Differential expression of miR-195 in esophageal squamous cell carcinoma and miR-195 expression inhibits tumor cell proliferation and invasion by targeting of Cdc42

Min-gen Fu 1,a,b, Shuo Li 1,a, Ting-ting Yu 3,a, Li-juan Qian 3,a, Ri-sheng Cao 3,a, Hong Zhu 3,a, Bin Xiao 3,a, Chun-hua Jiao 3,a, Na-na Tang 3,a, Jing-jing Ma 3,a, Jie Hua 3,a, Wei-feng Zhang 3,a, Hong-jie Zhang 3,a, Rui-hua Shi 3,a,*

1 The First Affiliated Hospital of Nanjing Medical University, Nanjing, China
2 Xinyu People’s Hospital, Jiangxi, China

**ABSTRACT**

MicroRNAs (miRNA) have played an important role in carcinogenesis. In this study, Agilent miRNA microarray was used to identify differentially expressed miRNAs in esophageal squamous cell carcinoma (ESCC) tissues and miR-195 was downregulated in ESCC compared with normal esophageal tissues. Moreover, Cdc42 was confirmed as target gene of miR-195. Ectopic expression of miR-195 in ESCC cells significantly downregulated Cdc42 by directly binding its 3'-untranslated regions, and induced G1 cell cycle arrest, leading to a significant decrease in cell growth, migration, and invasion in vitro. Therefore, our findings demonstrated that miR-195 may act as a tumor suppressor in ESCC by targeting Cdc42.

© 2013 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

### 1. Introduction

Esophageal cancer is the sixth leading cause of cancer-related deaths and the eighth most common cancer in the world [1]. Histologically, esophageal cancer can be divided into two main subtypes, i.e., esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). The incidence of ESCC is higher in East Asia, especially in China, while EAC incidence is higher in Western countries such as the USA [2]. These two types of esophageal cancer have relatively different etiologic characteristics and risk factors, but both are frequently diagnosed at the advanced stages of disease, which make cure extremely difficult. Thus, their overall 5-year survival rate is still less than 10–15% [3]. Therefore, it is urgent to discover molecular biomarkers for early tumor detection and novel therapeutic strategies.

To this end, our research has been focused on microRNAs (miRNA), which are a class of small, highly-conserved, single-stranded, non-coding RNAs with approximately 17–25 nucleotides. Functionally, they can target the 3'-untranslated region (UTR) of their target messenger RNAs to regulate their expression through direct mRNA degradation or they can block translation [4]. Thus, miRNAs participate in different biological and pathologic processes in the human body and play important regulatory roles in cell differentiation, proliferation, apoptosis, and metabolism [5]. Recent studies showed that aberrant expression of miRNAs can function as tumor suppressors or oncogenes [6–9] and many miRNAs have also been reported in the development and progression of ESCC [10,11]. For example, miR-375 was reported to be down-regulated in ESCC tissues and cell lines and can inhibit tumor growth and metastasis by repressing IGF1R expression [12]. miR-25 and miR-223 were up-regulated in ESCC tissues and cell lines and can inhibit tumor growth and metastasis by repressing IGFR expression [13-14].

In this study, we first used Agilent microarrays to identify differentially expressed miRNAs in ESCC tissues vs. the paired adjacent normal tissues. The detailed date on these three samples used for microarray analysis was showed in Table 1. We then chose one of these differentially expressed miRNAs, miR-195 for further study in ESCC tissues and cells as miR-195 has been shown to be up-regulated in breast cancer but down-regulated in colorectal cancer and squamous cell carcinoma of the tongue [15-17]. Bioinformatic studies have shown that miR-195 may target Cdc42, a member of Rho GTPase family, which has functions in cell polarity, proliferation, migration, and gene transcription [18]. Cdc42 was also up-regulated in many human cancers, including head and neck cancer [19], colorectal cancer [20], and ESCC [21]. Thus, we investigated the effects of miR-195 expression in the regulation of cell viability, colony formation, and gene expression in ESCC cells.
2. Materials and methods

2.1. Tissue samples

Surgically removed human ESCC and matched normal esophageal tissues (5 cm away from tumor) were collected and nine paired esophageal dysplasia tissues and adjacent normal tissues were obtained from patients who underwent endoscopic surgery from Jiangsu Province Hospital (Jiangsu, China). All patients had not received any treatment before surgery. All tissues were snap-frozen in liquid nitrogen immediately and then stored at −80 °C until use. The study was approved by the committees for ethical review of research of the First Affiliated Hospital of Nanjing

| Sample | Age, years | Sex | Clinical stage | Distant metastasis | Tumor size (cm) |
|--------|------------|-----|----------------|--------------------|-----------------|
| 1      | 55         | Male| I—II           | M0                 | 4*3*1.5         |
| 2      | 59         | Male| II—III         | M0                 | 4*2*1           |
| 3      | 71         | Female | II—III       | M0                 | 3.5*2*1.5       |

Table 1
Clinicopathological features of 3 ESCC patients for subjection of Agilent microarray analysis.

### Table 2
Differential expression of miRNAs in ESCC vs. normal tissues.

| Up-regulation     | Fold change | Down-regulation | Fold change |
|-------------------|-------------|-----------------|-------------|
| Hsa-miR-424       | 6.75        | Hsa-miR-195     | 2.63        |
| Hsa-miR-223       | 6.30        | Hsa-miR-133b    | 17.95       |
| Hsa-miR-21        | 3.93        | Hsa-miR-99a     | 4.01        |
| Hsa-miR-19a       | 2.79        | Hsa-miR-29c     | 2.12        |
| Hsa-miR-185       | 2.35        | Hsa-miR-145     | 10.72       |
| Hsa-miR-4306      | 2.31        | Has-miR-100     | 2.84        |
| Hsa-miR-20b       | 2.27        | Hsa-miR-1       | 9.54        |
| Hsa-miR-3651      | 2.21        | Hsa-miR-143     | 6.88        |
| Hsa-miR-106b      | 2.18        |                 |             |
| Hsa-miR-429       | 2.01        |                 |             |

Fig. 1. Differentially expressed miRNAs in esophageal squamous cell carcinoma as detected by microarray analysis. Three pairs of esophageal carcinoma and normal tissues were analyzed by Agilent microarrays. For each miRNA, yellow represents higher expression and blue represents lower expression than the average expression. T1, tumour1; N1, normal1; T2, tumour2; N2, normal2; T3, tumour3; N3, normal3.

Fig. 2. Taqman real-time PCR detection of miR-195 expression in ESCC, dysplasia, and normal tissues. (A) Loss of miR-195 expression in ESCC vs. normal tissues. N, normal; T, tumor. (B) miR-195 was not significantly down-regulated in dysplastic vs. normal tissues. N, normal; D, dysplasia. U6 mRNA served as an internal control. *P < 0.05.
Medical University. A written informed consent form was signed by each participant.

### 2.2. Cell lines and culture

Human ESCC cell lines TE13 and Eca109 were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). TE13 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Eca109 cells were cultured in Rosewell Park Memorial Institute (RPMI)-1640 medium with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Gaithersburg, MD, USA) in a humidified 5.0% CO₂ atmosphere at 37 °C.

### 2.3. miRNA microarray analysis

To profile differentially expressed miRNA in ESCC vs. normal tissues, we performed miRNA microarray analysis. In brief, total cellular RNA was first isolated from three pairs of ESCC tumor and corresponding distant non-cancerous tissues using a mirVana™ miRNA isolation kit (Applied Biosystem, Austin, TX, USA) and then reverse transcribed into cDNA probes using a miRNA complete labeling and Hyb kit (Agilent, Technologies, Inc., Santa Clara, CA, USA). After that, the labeled probes were hybridized onto three miRNA microarray (Agilent) according to the manufacturer's instructions. The arrays were scanned and the data were extracted and analyzed using Agilent Feature Extraction (v10.7) software and the Agilent GeneSpring software for data were normalized.

### 2.4. RNA isolation and qRT-PCR

Total RNA was isolated from tissue specimens and cell lines by using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and 10 ng of RNA samples were reverse transcribed into cDNA using a TaqMan MicroRNA reverse transcription kit. Real-time PCR was performed using standard TaqMan PCR reagents and TaqMan MicroRNA assays for hsa-miR-195 and U6 (Applied Biosystems). The U6 mRNA was used as an internal control for miR-195. Furthermore, RNA samples from ESCC cells were also reverse transcribed into cDNA by using a Reverse Transcriptase kit (TaKaRa, Dalian, China) for qRT-PCR detection of...

---

**Fig. 3.** Bioinformatic analysis of miR-195 targeting gene. 3' UTR of cdc42 mRNA contains a putative miR-195 target site. (A) Bioinformatics tools predicted that the binding region of miR-195 is localized at 23–29 nt of 3' UTR of Cdc42 mRNA. (B) Sequence analysis indicated that miR-195 target sequence at 23–29 nt of cdc42 3' UTR is highly conserved across different species.

**Fig. 4.** miR-195 regulation of Cdc42 expression by targeting its 3'UTR in ESCC cell lines. (A) Luciferase assay. The interaction of miR-195 with Cdc42 was confirmed by Luciferase assay in Eca109 cells. (B) Western blot. Expression of miR-195 or Cdc42 siRNA could inhibit expression of Cdc42 protein in Eca109 and TE13 cells. *P < 0.05. (C) Real-time PCR. Cdc42 mRNA was not regulated by miR-195 in Eca109 and TE13 cells.

**Fig. 5.** Effect of miR-195 mimics and inhibitor transfection on modulation of miR-195 expression in Eca109 and TE13 cell lines. **P < 0.01.
Cdc42 expression and GAPDH as an internal control using an SYBR Premix Ex Taq kit (Applied Biosystems). The Cdc42 primers were 5'-CCATCGGAAATGTTACCCGACTG-3' and 5'-CTCACCGTGTTATGCTCTTGTCA-3' and GAPDH primers were 5'-CGGACTCAACGGATTGGTGCTGAT-3' and 5'-AGCTTCTCTCAATGTTGAAGAC-3'. Each sample was run in triplicate. Expression levels of miRNA, mature mRNAs, or Cdc42 were calculated using the comparative CT method (2^{-\Delta\Delta C_T}).

2.5. Transient gene transfection into ESCC cell lines

hsa-miR-195 mimic (5'-UGACGACAGACAGAAUAUGGC-3' and 5'-CAUUACUGUGUCUGCUAUU-3') and negative control oligonucleotides (5'-UCCUCGGAACGUGUCAGCUTT-3' and 5'-ACGUGACGUCUCGAAATT-3'), miR-195 inhibitor (5'-GCCAUAAUUCUCUGUCUCUAU-3'), NC for miRNA-195 inhibitor (5'-CAUAAUUCUCUGUCUCAUU-3') and small interfering Cdc42 (si-Cdc42, 5'-UCCUACUUGAGAACUdtdtU-3' and 3'-dTdTGTGAGAUCUUAUUG-5') were purchased from Genepharma (Shanghai, China). ESCC cells were seeded and grown overnight and the next day, these RNA oligonucleotides were transiently transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. 48 h later, the cells were subjected to different experiments, as below.

2.6. Cell viability assay

TE13 and Eca109 cells transfected with or without transfection of aforementioned RNA oligonucleotides for 24 h, were seeded in 96-well plates (3000 cells/well) and cultured for up to 96 h. At the end of each experiment, reagents from the Cell Counting Kit-8 (CCK-8, Beyotime, Beijing, China) were added to each well, and the optical density (OD) was measured. The experiment was performed in 6 replicates and repeated once.

2.7. Colony formation assay

ESCC cells transfected with RNA oligonucleotides for 48 h and control ESCC cells were seeded into 6-well plates (300 cells/well) and cultured at 37°C for two weeks. At the end of the incubation

Fig. 6. Effect of miR-195 expression or Cdc42 knockdown on regulation of ESCC cell viability and clonogenicity. (A) Cell viability CCK-8 assay. Eca109 and TE13 cells were transfected with miR-195 mimic, Cdc42 siRNA, and miR-195 inhibitor grown for up to 4 days and then subjected to CCK-8 assay. (B) Colony formation assay. *P < 0.05.
period, the plates were washed twice with phosphate buffered saline (PBS) and fixed with methanol for 15 min before staining with 0.4% crystal violet at room temperature for 30 min. Plates were then washed with PBS and left to dry. The cell colonies with 50 cells or more were counted. Each assay was performed in triplicate.

2.8. Tumor cell migration and invasion assay

Tumor cell migration was assayed by using 24-well Transwell plates with 8 mm pore size (Millipore, Bedford, MA, USA). Briefly, cells after 48 h transfection were seeded into the top chambers with 0.2 ml culture medium containing $1 \times 10^5$ cells with 1% FBS, while 0.6 ml of 20% FBS-containing medium was added to the bottom chambers. After 48 h incubation at 37°C, cells that migrated onto the lower surface of the membrane were fixed with 95% ethanol for 15 min, stained with 0.4% crystal violet at room temperature for 30 min, and then counted in at least five random microscopic fields ($100 \times$). For the tumor cell invasion assay, the membranes were coated with Matrigel, and all other procedures were performed similarly as for the migration assay.

2.9. Flow cytometry

To analyze cell cycle distribution, we performed flow cytometry. In brief, the cells were transfected with the above named RNA oligonucleotides for 48 h and then harvested via trypsinization and washed with PBS twice, before fixing in 70% ethanol overnight at 4°C. The samples were then stained with propidium iodide (PI)/RNase and analyzed using fluorescence activated cell sorter (FACS, BD Biosciences, Mountain View, CA, USA) scan. The percentage of cells in G1/S/G2/M-phase was used to calculate the proliferative index.

2.10. Luciferase assay

To assay miR-195 binding to the Cdc42 3'-UTR region, we performed a luciferase assay. Specifically, we synthesized a fragment of the Cdc42 3'-UTR that contained the putative miR-195 binding sites and cloned downstream into the luciferase gene in the pGL3 luciferase vector (Invitrogen). For reporter assays, Eca109 cells were co-transfected with pGL3-3'-UTR-wt and miR-195 mimics or negative control oligonucleotides (NC) for 48 h. Firefly and Renilla luciferase activities were measured by Dual Luciferase.

Fig. 7. Regulation of cell cycle distribution by miR-195 expression. Eca109 (A) and TE13 (B) cells were transfected with miR-195 mimic or Cdc42 siRNA for 48 h and then subjected to flow cytometric analysis of cell cycle distribution. The results showed that miR-195 or Si-Cdc42 could induce cell cycle G1 arrest. *$P < 0.05$. 

M.-g. Fu et al. / FEBS Letters 587 (2013) 3471–3479

3475
Assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The data were normalized against the activity of the Renilla luciferase gene. Three independent experiments were performed in triplicate.

2.11. Protein extraction and Western blot

Total cell lysates were prepared by using a RIPA buffer (50 mM Tris–HCl pH 7.4, 10 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). Protein concentration was measured by the BCA method (Beyotime). Equal amounts of protein extracts were separated by 10% SDS–polyacrylamide gels and transferred onto PVDF membranes (Millipore). The membranes were then blocked with 5% non-fat milk powder at room temperature for 1 h and incubated with a primary antibody against Cdc42 or GAPDH (Cell Signaling Technology, Inc., Danvers, MA) at 4°C overnight and further incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Alpha Innotech (San Leandro, CA) imaging software was used to quantify Western blot data.

2.12. Statistical analysis

The data were summarized as mean ± S.D. and analyzed by using Student’s t test and Chi-square test using SPSS 16.0 software (SPSS, Chicago, IL). A P value less than 0.05 was considered to be statistically significant.

3. Results

3.1. miRNA expression profiling in human ESCC vs. normal tissues

In this study, we performed miRNA microarray analysis to profile differentially expressed miRNAs in ESCC vs. normal tissues. We detected expression of 1347 miRNAs in three pairs of ESCC vs. normal tissues. Taking a twofold difference as the cut-off point, we identified 10 up-regulated and 8 down-regulated miRNAs in these three pairs of ESCC vs. corresponding non-tumor tissues (Fig 1 and Table 2). Among these 18 differentially expressed miRNAs, many miRNAs had been shown in other studies to be differentially expressed in ESCC; for example, miR-223 was able to regulate migration and invasion by targeting Artemin in esophageal carcinoma cells [22] and miR-145 acts as a tumor-suppressor miRNA in ESCC by targeting FSCN1 [23]. Thus, we chose miR-195 for further study in ESCC.

3.2. Downregulation of miR-195 in ESCC tissues

Our study objective is to understand the molecular mechanisms responsible for ESCC progression; thus, we performed a bioinformatic search of TargetScan, miRDB, Miranda, and PicTar databases and found that miR-195 may target Cdc42, which controls diverse cellular functions including cell morphology, migration, endocytosis and cell cycle progression [24]. Thus, we further focused on miR-195 by detecting its expression in 18 pairs of ESCC tissues and 9 pairs of esophageal dysplasia tissues using Taqman Real-time PCR. We found that miR-195 expression was reduced in ESCC tissues (Fig. 2A, P < 0.05) but no statistically significant changes in dysplasia tissues were found compared to their corresponding normal tissues (Fig. 2B).

3.3. Expression of miR-195 inhibited Cdc42 expression in ESCC cell lines

Bioinformatic analysis of TargetScan, miRDB, Miranda, and PicTar databases showed that the miR-195 binding region was located at the 23–29 nt region of 3’UTR of Cdc42 gene and that the target sequences were highly conserved among different species (Fig. 3). To validate this, we performed a luciferase reporter assay and found that miR-195 transfection significantly decreased luciferase activities compared to the control oligonucleotide and pGL3-Cdc42-wt group. Fig. 4A (P < 0.05) suggested that Cdc42 is a target gene of miR-195. Moreover, we performed real-time PCR and western blot analysis of Cdc42 mRNA and protein levels respectively, in ESCC cells transfected with miR-195 mimic. Our data showed that Cdc42 protein levels were significantly reduced in miR-195-transfected tumor cells compared to the controls (Fig. 4B, P < 0.05). However, miR-195 did not modulate levels of Cdc42 mRNA in miR-195-transfected cells compared to the controls (Fig. 4C), indicating that this regulation is post-transcriptional.

3.4. Expression of miR-195 changed in ESCC cell phenotypes in vitro

As shown in Fig. 5, after transfected with miR-195 mimics and inhibitor, level of miR-195 expression in Eca109 and TE13 cells was up or downregulated compared to the control miRNAs.
We investigated the effects of miR-195 expression on regulation of tumor cell growth and found that overexpression of miR-195 or knockdown of Cdc42 expression slightly reduced viability of Eca109 and TE13 cells, whereas anti-miR-195 increased tumor cell viability (Fig. 5A). Moreover, the colony formation assay showed that miR-195 mimic or knockdown of Cdc42 expression suppressed colony forming efficiency of tumor cells, whereas anti-miR-195 increased colony forming efficiency of Eca109 cells (Fig. 6B).

In addition, the flow cytometry assay showed that tumor cells transfected with miR-195 mimics or Si-Cdc42 were arrested in the G1 phase of the cell cycle and that the S and G2/M phases were also significantly reduced compared to control cells (Fig. 7A and B).

3.5. Expression of miR-195 inhibited tumor cell migration and invasion

As displayed in Fig. 8, our data showed that tumor cell migration and invasion ability was significantly reduced in cells transfected with miR-195 mimics or Si-Cdc42 compared to the controls \((P < 0.05)\). In contrast, tumor cell migration and invasion ability was increased in cells transfected with anti-miR-195 oligonucleotides \((P < 0.05)\).

3.6. Expression of miR-195 suppressed Cdc42/ERK/Cyclin D1 signaling

To further investigate the underlying molecular events responsible for miR-195 actions, we analyzed the downstream signals of

---

**Fig. 9.** Regulation of gene expression by ectopic expression of miR-195 or Cdc42 knockdown. Eca109 and TE13 cells were transfected with miR-195b mimic or Cdc42 siRNA for 72 h and then subjected to Western blot analysis of gene expression. The quantitative data are shown in the graphs. *P < 0.05.
Cdc42 [such as extracellular signal-regulated kinase1/2 (ERK1/2), phosphorylation ERK1/2, and cyclin D1]. As shown in Fig. 9, phosphorylation levels of ERK1/2 and expression of cyclin D1 were decreased significantly in cells transfected with miR-195 mimics or Si-Cdc42, compared to cells transfected with control oligonucleotides.

4. Discussion

Recent studies have discovered many dysregulated miRNAs in human cancers, which play an important role in cancer development and progression [25,26]. Thus, in this study, we profiled differentially expressed miRNAs in three pairs of ESCC vs. distant non-tumor tissues. We found a large number of miRNA expressed in esophageal tissues and identified 18 differentially expressed miRNAs in ESCC tissues. miR-195 has been considered as a tumor suppressor in adenocarcinoma and hepatocellular cancer [27,28]. Thus, we chose miR-195 for further study and found that expression of miR-195 was down-regulated in ESCC and that expression of miR-195 inhibited tumor cell viability, migration, invasion, and colony formation in vitro. At the molecular level, miR-195 suppressed expression of Cdc42 protein, ERK1/2 phosphorylation and cyclin D1 expression. Our data indicate that altered expression of miRNAs could promote ESCC development and that lost miR-195 expression may contribute to ESCC progression.

To date, there are a number of published studies on miRNAs in esophageal cancer [29–31]. For example, Guo et al. [29] analyzed miRNA expression profile in 31 ESCC and adjacent normal tissue samples using miRNA microarrays. They found that the expression levels of some miRNAs were different between ESCC and normal tissues, and miR-103/107 expression was associated with poor survival [29]. Hu et al. [31] showed that all 10 analyzed miRNAs were altered in esophageal cancer tissues. Multivariate analysis showed an association between overall or disease-free survival of ESCC patients and miR-9, miR-16-2, miR-20, and miR-200a expression [31]. Another study showed that increased miR-21 expression in non-cancerous tissues of SCC patients was associated with worse prognosis of the patients [32]. However, the data from our current study did not show any overlap of these differentially expressed miRNAs with previous studies. The reason is not clear, but it may be because of different study population, technique used, and RNA sample preparation (for example, different levels of tumor and normal tissue mixture could affect the levels of miRNA expression).

Our current data showed downregulation of miR-195 expression in ESCC and in some esophageal dysplasia tissues compared to the matched distant normal tissues. However, the cause and mechanism responsible for down-regulation of miR-195 in ESCC remains unknown. Notably, miR-195 is a highly conserved miRNA cluster localized at chromosome 17P13.1 [33], which is a frequently deleted region in human cancers. In another aspect, Li et al. [34] showed that DNA methylation was responsible for miR-195 down-regulation in breast cancer. Thus, future studies will investigate the cause of lost miR-195 in ESCC.

Furthermore, our study showed that expression miR-195 reduced tumor cell viability, migration, invasion, and colony formation in vitro. At the molecular level, miR-195 suppressed expression of Cdc42 protein, ERK1/2 phosphorylation and cyclin D1 expression. Indeed, a previous study showed that Cdc42 was up-regulated in ESCC tissues and associated with lymph node metastasis [21]. Cdc42 functions by regulating cell motility, proliferation, and migration [24] by modulation of the PAK1, ERK1/2, JNK, and p38 gene pathways. Activation of Cdc42 can weaken c-Cbl-mediated EGFR degradation, promote EGFR hyperactivity, and induce proteasome degradation of p21[WF1], leading to an increase in cell proliferation and migration [35,36]. In addition, dominant-negative mutants of Cdc42 were able to control three major components of cellular signal transduction, namely, p53, Akt, and ERK1/2 signal cascades. In turn, this led to restraint of anchorage-independent growth and induced cell apoptosis [37]. In our study, we found that miR-195 was able to bind to the 3′ UTR of Cdc42 mRNA and suppressed expression of Cdc42 protein.

In conclusion, our study further showed that expression of miR-195 or knockdown of Cdc42 expression inhibited ERK1/2 phosphorylation and cyclinD1 expression in ESCC cells. Indeed, through binding to and activation of cyclin-dependent kinase 4 (cdk4)/cdk6, cyclinD1 can promote cell cycle progression [38]. Cyclin D1 is one of the D-type cyclins, reaches maximum activity in G1 period, and promotes cell cycle transition from G1 to S phase [39]. Welsh [40] found that Cdc42 contributed to promotion of cell cycle transition from G1 to S phase. Furthermore, we also found that miR-195 suppressed ESCC cell migration and invasion but we did not pursue the underlying signal transduction pathways. Thus, further investigation is needed to identify them.

In summary, our current study demonstrated that lost miR-195 expression contributed to ESCC progression through cell cycle arrest and inhibition of Cdc42 expression, which may be further evaluated as a novel target for ESCC therapy.

Acknowledgments

We thank Medjaden Bioscience Limited, Hong Kong, China, for assisting in preparation of this manuscript. This study was supported in part by a grant from the College graduate research and innovation projects of Jiangsu Province (No. CXZZ11_0705).

References

[1] Enzinger, P.C. and Mayer, R.J. (2003) Esophageal cancer. N. Engl. J. Med. 349, 2241–2252.
[2] Li, J.Y. (1982) Epidemiology of esophageal cancer in China. Natl. Cancer Inst. Monogr. 62, 113–120.
[3] Jermal, A., Siegel, R., Xu, J. and Ward, E. (2010) Cancer statistics, 2010. CA Cancer J. Clin. 60, 277–300.
[4] Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.
[5] Schmittgen, T.D. (2008) Regulation of microRNA processing in development, differentiation and cancer. J. Cell. Mol. Med. 12, 1811–1819.
[6] Liu, M., Lang, N., Qiu, M., et al. (2011) MiR-137 targets Cdc42 expression, induces cell cycle G1 arrest and inhibits invasion in colorectal cancer cells. Int. J. Cancer 128, 1269–1279.
[7] Song, Y.X., Yue, Z.Y., Wang, Z.N., et al. (2011) MicroRNA-148b is frequently down-regulated in gastric cancer and acts as a tumor suppressor by inhibiting cell proliferation. Mol. Cancer 10, 1.
[8] Zhang, X., Nie, Y., Du, Y., Cao, J., Shen, B. and Li, Y. (2012) MicroRNA-181a promotes gastric cancer by negatively regulating tumor suppressor KLF6. Tumour Biol. 33, 1589–1597.
[9] Yi, C., Wang, Q., Wang, L., et al. (2012) MiR-663, a microRNA targeting p21(WAF1/CIP1), promotes the proliferation and tumorigenesis of nasopharyngeal carcinoma. Oncogene 31, 4421–4433.
[10] Yuan, Y., Zeng, Z.Y., Liu, X.H., et al. (2011) MicroRNA-203 inhibits cell proliferation by repressing DeltaNp63 expression in human esophageal squamous cell carcinoma. BMC Cancer 11, 57.
[11] Tsuchiya, S., Fujiwara, T., Sato, F., et al. (2011) MicroRNA-210 regulates cancer cell proliferation through targeting fibroblast growth factor receptor-like 1 (FGFR1L1). J. Biol. Chem. 286, 420–428.
[12] Kong, K.L., Kwong, D.L., Chan, T.H., et al. (2012) MicroRNA-375 inhibits tumour growth and metastasis in esophageal squamous cell carcinoma through repressing insulin-like growth factor 1 receptor. Gut 61, 33–42.
[13] Xu, X., Chen, Z., Zhao, X., et al. (2012) MicroRNA-25 promotes cell migration and invasion in esophageal squamous cell carcinoma. Biochem. Biophys. Res. Commun. 421, 640–645.
[14] Kurashige, J., Watanabe, M., Iwatsuki, M., et al. (2012) Overexpression of microRNA-223 regulates the ubiquitin ligase FBXW7 in esophageal squamous cell carcinoma. Br. J. Cancer 106, 182–188.
[15] Zhang, H., Su, S.B., Zhou, Q.M. and Lu, Y.Y. (2009) Differential expression profiles of microRNAs between breast cancer cells and mammary epithelial cells. Ai Zhong 28, 493–499.
[16] Liu, L., Chen, L., Xu, Y., Li, R. and Du, X. (2010) MicroRNA-195 promotes apoptosis and suppresses tumorigenicity of human colorectal cancer cells. Biochem. Biophys. Res. Commun. 400, 236–240.
Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue. Clin. Cancer Res. 14, 2588–2592.

Etienne-Manneville, S. and Hall, A. (2002) Rho GTPases in cell biology. Nature 420, 629–635.

Abraham, M.T., Kuriakose, M.A., Sacks, P.G., et al. (2001) Motility-related proteins as markers for head and neck squamous cell cancer. Laryngoscope 111, 1285–1289.

Gomez, D.P.T., Valdes-Mora, F., Bandres, E., et al. (2008) Cdc42 is highly expressed in colorectal adenocarcinoma and downregulates ID4 through an epigenetic mechanism. Int. J. Oncol. 33, 185–193.

Feng, J.C., Liu, Q., Qin, X., et al. (2012) Clinicopathological pattern and Annexin A2 and Cdc42 status in patients presenting with differentiation and lymphnode metastasis of esophageal squamous cell carcinomas. Mol. Biol. Rep. 39, 1267–1274.

Li, S., Li, Z., Guo, F., et al. (2011) MiR-223 regulates migration and invasion by targeting Arterin in human esophageal carcinoma. J. Biomed. Sci. 18, 24.

Kano, M., Seki, N., Kikkawa, N., et al. (2010) MiR-145, miR-133a and miR-133b: tumor-suppressive miRNAs target FSCN1 in esophageal squamous cell carcinoma. Int. J. Cancer 127, 2804–2814.

Yang, L., Wang, L. and Zheng, Y. (2006) Gene targeting of Cdc42 and Cdc42GAP affirms the critical involvement of Cdc42 in filopodia induction, directed migration, and proliferation in primary mouse embryonic fibroblasts. Mol. Biol. Cell 17, 4675–4685.

Hammond, S.M. (2006) MicroRNAs as oncogenes. Curr. Opin. Genet. Dev. 16, 4–9.

Chen, C.Z. (2005) MicroRNAs as oncogenes and tumor suppressors. N. Engl. J. Med. 353, 1768–1771.

Soon, P.S., Tacon, L.J., Gill, A.J., et al. (2009) MiR-195 and miR-483-5p identified as predictors of poor prognosis in adenocortical cancer. Clin. Cancer Res. 15, 7694–7699.

Xu, T., Zhu, Y., Xiong, Y., Ge, Y.Y., Yun, J.P. and Zhuang, S.M. (2009) MicroRNA-195 suppresses tumorigenicity and regulates G1/S transition of human hepatocellular carcinoma cells. Hepatology 50, 113–121.