is a frequent diagnosis in women worldwide; however, its incidence is decreasing in developed countries thanks to early diagnosis (1,2). Although comprehensive cervical cancer genomic profiles have been reported (3), the underlying genomic mechanisms remain poorly understood. MicroRNAs (miRNAs) represent a family of abundant endogenous, noncoding RNA molecules, ~22 nucleotides in length (4,5), which post-transcriptionally repress the expression of target genes by typically binding the 3'untranslated regions (3'UTRs) of messenger RNA (mRNA) (6,7). It is becoming increasingly evident that now miRNAs play a role in virtually every pathophysiological process (8,9). One particular miRNA, namely miR-150, reportedly influences the progression of many cancer types (10-17). Notably, the expression of miR-150 in cervical carcinoma is significantly higher than that in normal cervical tissue from healthy donors, and its expression in cervical tissue correlates with cancer stage progression (18). Moreover, the overexpression of miR-150 promotes proliferation in cervical cancer by targeting *FOXO4*, *P2RX7*, and *SRCIN1* (18-20). FOXO4 regulates many cellular pathways, including oxidative stress signaling, longevity, insulin signaling, cell cycle progression, and apoptosis (21). In addition, FOXO4 regulates the expression of cyclin D1, p27, BIM, and FASL (21). The activation of the P2X7 receptor mediates apoptosis via the caspase-9 mitochondrial pathway (22). SRCIN1 is another tumor suppressor that plays a vital role in tumor cells (23). Furthermore, microRNA-150 promotes cell proliferation, migration, and invasion of cervical cancer by targeting *PDCD4* (24). However, the exact identities of the molecular targets of miR-150 in cervical cancer remain uncertain.

We predicted that miR-150 binds the 3'UTR of *CDKN1B* using the TargetScan database (http://www.targetscan.org/). The *CDKN1B* gene encodes the cyclin-dependent kinase inhibitor 1B (p27^{Kip1}), which integrates extracellular signals that negatively regulate the cell cycle progression at the G1 stage (25,26). In this study, we aimed to investigate the interaction between miR-150 and CDKN1B, via dual-luciferase reporter assay in the HeLa human cervical cancer cell line. We also carried out oligonucleotide transfection and western blot analysis to examine the effect of miR-150 on HeLa cell proliferation.

Materials and methods

Cell culture. The HeLa human cervical cancer cell line was obtained from the RIKEN Cell Bank (Tsukuba, Japan) and

cultured in complete Dulbecco's Modified Eagle's medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% antibiotic/antimycotic solution (Thermo Fisher Scientific, Inc.) at 37°C in a humidified 5% CO_2 incubator.

Dual-luciferase reporter assay. To explore the mechanism through which miR-150 promotes tumor progression, we used TargetScan as a miRNA target prediction algorithm. Based on the frequencies of miR-150 sites in the 3'UTRs of mRNAs, more than 100 mRNAs were predicted to be regulated by miR-150. In this study, we focused on *CDKN1*, which encodes a cell cycle regulator. As shown in Fig. 1, this analysis revealed putative 8- and 7-mer binding sites for miR-150 in the 3'UTR of the *CDKN1B* transcript. The restriction sites in the psiCHECK-2 vector multiple cloning region are *Sgf*I, *Xho*I, *Pme*I, and *Not*I. Therefore, two pairs of wild-type and mutant 3'UTR sequences of *CDKN1B* with *Xho*I and *Not*I restriction enzyme digestion sites were chemically synthesized (Table I). The oligo DNAs of the paired sequences were annealed and digested by *Xho*I and *Not*I restriction enzymes. These synthesized sequences were integrated into the psiCHECK-2 vector (Promega Corporation), and the recombinant plasmids were transformed into One Shot chemically competent *E. coli* (Thermo Fisher Scientific, Inc.) and purified according to the manufacturer's instructions. The recombinant plasmids were named WT1, MUT1, WT2, and MUT2 (Table I). HeLa cells were co-transfected with the recombinant plasmids and 100 nM miR-150 mimics or miRNA control for 48 h using Lipofectamine 3000 Transfection reagent (Thermo Fisher Scientific, Inc.). After transfection, both firefly and *Renilla* luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega Corporation). Luciferase activities were normalized to *Renilla* luciferase. This analysis was performed in triplicate.

Oligonucleotide transfection. HeLa cells were transfected with 100 nM hsa-miR-150-5p mimics (miR-150 mimics), 100 nM hsa-miR-150-5p inhibitors (miR-150 inhibitors), or 100 nM miRNA control (Bioneer) for 72 h using Lipofectamine RNAiMAX Transfection reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions.

Reverse-transcription quantitative (RT-q) PCR. The expression level of miR-150 and *CDKN1B* mRNA was quantified by real-time PCR. Total RNA from transfected HeLa cells was extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity and quality of total genomic RNA were evaluated using the NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Inc.). For miRNA analysis, TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) was used according to the manufacturer's protocol. The program was the following: 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. Quantitative PCR (qPCR) on miRNA was performed using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Inc.) and TaqMan miRNA assays (Thermo Fisher Scientific, Inc.) for hsa-miR-150-5p (Assay ID, 000473) and RNU6B (Assay ID, 001093) in accordance with the manufacturer's instructions. RNU6B was used as a reference gene. The cycling conditions were as follows; 95°C for 20 sec, followed

by 40 cycles at 95°C for 1 sec and 60°C for 20 sec. For mRNA analysis, the extracted total RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Real-time PCR was performed using the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, starting with 100 ng of total RNA. The cycling conditions were as follows; 95°C for 20 sec, followed by 40 cycles at 95°C for 3 sec and 60°C for 30 sec. Primer sequences are shown in Table II. *GAPDH* was quantified as a reference gene. All real-time PCR experiments were conducted with a StepOnePlus instrument (Thermo Fisher Scientific, Inc.). The average cycle threshold value for the housekeeping gene was used to normalize the raw cycle threshold data and calculate ΔCq. The $2^{-\Delta\Delta Cq}$ method was used to determine the relative expression levels of miR-150 and *CDKN1B*. All experiment was performed in triplicate.

Western blotting. Transfected HeLa cells were homogenized in cold EzRIPA Lysis buffer (ATTO). A 100-μg protein extract was separated by SDS-PAGE using a 10% polyacrylamide gel, and the proteins were transferred onto polyvinylidene difluoride membranes (Merck Millipore). After blocking with 5% non-fat dry milk at room temperature for 1 h, the membranes were incubated with primary antibodies against p27^{Kip1} (1:1,000; cat. no. 3686; Cell Signaling Technology, Inc.) and β-actin (1:1,000; cat. no. 8457; Cell Signaling Technology, Inc.) overnight at 4°C. Subsequently, the membranes were washed and exposed to goat anti-rabbit-IgG HRP (1:1,000; cat. no. 7074, Cell Signaling Technology, Inc.) for 1 h at room temperature, which was followed by imaging with a chemiluminescent substrate. This assay was performed in triplicate.

Cell cycle analysis. To analyze the cell cycle, transfected cells were collected, washed with phosphate-buffered saline (PBS), and fixed in cold 70% ethanol at 4°C overnight. Fixed cells were washed with PBS and incubated with 250 μg/ml RNase A and 50 μg/ml propidium iodide for 30 min. The DNA content was determined using a Cell Lab Quanta SC flow cytometer (Beckman Coulter) and the cell cycle profile was determined with ModFit LT, which is the most comprehensive flow cytometric DNA cell cycle analysis software available (Verity Software House). This assay was performed in triplicate.

Cell proliferation assay. Cell proliferation of transfected HeLa cells was determined using the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8) assay with the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories) every 24 h according to the manufacturer's instructions. CCK-8 solutions were added to the cell culture, and cells were incubated at 37°C for 2 h. Later, the absorbance was measured at 450 nm. This assay was performed in triplicate.

Statistical analysis. Results are expressed as the mean ± standard deviation. Statistical significance for the experiments was determined using Dunn's test, a Mann-Whitney's U test, or a one-way factorial ANOVA test. $P < 0.05$ was considered to indicate a statistically significant difference. StatMate V software was used to perform statistical analyses.

Table I. Chemically synthesized sequences for dual-luciferase reporter assay

Sequence		XhoI site	3'UTR of CDKN1B	NotI site	
WT-1 (5'-3')	GG	CTCGAG	AAGUUUAUUCUCAUUUGGGAGA	GCGGCCGC	GG
WT-1 (3'-5')	CC	GAGCTC	UUCAAAUAAGAGUAAACCCUCU	CGCCGGCG	CC
MUT-1 (5'-3')	GG	CTCGAG	AAGUUUAUUCUCAUAACCCUCU	GCGGCCGC	GG
MUT-1 (3'-5')	CC	GAGCTC	UUCAAAUAAGAGUAUUGGGAGA	CGCCGGCG	CC
WT-2 (5'-3')	GG	CTCGAG	AAAAUCCGAGGUGCUUGGGAGU	GCGGCCGC	GG
WT-2 (3'-5')	CC	GAGCTC	UUUUAGGCUCCACGAACCCUCA	CGCCGGCG	CC
MUT-2 (5'-3')	GG	CTCGAG	AAAAUCCGAGGUGCAACCCUCU	GCGGCCGC	GG
MUT-2 (3'-5')	CC	GAGCTC	UUUUAGGCUCCACGUUGGGAGA	CGCCGGCG	CC

WT, wildtype; MUT, mutant; UTR, untranslated region.

Table II. Primer sequences for reverse-transcription quantitative PCR.

Gene name	Orientation	Primer sequences
CDKN1B	Forward	5'-GGCCTCAGAAGACGTCAAAC-3'
	Reverse	5'-CAGGATGTCCATTCCATGAAG-3'
GAPDH	Forward	5'-GCACCGTCAAGGCTGAGAAC-3'
	Reverse	5'-TGGTGAAGACGCCAGTGGA-3'
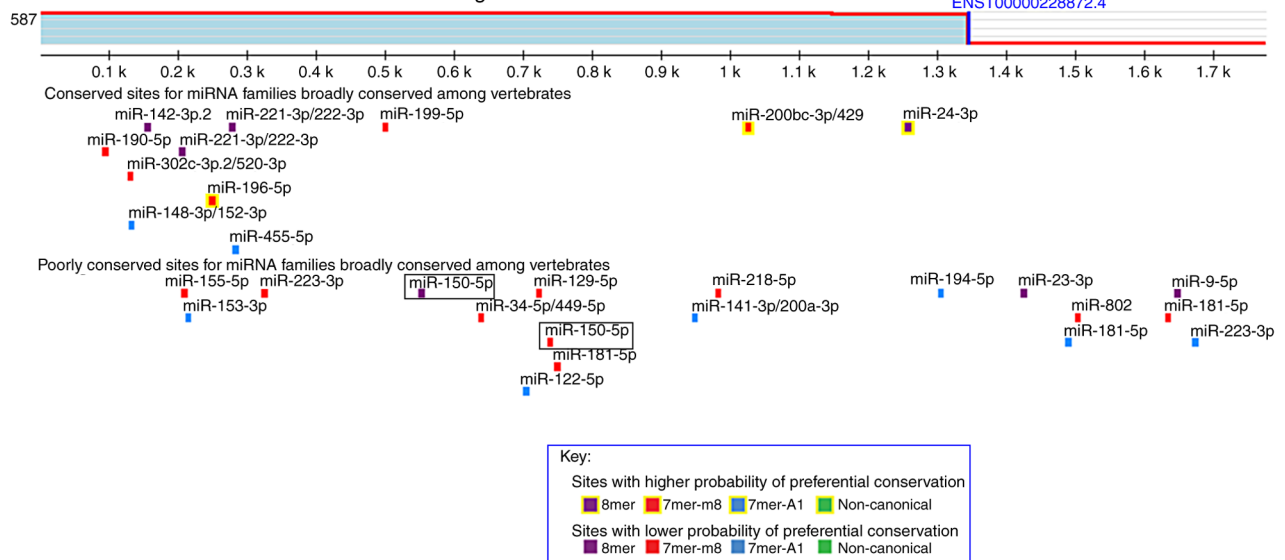
Figure 1. Predicted binding site between miR-150 and 3'UTR of CDKN1B based on TargetScan 7.2 database. miR, microRNA; UTR, untranslated region.

Results

miR-150 directly targets the 3'UTR of CDKN1B in transfected HeLa cells. miRNAs repress the expression of target genes by binding the 3'UTR of target genes. The target sites of miRNA-150 were investigated using the TargetScan miRNA target prediction database. *CDKN1B* is a predicted target gene of miR-150. Two predicted miR-150-binding sites in the 3'UTR of *CDKN1B* were identified. To verify these two binding sites, we designed recombinant plasmids, including wild-type or mutant 3'UTR sequences of *CDKN1B* (Fig. 2A and B). Then, dual-luciferase reporter assays were performed to investigate whether miR-150 could influence the expression of luciferase via binding to the predicted sequences. Our results showed that miR-150 directly downregulated *CDKN1B* in the HeLa cells (Fig. 2C and D). Conversely, there was no effect on the reporters with mutant sequences.

miR-150 reduces CDKN1B mRNA expression and p27^{Kip1} protein expression in transfected HeLa cells. To investigate

A

WT1   AAGUUUAUUCUCAU**UUGGGAGA**

miR-150 GUGACCAUGUUCCC**AACCCUCU**

MUT1   AAGUUUAUUCUCAU**AACCCUCU**

B

WT2   AAAAUCCGAGGUGC**UUGGGAG**U

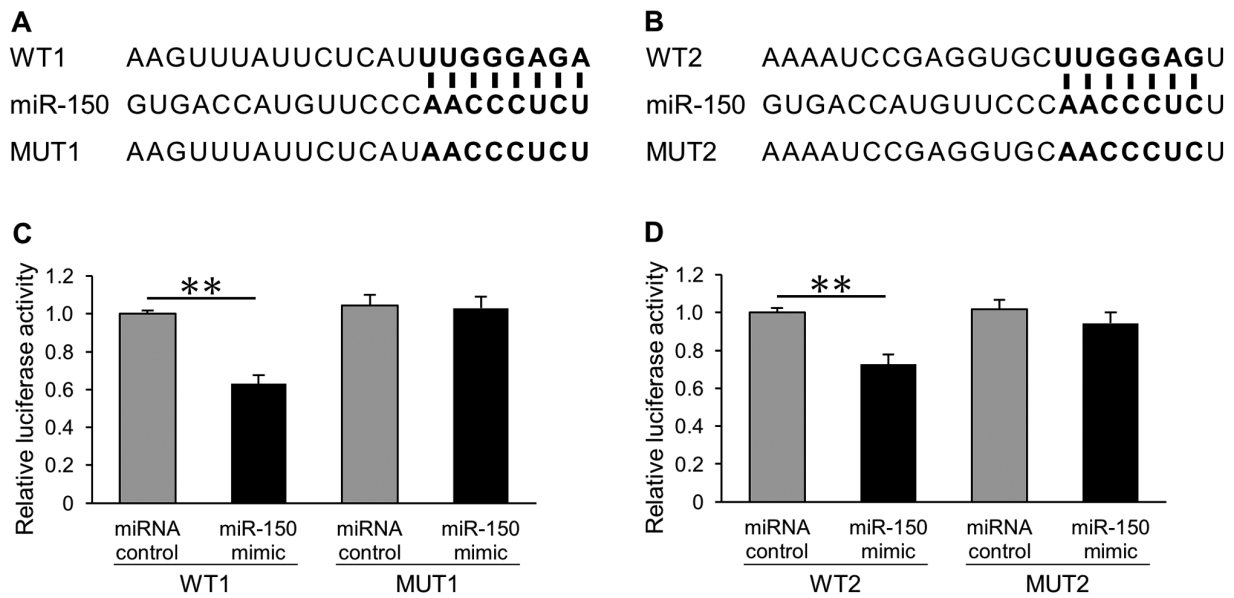miR-150 GUGACCAUGUUCCC**AACCCUC**U

MUT2   AAAAUCCGAGGUGC**AACCCUC**U

Figure 2. miR-150 directly targets the 3'-UTR of *CDKN1B*. (A) Sequences of the miR-150 binding sites in the 3'-UTR of wild-type CDKN1B (WT1) and a CDKN1B mutant containing eight mutated nucleotides in the 3'-UTR of CDKN1B (MUT1). The predicted miR-150-binding sites are presented in bold. (B) Sequences of the miR-150 binding sites in the 3'UTR of wild-type CDKN1B (WT2) and a CDKN1B mutant containing 7 mutated nucleotides in the 3'UTR of CDKN1B (MUT2). The predicted miR-150-binding sites are presented in bold. (C) Luciferase activity assays of the WT1 or MUT1 reporter in HeLa cells transfected with miR-150 mimics or miRNA controls. (D) Luciferase activity assays of the WT2 or MUT2 reporter in HeLa cells transfected with miR-150 mimics or miRNA controls. Firefly and *Renilla* luciferase activities were measured. Luciferase activities were normalized to *Renilla* luciferase. Significant differences were assessed using the Mann-Whitney U test. **P<0.01. Bars represent the mean ± standard deviation of three experiments. miR, microRNA; UTR, untranslated region; WT, wild-type; MUT, mutant; miR, microRNA.
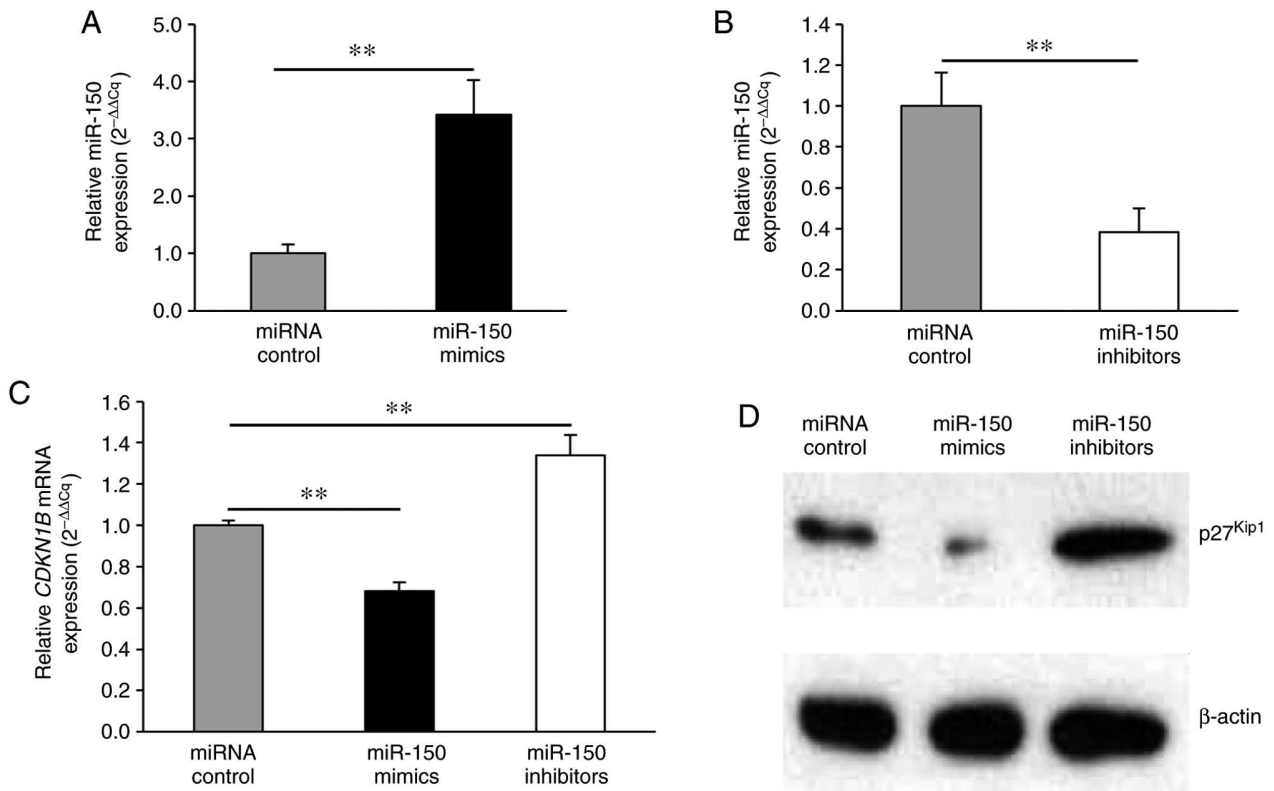

Figure 3. miR-150 downregulates *CDKN1B* mRNA and p27^Kip1 expression. Total RNA and protein were extracted from transfected HeLa cells. (A) HeLa cells were transfected with miRNA controls or miR-150 mimics. (B) HeLa cells were transfected with miRNA controls or miR-150 inhibitors. Average cycle threshold value for the endogenous control RNU6B was used to normalize the raw cycle threshold data and calculate ΔCq. The $2^{-\Delta\Delta Cq}$ method was used to determine the relative expression levels of miR-150. Significant differences were assessed using the Mann-Whitney U test. (C) The average cycle threshold value for the housekeeping gene *GAPDH* was used to normalize the raw cycle threshold data and calculate ΔCq. The $2^{-\Delta\Delta Cq}$ method was used to determine the relative expression levels of *CDKN1B*. Significant differences were assessed using Dunn's test. **P<0.01. Bars represent the mean ± standard deviation of three experiments. (D) Representative western blot analysis shows p27^Kip1 protein expression in transfected HeLa cells. β-actin was used as a loading control for western blot analysis. miR, microRNA.
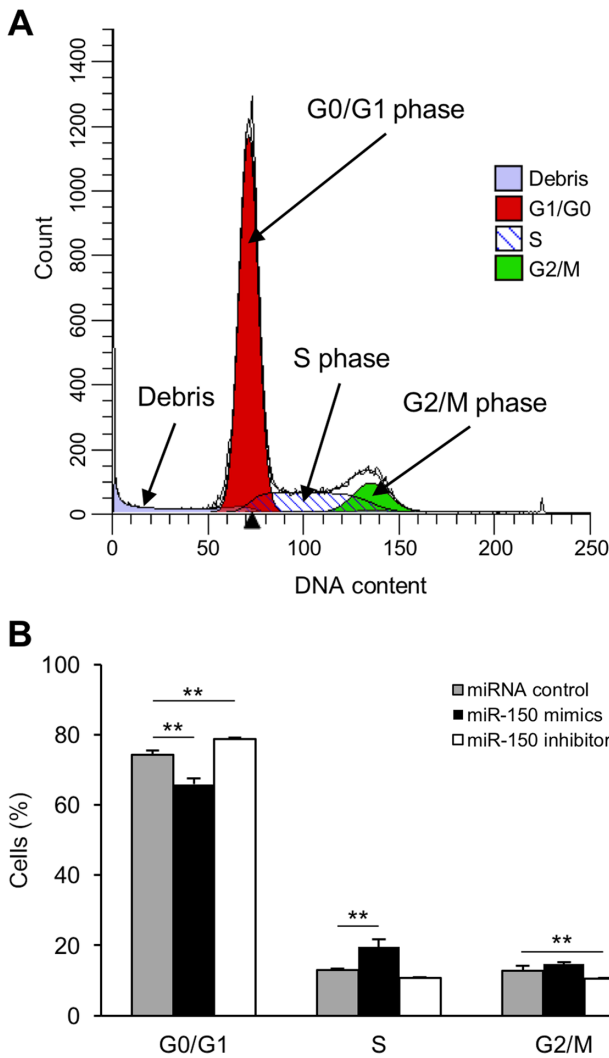
A

B

Figure 4. miR-150 promotes cell cycle progression. HeLa cells were transfected with miR-150 mimics, miR-150 inhibitors or miRNA controls for 72 h, and the number of cells in each phase of the cell cycle was measured using a flow cytometer and determined with ModFit LT software. (A) Representative DNA content graphic profiles of the cell cycle are presented. ModFit LT program allows to scan the histogram for peaks and to determine the number of cell cycles based on the sizes and positions. Debris is excluded from cell cycle determination by ModFit LT algorithm. The X-axis shows the DNA content. The red region, diagonal line, and green area indicate the G0/G1 phase, S phase, G2/M phase, respectively. (B) The percentages of cells in different cell cycle phases were analyzed and presented as a histogram. Significant differences were assessed using Dunn's test. **P<0.01. Bars represent the mean ± standard deviation of three experiments. miR, microRNA.

the molecular details of miR-150 effects, HeLa cells were transfected with miR-150 mimics, miR-150 inhibitors, or miRNA control. Firstly, qPCR for miRNA showed that miR-150 expression levels were significantly higher and lower in the miR-150 mimic-transfected and miR-150 inhibitor-transfected cells, respectively, relative to the controls (Fig. 3A, B). Since miR-150 directly targeted the 3'UTR of the *CDKN1B* gene, the expression levels of *CDKN1B* mRNA and p27^{Kip1} protein were evaluated after the transfection of miR-150 mimics or inhibitors using qPCR and western blot analysis, respectively. miR-150 mimics significantly decreased *CDKN1B* mRNA expression levels (Fig. 3C). In contrast, miR-150 inhibitors significantly upregulated
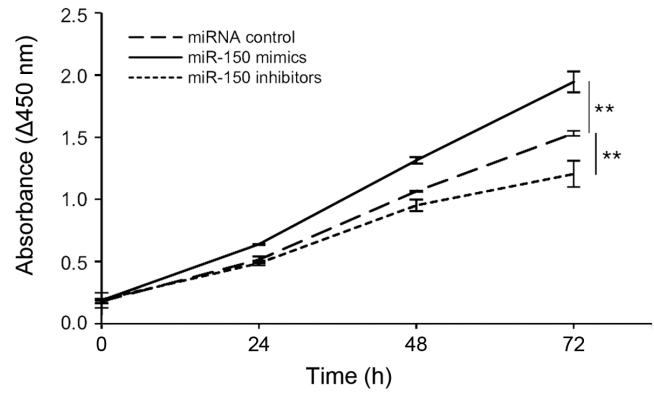

Figure 5. miR-150 promotes cell proliferation. HeLa cells were transfected with miR-150 mimics, miR-150 inhibitors or miRNA controls for 72 h, and then, cell proliferation was determined using CCK-8 every 24 h. Significant differences were assessed using a one-way factorial ANOVA test. **P<0.01. Bars represent the mean ± standard deviation of three experiments. miR, microRNA.

CDKN1B mRNA expression levels (Fig. 3C). Consistent with the RT-PCR results, the protein levels of p27^{Kip1} were reduced by miR-150 mimics and increased by miR-150 inhibitors (Fig. 3D).

miR-150 facilitates cell cycle progression in transfected HeLa cells. To investigate the role of miR-150 in cervical cancer cells, we analyzed cell cycle progression in transfected HeLa cells using a flow cytometer (Fig. 4A). There was an increased number of cells in the S phase in miR-150 mimic-transfected cells compared to that in miRNA control-transfected cells (Fig. 4B). In contrast, transfection of miR-150 inhibitors induced G0/G1 phase arrest (Fig. 4B).

miR-150 promotes cell proliferation in transfected HeLa cells. We hypothesized that miR-150 levels are involved in cell proliferation in cervical cancer. The proliferation of transfected HeLa cells was determined by measuring the absorbance at 450 nm based on a colorimetric assay. Our results showed that the overexpression of miR-150 led to a significant increase in cell proliferation, whereas expressing miR-150 inhibitors led to a significant decrease in HeLa cell proliferation (Fig. 5).

Discussion

miRNAs play critical roles in many diverse biological and pathological processes, such as cell proliferation, migration, apoptosis, and the pathogenesis of cancer (8,9). Among them, recent studies have reported the influence of miR-150 on many types of cancers (10-17). Previous studies have reported decreased levels of miR-150 in colorectal cancer (27,28), liver cancer (29), pancreatic cancer (12), esophageal squamous cell carcinoma (30), and ovarian cancer (14). In contrast, the upregulated expression of miR-150 has been found in breast cancer (13), non-small cell lung cancer (31-33), gastric cancer (34), and prostate cancer (15). A previous report indicated that miR-150 expression levels are significantly higher in cervical carcinoma cells than in para-carcinoma tissues (18). Moreover, the expression of miR-150 in samples from patients with cervical carcinoma significantly exceeded that of normal

cervical tissue from healthy donors. Moreover, miR-50 expression correlated with stage progression (18). However, results depict a controversial picture regarding the targets of miR-150 in cervical cancer. Moreover, the consensus regarding a hypothetical influence of miR-150 on cervical cancer is lacking.

In the present study, we analyzed the effect of miR-150 on cervical cancer cells *in vitro*. We first investigated the target sites of miRNA-150 using the TargetScan miRNA target prediction database and predicted two binding sites on the 3'UTR of *CDKN1B*. Further, the results of dual-luciferase reporter assays showed that miR-150 directly bound and downregulated *CDKN1B* in HeLa cells. Notably, miR-150 significantly decreased *CDKN1B* mRNA expression levels, and western blot analysis showed that the transfection of miR-150 mimics reduced p27^{Kip1} protein levels. The p27^{Kip1} protein transduces extracellular signals of cell cycle regulators that negatively regulate G1 cell cycle progression (25,26).

To investigate the role of miR-150 in HeLa cells, we analyzed cell cycle progression by flow cytometry. In miR-150 mimic-transfected cells, a higher proportion of cells was in the S phase. Moreover, the transfection of miR-150 inhibitors induced cell cycle arrest at the G1/G0 phase. These results suggested that miR-150 promotes cell cycle progression from the G0/G1 to S phase in cervical cancer cells by suppressing the p27^{Kip1} function.

We then measured transfected HeLa cell numbers using CCK-8, via a sensitive colorimetric assay for the determination of cell proliferation. The cells transfected with miR-150 mimics proliferated faster than control cells, whereas the cells transfected with miR-150 inhibitors grew slower than control cells. These findings suggest that miR-150 promotes cell cycle progression and cell proliferation by suppressing p27^{Kip1} expression. Our results validated previously reported direct targets of miR-150 in cervical cancer, namely *FOXO4*, *P2RX7*, and *SRCIN1* (18-20). However, our results also suggest that miR-150 promotes proliferation by directly targeting *CDKN1B*. Considering the well-known pleiotropic effects of miRNA, a single miRNA could bind several hundred target mRNAs (35). Although the present study provides a clearer understanding of the function and molecular mechanism of miR-150 in HeLa cells, more comprehensive *in vitro* and *in vivo* studies are needed to decipher the role of miR-150 in cervical cancer.

In conclusion, our results indicate that miR-150 promotes cell cycle progression and cell proliferation of HeLa human cervical cancer cells by directly suppressing p27^{Kip1} expression. We identified *CDKN1B* as a novel target of miR-150 in cervical cancer.

Authors' contributions

WO conceived and designed all the experiments. WO and KH performed the experiments. HT, KI, YY, AK, TN and NY analyzed the experimental data. TN and NY contributed to writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Kagawa Prefectural University of Health Sciences, Japan.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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