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*Original Article*

# miR-335 inhibits cell proliferation, migration and invasion in HeLa cervical cancer cells

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**Abstract:** miR-335 is associated with the presence and progress of multiple tumors, its particular effects on cervical carcinoma, however, remain unclear. This study aims at exploring the particular effects of miR-335 on proliferation, cell cycle, migration and invasion of HeLa cell line. miR-335 expression was quantified by qRT-PCR in 19 pairs of cervical carcinoma and para-carcinoma formalin-fixed paraffin-embedded (FFPE) specimens. The HeLa cells were transfected with miR-335 mimics, mimics NC, inhibitor and inhibitor NC, then confirmed by qRT-PCR. The cells viability was tested by CCK8 assay; cell cycle was analyzed by flow cytometry; invasion and migration capacity were observed by Transwell assays and wound-healing assay. Bioinformatics was utilized to predict miR-335 target genes and analyze the function and signal pathway of the target genes. Results show that miR-335 expression was lower in carcinoma tissues than in para-carcinoma tissues ($P<0.01$). HeLa cells had abatements in proliferation, invasion and migration after the transfection of miR-335 mimics, and were arrested in S phase ($P<0.05$). 35 target genes were predicted, these genes were found to be mainly enriched in GTPase binding, cell division, cell apoptosis, cell migration and regulation of transcription, as well as focal adhesion and focal adhesion. In conclusion, miR-335 is associated with presence and progress of the cervical cancer. The target genes of miR-335 closely related to multiple biological processes and signal pathways which is critical in cancer.

**Keywords:** Cervical cancer, miR-335, HeLa cell, proliferation, migration, invasion

## Introduction

Cervical cancer is one of the top 3 mortal malignancies in women [1]. Human papillomavirus (HPV) is confirmed a necessary precipitating factor of cervical cancer, yet not a sufficient condition. Factors of immunity, genetics, epigenetics or environment are also involved in the situation [2].

MicroRNAs (miRNA) are single-stranded noncoding RNAs that contain about 20-23 nucleotides which can match 3'UTRs of the mRNA thus regulate the expression or degrade the mRNA [3]. miRNA regulates approximately 30% of human genes, which is vital in human development, physiological conditions and ailment progress [4, 5].

miR-335 is located in 7q32.2 of human chromosomes. Current evidences have indicated that abnormal expression of miR-335 is related to multiple tumors such as pancreatic cancer, ovary cancer, breast cancer, colorectal cancer et al. [6-10]. miR-335 could downregulate Bcl-2-like protein 2 (BCL2L) expression, which consequently inhibits expression of 72 kDa type IV collagenase (MMP-2), playing a role of anti-oncogene of the metastasis and invasion procedure in ovary cancer [11]. In metastatic cell lines of breast cancer, miR-335 suppresses metastasis and migration of malignant cells via targeting down-regulation of expressions of the progenitor cell transcription factor SOX-4 and extracellular matrix component tenascin- C [12]. Gong M et al. [13] proved that overexpression of miR-335 in SBC-5 cells significantly restrains cell migration, invasion, proliferation, colony formation, and osteoclast induction. miR-335 is also involved in the regulation of cell growth and apoptosis [6], and participates in

inhibitory mechanism of cell migration and invasion [11].

It is substantiated by recent researches that miR-335 expression is lowered in cervical carcinoma tissue which deteriorates prognosis and survival rate [14]. But the mechanism of miR-335 regulation in cervical cancer, and its impact on HeLa cell line proliferation, cell cycle, invasion and migration remain unclear.

In this research, expression of miR-335 in both cervical carcinoma tissue and para carcinoma tissue FFPE were calculated, which resulted a lowered expression of miR-335 in cervical carcinoma. miR-335 mimics and inhibitor and their controls are transfected into HeLa cells respectively before testing of biological behaviors of each group. The outcomes showed that upregulated miR-335 expression inhibited proliferation, invasion and migration of HeLa cells, arrested the cells in S phase; and the opposite results were found in downregulated group. These results suggest that miR-335 has an anti-oncogene effect in the progress of occurrence and development of cervical carcinoma.

## Materials and methods

### *clinical samples*

A total of 19 pair of cervical cancer and adjacent normal cervical tissue FFPE blocks were recruited from cervical cancer patients who have been diagnosed by Nanfang Hospital (Guangzhou, China), and received no prior chemotherapy or radiotherapy before surgeries. Specimen collection was performed upon written informed consent and this study was approved by the ethics committee of the hospital.

### *Cell culture and transfection*

Human cervical cancer cells line HeLa were cultured in DMEM (Hyclone, USA) with 10% FBS (Gibco, USA) at 37°C in 5% $CO_2$. The miR-335 mimics, mimics NC, inhibitor and inhibitor NC were synthesized and purified by Shanghai GenePharma (Shanghai, China). When the cells were 80% confluent, 50 nM corresponding miRNAs were transfected into HeLa cells using LipofectamineTM 2000 (Invitrogen, USA) according to the manufacturer's instructions. 24 h to 48 h after transfection, the cells were harvested for further experiments.

### *Isolation of small RNA from FFPE tissues and cells*

miRNAs were isolated from cells or tissues using the RNAiso for Small RNA reagent (TaKaRa, Dalian, China), according to the manufacturer's protocol. For FFPE samples, the blocks were cut and placed into anuclease-free microcentrifuge tube. Xylene was added for deparaffinization. Samples were left at 57°C for 10 min and then centrifuged at 10000 rpm for 10 min at room temperature. Supernatants were removed, and washed with 100% ethanol for twice, and then samples were dried at 37°C. Each sample we added 1 ml of the RNAiso for Small RNA reagent for small RNA isolation, following the manufacturer's protocol. Isolated small RNA was stored at -80°C. Small RNA concentrations were defined spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (NanoDrop, DE, USA).

### *Quantitative real-time reverse transcription-PCR (qRT-PCR)*

The expression level of miR-335 was quantified by qRT-PCR. miRNA reverse transcriptase reactions were done using PrimeScript RT-PCR Kit (TaKaRa, Dalian, China), real-time PCR was done using SYBR Green assays (TaKaRa, Dalian, China) and ABI Prism® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. U6 snRNA was used as the reference gene for normalization of expression. The sequences of the primers used for qRT-PCR in this study are as follows: miR-335 forward 5'-TCAAGAGCAATAACGAAAAATGT-3' and miR-335 reverse 5'-GCTGTCAACGATACGCTACGT-3 and U6 forward 5'-CGCTTCGGCAGCACATATAC-3' and U6 reverse 5'-TTCACGAATTTGCGTGTCAT-3'. The relative expression levels of miR-335 were calculated and quantified using the $2^{-\Delta\Delta CT}$ method. Each sample was examined in triplicate.

### *Proliferation assay*

The viability of cells was evaluated by CCK-8 assay. Cells were plated into 96-well plates at a density of $5 \times 10^3$ per well, in three replicate wells. We added 10 μl of CCK-8 solution (KeyGEN, Nanjing, China) to each well daily for consecutive 4 days, and the cells were incubated for an additional 1.5 h at 37°C. Optical density was measured at a wavelength of 450 nm to estimate viable cell numbers.

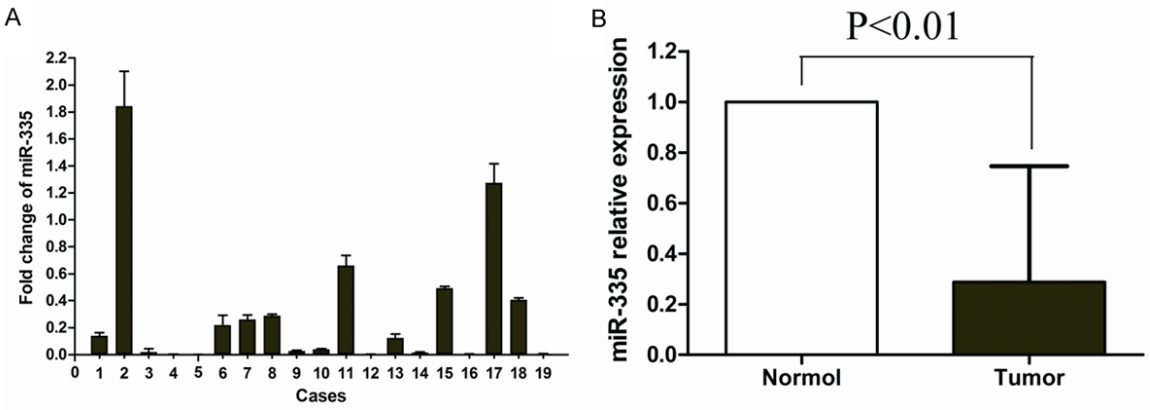


**Figure 1.** miR-335 expression is downregulated in cervical cancer tissues. A. qRT-PCR results of miR-335 relative expression levels in 19 pairs of cervical cancer and para-cancer tissues. B. miR-335 relative mean expression levels in cervical cancer and para-cancer tissues.

*Cell cycle analysis*

The cell cycle was analyzed by using a cell cycle detection kit (KeyGEN, Nanjing, China). Cells were harvested at 48 h after transfected, and fixed in cold 70% ethanol, stored at 4°C. The fixed cells were washed with PBS, and then added 100 μl RNase A. After incubation for 30 min at 37°C, 400 μl PI added for 30 min. The cell cycle was determined by FACS caliber (BD Bioscience Mountain View, USA) and Cell Quest software (Becton-Dickinson, USA).

*Invasion and migration assays*

To determine cell invasion and migration capacity, transwell cell Matrigel invasion and migration assays were performed. For invasion assays, $3.5 \times 10^5$ cells were placed into the top chamber (8.0-mmpores) (Coster, NY) with 70 μl Matrigel (BD Bioscience, USA). For migration assays, $6.0 \times 10^4$ cells were placed into the top chamber of each insert. The cells were resuspended in the FBS-free medium, media with 10% FBS was added to the lower chamber as a chemoattractant for 24 h. The cells that had invaded or migrated to the bottom of the membrane were then fixed with methyl alcohol and stained in 0.1% crystal violet. 5 visual fields of each insert were randomly chosen and imaged (× 200 magnification). In addition, wound-healing assay was carried out to confirm the transwell cell migration assays. Cells were seeded into a 6-well plate, after transfection and the cell monolayer was subjected to a mechanical scratch wound induced using a pipette tip. Then the images of wound area were captured at 0 h, 24 h and 48 h (× 4 magnification).The width was measured by IPP 6.0 software. The percent migration calculating formula is as follows: [Δwidth/width (0 h)] × 100.

*miR-335 target genes prediction and functional enrichment analysis*

Predicting miR-335 target genes was performed using the commonly used miRNA target prediction on-line toolsDIANA-microT (http://diana.imis.athena-innovation.gr/DianaTools/), PicTar (http://pictar.mdc-berlin.de/), miRDB (http://www.mirdb.org/miRDB/) and TargetScan (http://www.targetscan.org/) [15]. In order to improve the prediction accuracy, we intersected the results of the several predicted targets. We also found the experimentally confirmed miR-335 targets by experimentally validated miRNA-mRNA interaction databases miRTarBase (http://mirtarbase.mbc.nctu.edu.tw/) [16] and published literature, combined predicted target genes as miR-335 targets set for the following study.

To identify the biological function and the pathways of miR-335 target genes, the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Functional Annotation Tool (http://david.abcc.ncifcrf.gov) [17] was used to find out Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) terminology. Genes were classified using functional annotation, choose human genome as the background, classification stringency was set at medium level.

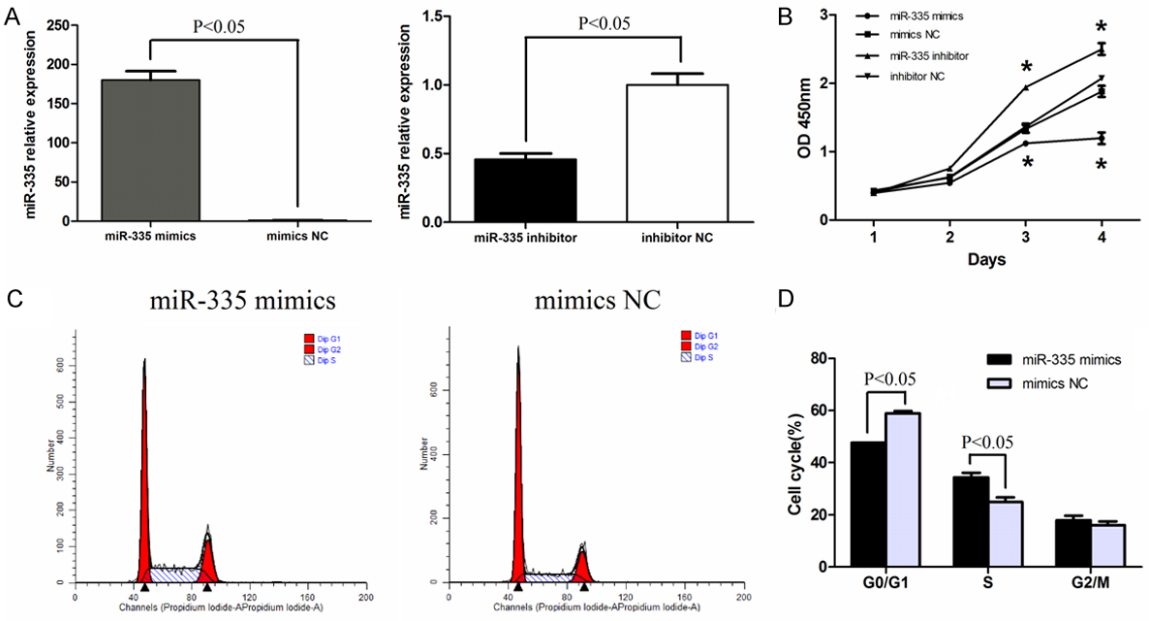


**Figure 2.** miR-335 inhibits HeLa cells proliferation and induces S arrest. A. Expression of miR-335 was confirmed by qRT-PCR after infection with miR-335 mimics, mimics NC, inhibitor and inhibitor NC respectively. B. Cells proliferation was evaluated by CCK-8 assay. *P<0.05. C, D. Cell cycle was analyzed by flow cytometry analysis.

*Statistical analysis*

Statistics were analyzed using the SPSS20.0 statistical software package (SPSS Inc., Chicago, IL, USA). Differences between groups were analyzed using the Student's t-test to calculate the *p*-values. *p*-values of P<0.05 was considered significant. All data representations include mean ± SE.

## Results

*miR-335 expression is downregulated in cervical cancer tissues*

Expression of miR-335 were measured by qRT-PCR in 19 pairs of cervical cancer and para-cancer tissues, which showed significantly lowered quantities of miR-335 in cancer tissues than in para-cancer tissues in 17 pairs, while higher quantities in the rest 2 pairs (**Figure 1A**). miR-335 relative mean expression levels were down-regulated in cervical cancer tissues (**Figure 1B**).

*miR-335 inhibits HeLa cells proliferation and induces S arrest*

Given that miR-335 is downregulated in cervical cancer tissues, we proceeded to explore the biological function of miR-335 in cervical cancer. miR-335 mimics, mimics NC, inhibitor and inhibitor NC were transfected in HeLa cells, respectively. Expression of miR-335 in transfected HeLa cells was quantified by qRT-PCR. (**Figure 2A**). miR-335 mimics and miR-335 inhibitor transfection resulted in an elevation or reduction of mature miR-335 in HeLa cells respectively. CCK8 test indicated that cells in miR-335 mimics group had a decreased proliferation rate after 3rd day compared with the control group, while cells in miR-335 inhibitor group demonstrated a higher proliferation rate after 3rd day compared with the control group. These outcomes demonstrate that miR-335 inhibits HeLa cells proliferation (**Figure 2B**). Cell cycle alteration in each group was tested by flow cytometry. The consequence showed cells of S phase gained a higher percentage in miR-335 mimics group than that in mimics NC group (**Figure 2C** and **2D**), indicating upregulating miR-335 expression arrests HeLa cells in S phase.

*miR-335 inhibits HeLa cells invasion and migration*

To explore the function of miR-335 expression on the regulation of cellular invasion and migra-

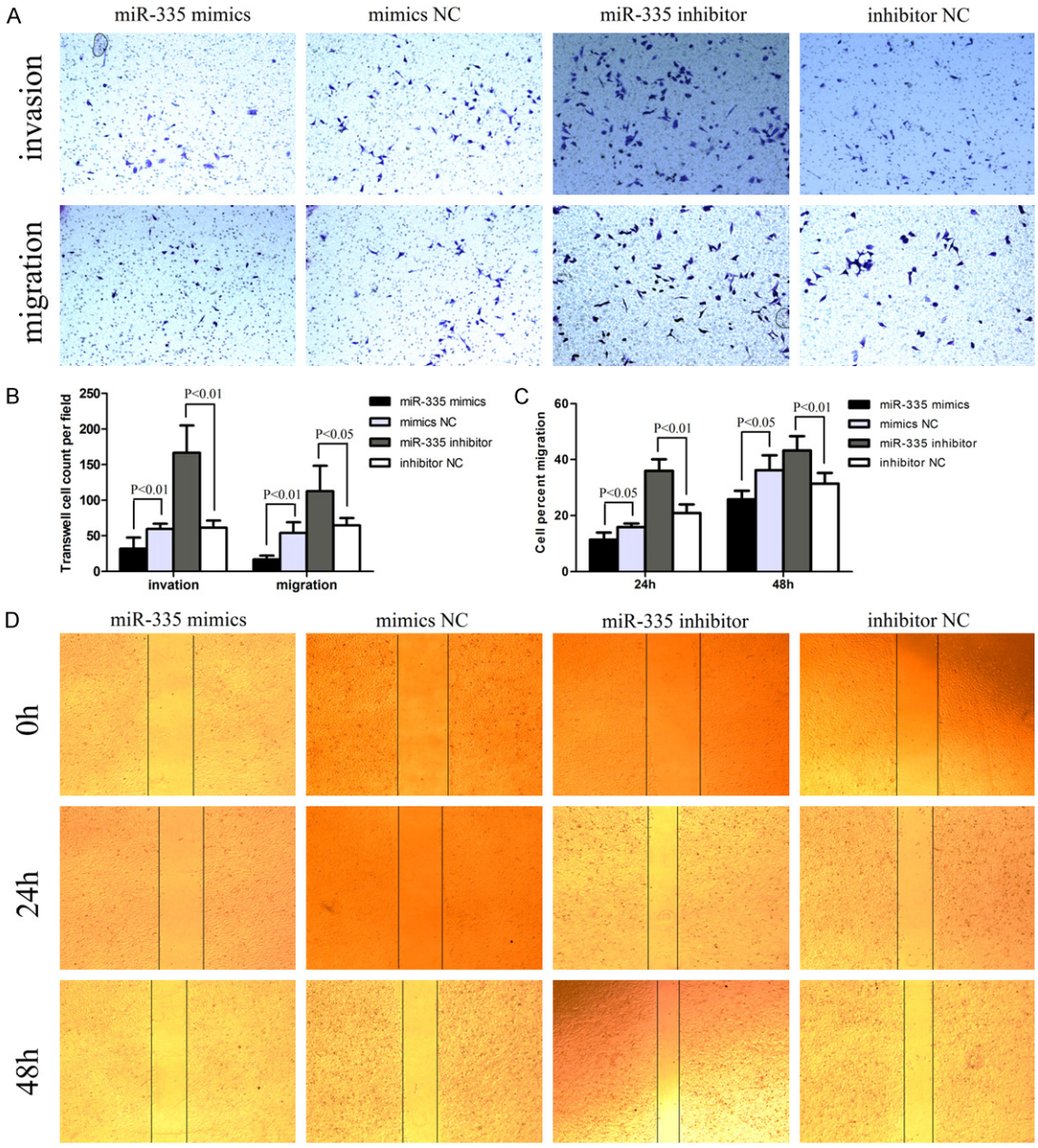


**Figure 3.** miR-335 inhibits HeLa cells invasion and migration. A, B. Transwell invasion and migration assays. Invaded or migrated cells were imaged at x200 magnification. C, D. Wound-healing assay. The wound area was captured at 0 h, 24 h and 48 h at × 4 magnification.

tion, transwell invasion and migration assays were performed. We found that permeated cell count of miR-335 mimics group was less than that of mimics NC group, permeated cell count of miR-335 inhibitor group outnumbered that of inhibitor NC group (**Figure 3A** and **3B**). These data suggest that miR-335 inhibits HeLa cells invasion and migration. Furthermore, the effect of miR-335 on inhibiting HeLa cells migration was supported by the wound-healing assay (**Figure 3C** and **3D**).

*miR-335 target genes closely related to multiple biological processes*

166, 137, 251 and 2308 miR-335 target genes were predicted through DIANA-microT, PicTar, miRDB and TargetScanin respectively, con-

**Table 1.** Potential miR-335 target genes

| NO. | genes | Predicted online | miRTarBase | Published literature |
|---|---|---|---|---|
| 1 | ARPC5L | | √ | |
| 2 | ATP1B1 | √ | | |
| 3 | BCL2L2 | | | Wang H, et al. 2013. |
| 4 | BIRC5 | | √ | Zu Y, et al. 2013. |
| 5 | DAAM1 | | √ | |
| 6 | DAAM2 | | | Lynch J, et al. 2013. |
| 7 | ETF1 | √ | | |
| 8 | FMN2 | | | Lynch J, et al. 2013. |
| 9 | FMNL3 | | | Lynch J, et al. 2013 |
| 10 | FMR1 | √ | | Gong X, et al. 2014. |
| 11 | HOXD8 | √ | | |
| 12 | LOC728519 | | | |
| 13 | LRG1 | | √ | Lynch J, et al. 2012. |
| 14 | MAP2 | √ | | |
| 15 | MAPK1 | | √ | Lynch J, et al. 2012. |
| 16 | MERTK | | | Valastyan S, et al. 2009. |
| 17 | MET | | | Gao Y, et al. 2015. |
| 18 | POU5F1 | | | Gao L, et al. 2014. Schoeftner S, et al. 2013. |
| 19 | PAX6 | | | Cheng Q, et al. 2014. |
| 20 | PTPRN2 | | √ | Valastyan S, et al. 2009. |
| 21 | RASA1 | | √ | Lu Y, et al. 2015. |
| 22 | RB1 | | √ | Shi L, et al. 2012. Scarola M, et al. 2010. |
| 23 | RBBP8 | | | Martin NT, et al. 2013. |
| 24 | ROCK1 | | | Liu H, et al. 2015. Wang Yet al. 2013. Lynch J, et al. 2012. |
| 25 | RSBN1 | √ | | |
| 26 | RUNX2 | | √ | |
| 27 | SOX4 | | √ | Hasegawa S, et al. 2015. Valastyan S, et al. 2009. |
| 28 | SP1 | | | Wang H, et al. 2013. |
| 29 | TFF2 | | | |
| 30 | TNC | | √ | |
| 31 | TRIM29 | | | Zhou XM, et al. 2016. |
| 32 | UBE2F | | √ | |
| 33 | UBE2G1 | √ | | |
| 34 | ZEB2 | | | Sun Z, et al. 2014. |
| 35 | ZMPSTE24 | √ | | |

tained 8 overlapping genes. We got 15 and 21 miR-335 target genes which have confirmed by experiments in miRTarBase and research papers respectively (**Table 1**). Taking the 3 results together, a total of 35 target genes were collected for further functional enrichment analysis.

GO and KEGG enrichment analysis can be used to analyze the mainly functions and signal transduction pathways of multiple genes during metabolic processes (**Table 2**). According to the results of the functional enrichment analysis by DAVID, miR-335 target genes were most highly enriched in GTPase binding, cell division, cell apoptosis, cell migration and regulation of transcription, as well as focal adhesion and focal adhesion. MAPK1, ROCK1, BIRC5 and SP1 involved more annotation cluster, may provide some insights into biological processes in cancer.

## Discussion

High-risk HPV infection induced aberrant regulation of cellular oncogenic and tumor suppres-

**Table 2.** DAVID functional enrichment analysis (P<0.05)

| Category | Term | Count (%) | *P* value | Genes |
|---|---|---|---|---|
| Annotation Cluster 1 Enrichment Score: 3.63 | | | | |
| MF | GTPase binding | 6 (17.14) | <0.001 | FMNL3, ROCK1, BIRC5, DAAM1, DAAM2, RASA1 |
| MF | Enzyme binding | 9 (25.71) | <0.001 | MAPK1, FMNL3, ROCK1, SP1, BIRC5, RB1, DAAM1, DAAM2, RASA1 |
| MF | Ras GTPase binding | 5 (14.29) | <0.001 | FMNL3, ROCK1, BIRC5, DAAM1, DAAM2 |
| MF | Small GTPase binding | 5 (14.29) | <0.001 | FMNL3, ROCK1, BIRC5, DAAM1, DAAM2 |
| MF | Rho GTPase binding | 4 (11.43) | <0.001 | FMNL3, ROCK1, DAAM1, DAAM2 |
| BP | Actin cytoskeleton organization | 5 (14.29) | 0.002 | FMN2, FMNL3, ROCK1, DAAM1, DAAM2 |
| BP | Actin filament-based process | 5 (14.29) | 0.002 | FMN2, FMNL3, ROCK1, DAAM1, DAAM2 |
| BP | Cytoskeleton organization | 6 (17.14) | 0.003 | FMN2, FMNL3, ROCK1, MAP2, DAAM1, DAAM2 |
| MF | Cytoskeletal protein binding | 6 (17.14) | 0.003 | FMN2, FMNL3, ARPC5L, BIRC5, DAAM1, DAAM2 |
| MF | Actin binding | 5 (14.29) | 0.004 | FMN2, FMNL3, ARPC5L, DAAM1, DAAM2 |
| Annotation Cluster 2 Enrichment Score: 1.59 | | | | |
| BP | Cell division | 5 (14.29) | 0.005 | FMN2, ROCK1, BIRC5, RB1, RASA1 |
| CC | Cytoskeleton | 7 (20.00) | 0.012 | MAPK1, FMN2, ROCK1, ARPC5L, MAP2, BIRC5, RB1 |
| CC | Intracellular non-membrane-bounded organelle | 9 (25.71) | 0.025 | MAPK1, FMN2, ROCK1, SP1, ARPC5L, MAP2, FMR1, BIRC5, RB1 |
| CC | Non-membrane-bounded organelle | 9 (25.71) | 0.025 | MAPK1, FMN2, ROCK1, SP1, ARPC5L, MAP2, FMR1, BIRC5, RB1 |
| CC | Microtubule cytoskeleton | 4 (11.43) | 0.046 | ROCK1, MAP2, BIRC5, RB1 |
| Annotation Cluster 3 Enrichment Score: 1.41 | | | | |
| BP | Cytokinesis | 4 (11.43) | <0.001 | FMN2, ROCK1, BIRC5, RASA1 |
| BP | Cell division | 5 (14.29) | 0.005 | FMN2, ROCK1, BIRC5, RB1, RASA1 |
| BP | Negative regulation of apoptosis | 5 (14.29) | 0.009 | ROCK1, SOX4, BCL2L2, BIRC5, RASA1 |
| BP | Negative regulation of programmed cell death | 5 (14.29) | 0.010 | ROCK1, SOX4, BCL2L2, BIRC5, RASA1 |
| BP | Negative regulation of cell death | 5 (14.29) | 0.010 | ROCK1, SOX4, BCL2L2, BIRC5, RASA1 |
| BP | Regulation of apoptosis | 5 (14.29) | 0.039 | MAPK1, ROCK1, SOX4, BCL2L2, BIRC5, RASA1 |
| BP | Regulation of programmed cell death | 5 (14.29) | 0.040 | MAPK1, ROCK1, SOX4, BCL2L2, BIRC5, RASA1 |
| BP | Regulation of cell death | 5 (14.29) | 0.041 | MAPK1, ROCK1, SOX4, BCL2L2, BIRC5, RASA1 |
| Annotation Cluster 4 Enrichment Score: 1.37 | | | | |
| BP | Cell migration | 4 (11.43) | 0.027 | ROCK1, MET, PAX6, ZEB2 |
| BP | Localization of cell | 4 (11.43) | 0.035 | ROCK1, MET, PAX6, ZEB2 |
| BP | Cell motility | 4 (11.43) | 0.035 | ROCK1, MET, PAX6, ZEB2 |
| Annotation Cluster 5 Enrichment Score: 1.29 | | | | |
| BP | Cell division | 5 (14.29) | 0.005 | FMN2, ROCK1, BIRC5, RB1, RASA1 |
| CC | Cytoskeleton | 7 (20.00) | 0.012 | MAPK1, FMN2, ROCK1, ARPC5L, MAP2, BIRC5, RB1 |
| BP | Cell cycle checkpoint | 3 (8.57) | 0.019 | BIRC5, RB1, RBBP8 |
| BP | Interphase of mitotic cell cycle | 3 (8.57) | 0.025 | BIRC5, RB1, RBBP8 |
| Annotation Cluster 6 Enrichment Score: 1.27 | | | | |
| MF | Transcription factor activity | 9 (25.71) | 0.001 | HOXD8, SP1, POU5F1, TRIM29, PAX6, SOX4, ZEB2, RB1, RUNX2 |
| MF | Transcription regulator activity | 9 (25.71) | 0.010 | HOXD8, SP1, POU5F1, TRIM29, PAX6, SOX4, ZEB2, RB1, RUNX2 |
| BP | Positive regulation of transcription | 6 (17.14) | 0.010 | MAPK1, SP1, PAX6, SOX4, RB1, RUNX2 |
| BP | Positive regulation of gene expression | 6 (17.14) | 0.011 | MAPK1, SP1, PAX6, SOX4, RB1, RUNX2 |
| BP | Positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process | 6 (17.14) | 0.015 | MAPK1, SP1, PAX6, SOX4, RB1, RUNX2 |
| BP | Positive regulation of nitrogen compound metabolic process | 6 (17.14) | 0.017 | MAPK1, SP1, PAX6, SOX4, RB1, RUNX2 |
| BP | Positive regulation of macromolecule biosynthetic process | 6 (17.14) | 0.018 | MAPK1, SP1, PAX6, SOX4, RB1, RUNX2 |
| BP | Positive regulation of cellular biosynthetic process | 6 (17.14) | 0.021 | MAPK1, SP1, PAX6, SOX4, RB1, RUNX2 |
| BP | Regulation of RNA metabolic process | 10 (28.57) | 0.021 | HOXD8, SP1, POU5F1, PAX6, SOX4, ZEB2, RB1, RUNX2, RASA1, RBBP8 |
| BP | Positive regulation of biosynthetic process | 6 (17.14) | 0.022 | MAPK1, SP1, PAX6, SOX4, RB1, RUNX2 |
| BP | Positive regulation of transcription, DNA-dependent | 5 (14.29) | 0.025 | SP1, PAX6, SOX4, RB1, RUNX2 |
| CC | Intracellular non-membrane-bounded organelle | 9 (25.71) | 0.025 | MAPK1, FMN2, ROCK1, SP1, ARPC5L, MAP2, FMR1, BIRC5, RB1 |

| | | | | |
|---|---|---|---|---|
| CC | Non-membrane-bounded organelle | 9 (25.71) | 0.025 | MAPK1, FMN2, ROCK1, SP1, ARPC5L, MAP2, FMR1, BIRC5, RB1 |
| BP | Positive regulation of RNA metabolic process | 5 (14.29) | 0.026 | SP1, PAX6, SOX4, RB1, RUNX2 |
| BP | Regulation of transcription from RNA polymerase II promoter | 6 (17.14) | 0.027 | HOXD8, SP1, PAX6, RB1, RUNX2, RBBP8 |
| MF | Sequence-specific DNA binding | 5 (14.29) | 0.035 | HOXD8, SP1, POU5F1, PAX6, ZEB2 |
| BP | Chordate embryonic development | 4 (11.43) | 0.043 | SP1, PAX6, ZEB2, RBBP8 |
| BP | Embryonic development ending in birth or egg hatching | 4 (11.43) | 0.044 | SP1, PAX6, ZEB2, RBBP8 |
| BP | Positive regulation of macromolecule metabolic process | 6 (17.14) | 0.049 | MAPK1, SP1, PAX6, SOX4, RB1, RUNX2 |
| Annotation Cluster 7 Enrichment Score: 1.20 | | | | |
| MF | Sequence-specific DNA binding | 5 (14.29) | 0.035 | HOXD8, SP1, POU5F1, PAX6, ZEB2 |
| BP | Anterior/posterior pattern formation | 3 (8.57) | 0.043 | HOXD8, PAX6, ZEB2 |
| Annotation Cluster 8 Enrichment Score: 1.20 | | | | |
| CC | Cell projection | 5 (14.29) | 0.018 | MAPK1, MET, MAP2, MERTK, RASA1 |
| CC | Cell fraction | 6 (17.14) | 0.018 | MAPK1, MET, MAP2, FMR1, ZMPSTE24, MERTK |
| Pathway | | | | |
| KEGG | Axon guidance | 4 (11.43) | 0.007 | MAPK1, ROCK1, MET, RASA1 |
| KEGG | Melanoma | 3 (8.57) | 0.020 | MAPK1, MET, RB1 |
| KEGG | Focal adhesion | 4 (11.43) | 0.023 | MAPK1, ROCK1, TNC, MET |
| KEGG | Colorectal cancer | 3 (8.57) | 0.028 | MAPK1, MET, BIRC5 |
| KEGG | TGF-beta signaling pathway | 3 (8.57) | 0.030 | MAPK1, ROCK1, SP1 |

sive miRNAs. According to the latest report, a large number of miRNAs are downstream targets of the transcription factors, such as E2F, p53, and c-Myc. These factors can be regulated by oncogenic HPV E6 and E7. Cervical cancer represents a powerful tumor model for researching how viral E6 and E7 oncoproteins deregulate the expression of the miR-15/16 cluster, miR-17-92 family, miR-21, miR-34a, miR-125b, miR-203 and miR-335 [18].

Profiling analysis and functional experiments have identified that miRNAs can function as tumor suppressors or oncogenes [19]. Emerging evidence has proven the association of cervical cancer to multiple miRNAs. Genomic profiles and researches have verified the existence of abnormal expression of cellular oncogenic or tumor suppressive miRNAs in cervical cancer tissues and cell lines [20, 21]. These abnormalities are related to the tumorigenesis and tumor progression of cervical cancer. miRNAs may influence the expression of papillomavirus genes in different pathway by targeting viral RNA transcripts. miR-34a acts as tumor suppressor inhibits cancer growth when directly regulated by p53. miR-125b is an inhibitor in immune response and cell apoptosis. Additionally, both miR-203 and miR-125b regulate DNA replication of Human papillomavirus (HPV) [22]. miR-34a and miR-125b could regulate HPV expression, thus promote persistent infection and progress of cervical cancer [23]. miR-200a has influence on metastasis of cervical cancer. Expression levels of miR-200a and miR-9 may be an indicator for survivals of cervical cancer patients [24]. Treatments that inhibit miR-21 expression or enhance miR-143 expression in vitro are likely to be new protocol for cervical cancer [25].

This study is aiming at discovering a potential miRNA target for diagnosis or treatment of cervical carcinoma. Existing researches indicate a lowered expression of miR-335 in multiple cancers such as esophageal carcinoma, gastric carcinoma and epithelial ovary carcinoma [9, 26, 27]. Other studies show that miR-335 expression is upregulated in glial tumors and colorectal cancer [28, 29]. We confirmed a lower expression of miR-335 in cervical cancer sections than in para-carcinoma tissue sections, in accordance to previous experiment where 135 pairs of cervical cancer and paracancer tissue sections are tested by Wang et al. [14].

An amount of experiments have verified that miRNA gives a great impact on biological cell behaviors such as migration, proliferation, cycle and apoptosis [30]. Metastasis is the great majority reason for cancer death [31]. miR-335 is one of the first metastasis-suppressing miRNAs discovered through expression profiling analysis of breast cancer metastatic and non-metastatic cell lines [12, 32].

miR-335 was confirmed to be low-expressed in osteosarcoma tissues and cell lines, inhibited osteosarcoma cells migration, invasion and apoptosis [33-35]. miR-335 was able to suppress cell proliferation and invasion of clear cell renal cell carcinoma cells [36]. However upregulated miR-335 in ovary cancer and breast cancer cells can inhibit invasion but has no effects on proliferation [11, 37]. These conclusions suggest miR-335 is an exclusive invasion suppressor in certain tumors. A study by Xu et al. confirmed that miR-335 suppressed gastric cancer cell invasion and metastasis in vitro and in vivo without significant effects on cell proliferation [11, 37]. While, Yang et al. reported that miR-335 could inhibit growth, apoptosis, and invasion of gastric cancer cells [39]. It is proved that miR-335 was overexpressed in meningiomas and increased cell growth and inhibited cell cycle arrest in the G0/G1 phase [40]. The expression level of miR-335 and its function are different in various kinds of cancers, and may controversial in the same kind of tumor. However, the roles of miR-335 in cervical cancer remain unknown. In this study, we have transfected HeLa cells with miR-335 mimics and miR-335 inhibitor, discovering upregulated miR-335 expression inhibits the cell migration as well as cell proliferation and arrests HeLa cells in S phase.

The molecular mechanism of miRNAs regulate cell growth is regulating target genes expression and cell signaling pathway. One miRNA may target multiple genes, and a single gene can be regulated by multiple miRNAs [41, 42]. miR-335 has multiple target genes which are associated with cell migration, invasion and other cellular activities [43]. It was demonstrated that miR-335 controls cell proliferation via regulating Rb1 in a p53-dependent manner [44]. miR-335 modifies the expression of antiapoptotic genes BCL2L and specificity protein 1 (SP1) thus accelerate apoptosis of ovarian cancer cells and lung cancer cell [11, 45]. Studies have shown miR-335 down regulates its target MET protein level leading to a decreasing hepatocyte growth factor (HGF) induced phosphorylation of c-Met, and subsequently negatively regulating the HGF/c-Met pathway to suppresses breast cancer cell migration [37]. Tripartite motif-containing 29 (TRIM29) has been reported as the direct target of miR-335-5p and miR-15b-5p, which induces proliferation, EMT and metastasis of nasopharyngeal carcinoma through the PTEN/AKT/mTOR signaling pathway [46]. These researches indicate that miR-335 plays an important role in the development of tumor.

It is persuasive to confirm the interact functions between miRNA and its target genes through laboratory experiment, however, bioinformatics approaches are efficiently and promising in identifying the potential miRNA target genes and enriching the functions of the target genes [47]. The results of DAVID analysis provide some available clues for the function of miR-335 target genes in biological processes. miR-335 target genes closely related to multiple biological processes such as cell division, cell apoptosis, cell migration, which were verified by experiment in the present paper. It is worth noting that the targets MAPK1, ROCK1, BIRC5 and SP1 involved most of the annotation cluster, may play essential roles in cancer.

Research has demonstrated that miR-335 directly targets and down-regulates genes in the TGF-beta non-canonical pathways members, including Rho/Rho-associated serine-threonine protein kinase 1 (ROCK1), mitogen-activated protein kinase 1 (MAPK1) and putative member leucine-rich alpha-2-glycoprotein 1 (LRG1), then reduces phosphorylation levels of the motor protein myosin light chain (MLC) resulting in inhibition of the invasive and migratory potential of neuroblastoma cells [48]. The phenomenon that miR-335 suppresses migration and invasion by targeting ROCK1 has also been verified in osteosarcoma and hepatocellular carcinoma [49, 50]. In the present researches, significantly recurrent somatic MAPK1 mutations in squamous cell cervical cancers and the high expression of MAPK1 in the cervical cancer tissue were observed; down-regulated MAPK1 expression can inhibit the invasion and metastasis of cervical cancer HeLa cells [51, 52]. The activation of RhoC/ROCK1 signaling pathways is likely involved in the cell invasion and migration of cervical squamous cell carcinoma [53]. Combined the conclusion above researches and our own study, we can deduce that the two genes MAPK1 and ROCK1 influence invasion and metastasis of cervical cancer may also affected targeted regulation by miR-335, and the influence on cervical cancer may also involve cell apoptosis, cytoskeleton and focal adhesion.

BIRC5 have identified differentially expressed in cervical cancer involved in cell cycle regulation [54]. BIRC5 as an important cervical cancer target of miR-218, altered tumor clonogenicity, migration, and invasion [55]. Existing research shows that SP1 is a direct target of miR-129-5p in Hela cells, and induced expression of miR-129-5p by IFN-β suppress the progression of cervical cancer by down-regulating HPV-18 E6 and E7 expression [56]. SP1 may activate LMX1A expression upon oncogenic stress during cervical cancer development [57]. Modulation of SP1 by mithramycin A may be a novel therapeutic strategy for cervical cancer [58]. All of the above findings suggest that BIRC5 and SP1 play important roles in the development and treatment of cervical cancer. Considering the two genes are potential targets of miR-335, we may provide clues to discover the mechanism of miR-335 and its target genes in cervical cancer by associating with the function of miR-335. Next, we can expand in-depth study.

As a conclusion, our experiments suggest miR-335 expression is decreased in cervical carcinoma, and miR-335 could inhibit proliferation, invasion and migration of the cancer cells, and arrest the cells in S phase. Our bioinformatics analysis speculate miR-335 target regulating multiple genes, and play a key role in inhibiting the genesis and progression of cervical carcinoma involves a number of biological processes, hence a potential target for diagnosis or treatment of cervical carcinoma.

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## Disclosure of conflict of interest

None.

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