MicroRNA-133b inhibits cell proliferation and promotes apoptosis by targeting cullin 4B in esophageal squamous cell carcinoma

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Received May 21, 2017; Accepted December 8, 2017

DOI: 10.3892/etm.2018.5906

Abstract. microRNAs (miRs) serve a role as modulators during carcinogenesis. It has been demonstrated that the expression of miR-133b decreases in a variety of tumor tissues and cell lines and serves a suppressive role in the proliferation and apoptosis of different types of tumor cells. However, its effect on esophageal squamous cell carcinoma (ESCC) cells remains unclear. In the current study, the expression of mature miR-133b was measured using reverse transcription-quantitative polymerase chain reaction and the results indicated that miR-133b was significantly downregulated in ESCC tissues and various ESCC cell lines. The overexpression of miR-133b significantly inhibited the proliferation and promoted the apoptosis of KYSE150 and Eca-109 cells. Furthermore, it was demonstrated that cullin 4B (CUL4B) promotes ESCC cell proliferation and inhibits apoptosis by activating the protein kinase B/glycogen synthase kinase 3β/β-catenin pathway. Taken together, these results demonstrate that miR-133b/CUL4B serves a tumor suppressive role during ESCC progression and may therefore be used as a potential target to treat patients with ESCC.

Introduction

Esophageal cancer is the eighth most common cancer worldwide, with ~480,000 new cases and 400,000 associated mortalities per year (1). Histologically, there are two main forms of esophageal cancer: Esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC), which represents 90% of all esophageal cancer cases (2). Currently, surgery is the only way to treat patients with esophageal cancer and the overall 5-year survival rate is 14-18%. This is due to the fact that surgery can only be performed in a limited number of patients, as in many cases tumors are inoperable (3). Although genetic and epigenetic alterations underpin the development of ESCC, the molecular mechanisms underlying neoplastic progression remain unclear (4,5). Therefore, improving understanding of the molecular biology of ESCC is critical to facilitate the development of more effective diagnostic and therapeutic strategies for ESCC.

MicroRNAs (miRs) are small non-coding RNAs ~22 nucleotides long that bind to the 3’-untranslated region (3’-UTR) of target mRNA (6). Previous studies have demonstrated that different types of miRs regulate the proliferation, apoptosis, invasion, metastasis and the epithelial-mesenchymal transition of tumor cells (7,8). The aberrant expression of miRs has also been identified in ESCC (9,10). The upregulated expression of miR-21 may promote cell proliferation, migration and resistance to apoptosis via the phosphatase and tension homolog/phosphoinositide 3 kinase/protein kinase b signaling pathway in ESCC (11). miR-let-7 is downregulated in ESCC and is considered to be a tumor suppressor, as it regulates the interleukin-6/signal transducer and activator of transcription 3 signaling pathway during neoplastic progression (12). Additionally, it has been demonstrated that miR-142-3p expression is associated with histological differentiation and may be a potential independent prognostic factor in patients with ESCC following surgery (13). It has been demonstrated that miR-133b decreases the invasiveness of esophageal cancer by inhibiting the expression of fascin actin-bundling protein 1 (FSCN1) (14). However, the molecular mechanisms underlying the function of miR-133b in proliferation and apoptosis of esophageal cancer cells remains unknown.

The results of the current study demonstrated that miR-133b expression was downregulated in ESCC tissues and cell lines and its low expression was associated with the clinicopathological features of patients with ESCC. Overexpression of miR-133b in ESCC cell lines decreased cell proliferation and promoted cell apoptosis. Cullin 4B (CUL4B) has been identified as a direct target of miR-133b and it was demonstrated that miR-133b functions as a tumor suppressor by negatively regulating CUL4B expression. Furthermore, it was demonstrated that CUL4B promotes ESCC cell proliferation and inhibits apoptosis by activating the protein kinase B/glycogen synthase kinase 3β/β-catenin (AKT/GSK3β/β-catenin) pathway. Thus, the results of the present study suggest that miR-133b/CUL4B may be a promising therapeutic target for ESCC.
Materials and methods

Tissue collection. Primary ESCC tissues and adjacent non-tumor tissues samples (>5 cm from the edge of tumor) were obtained from 47 untreated patients (27 males and 20 females; mean age of 57 years) undergoing primary surgical resection at the Department of Cardiothoracic Surgery, Jinling Hospital (Nanjing, China) between January 2014 and August 2015. The clinical staging of tumors was performed using the seventh edition of the American Joint Committee on Cancer Staging Manual (15). Samples were snap-frozen in liquid nitrogen prior to RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The current study was approved by the Human Ethics Committee of Jinling Hospital and written informed consent was obtained from each study participant.

Cell lines and cell culture. Six human ESCC cell lines (TE-1, TE-8, KYSE150, KYSE450, Eca-109 and EC9706) and the normal human esophageal epithelial cell line HEECs (cat. no. BC05030029) were purchased from the Shanghai Institute of the Chinese Academy of Sciences (Shanghai, China). Another normal human esophageal epithelial cell line Het-1A was purchased from American Type Culture Collection (Manassas, VA, USA). TE-1, TE-8, Eca-109, EC9706 and Het-1A cells were cultured in Roswell Park Memorial Institute medium-1640 (Thermo Fisher Scientific Inc., Waltham, MA, USA), and KYSE150, KYSE450 and HEECs cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.). These media were supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.). Cultures were incubated for 72 h prior to collecting samples for subsequent western blotting and RT-qPCR analysis.

RT-qPCR. Tissues samples and culture cells were treated with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for total RNA extraction, following the manufacturer's protocols. For miR-133b quantification, 100 ng total RNA was reverse transcribed using the specific stem-loop RT primer and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used. The cDNA were quantified using a TaqMan MicroRNA assay (Applied Biosystems; Thermo Fisher Scientific, Inc.) and normalized to U6 RNA levels. The qPCR reaction conditions were as follows: Initial denaturation at 95°C for 10 min and subsequently 40 cycles of 95°C for 15 sec and 60°C for 1 min. For CUL4B mRNA analysis, cDNA was synthesized from 1 µg total RNA using PrimeScript™ RT reagent kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). qPCR was performed using a TaqMan RT-qPCR assay (Applied Biosystems; Thermo Fisher Scientific, Inc.) and normalized to GAPDH mRNA levels. The qPCR reaction conditions were as follows: Initial denaturation at 95°C for 5 min and subsequently 40 cycles of 95°C for 15 sec, 60°C for 15 sec and 70°C for 20 sec. Each sample was analyzed in triplicate using an ABI 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative expression levels were evaluated using the 2-ΔΔCT method (16). The following primers were used for qPCR: miR-133b forward, 5’-TTTTGTTCCCTTCAACAGCCT-3’ and reverse, 5’-GTCAGGCGTCCAGGT-3’; and CUL4B forward, 5’-GGGAAAGAAGGTGTTGAA-3’ and reverse, 5’-TCGATAGCCGCGTGTAG-3’. The primers used for the internal controls were as follows: U6 forward, 5’-CTCCGAGGCAGAGCA-3’ and reverse, 5’-AACGTCCTACAGATTGGCT-3’; and GAPDH forward, 5’-TCGACTCAACAGCGACACCCA-3’ and reverse, 5’-ACCTCGTTGCTGTAAGCAG-3’.

miR-133b transfection. The mature miR-133b sequence (5’-UUUGGGUCUCCCUACCGCUA-3’) was obtained from the miRBase database (http://www.mirbase.org/). Subsequently, 0.5 µg miR-133b cDNA sequence was synthesized and inserted into the pcDNA3.1 vector to generate a human pcDNA-miR-133b plasmid. For transfection, KYSE150 or Eca-109 cells (~5x10^5/well) were seeded in 6-well plates. After 24 h, the cells were transfected with 3 µg pcDNA-miR-NC control vector or pcDNA-miR-133b plasmids using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cultures were incubated for 72 h prior to cell proliferation and apoptosis assays.

Cell proliferation assay. Cell proliferation was determined by culturing ~5x10^6 cells/well on 96-well plates and cells were transiently transfected with 2 µg pcDNA-miR-133b/miR-NC or pcDNA-CUL4B empties plasmids (FulenGen, Guangzhou, China) using Lipofectamine™ 2000 after 72 h. 10 µl Cell Counting kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well and cells were incubated for 1 h at 37°C. Optical density was then detected at a wavelength of 450 nm using an Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Each sample was analyzed in triplicate and data analysis was performed using the mean of the results.

Cell apoptosis assay. Cell apoptosis was analyzed using an Annexin V-Fluorescein isothiocyanate (FITC) Apoptosis Detection kit (Beyotime Institute of Biotechnology, Haimen, China). Trypsinized cells were washed three times with PBS and stained with FITC-labeled anti-Annexin liposome and propidium iodide in the dark for 10 min at room temperature. Cells were then analyzed using the BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo 7.6.1 software (FlowJo LLC, Ashland, OR, USA).

Western blot analysis. Transfected cells were washed once in PBS and lysed in radioimmunoprecipitation lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China) and protein concentration was determined using a BCA protein assay kit. A total of 50 µg of protein was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto 0.22 µm polyvinylidene difluoride membranes, which was incubated with 5% fat-free skimmed milk in Tris-buffered saline containing 0.05% Tween-20 for 1 h at room temperature. Membranes were incubated overnight at 4°C with rabbit anti-CUL4B (cat. no. ab157103; 1:1,000; Abcam, Cambridge, UK), β-catenin (cat. no. ab32572; 1:1,000; Abcam), GSK-3β (cat. no. 9315; 1:1,000; Cell Signaling Technology, Danvers, MA, USA), phosphorylated (p)-GSK-3β (510 ATP (cat. no. ab57545; 1:1,000; Abcam) and β-actin (cat. no. sc-130656; 1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Membranes were incubated with specific antibodies (1:1,000 dilution) overnight at 4°C. After washing, membranes were incubated with horseradish peroxidase-labeled secondary antibodies (1:1,000 dilution) for 1 h at room temperature. Membranes were then developed using an Enhanced Chemiluminescence kit (GE Healthcare, Little Chalfont, UK), and images were captured using a ChemiDoc Imaging System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Results

miR-133b expression. RT-qPCR analysis revealed that miR-133b levels were significantly higher in tumors when compared to adjacent normal tissues (Fig. 1A). Additionally, the results from western blot analysis demonstrated lower expression of CUL4B in tumors compared to surrounding normal tissues (Fig. 1B). Therefore, miR-133b was selected for overexpression experiments in vitro.
Luciferase reporter assays. The 3'-UTR of CUL4B with wild-type or mutant binding sites for miR-133b was amplified and subcloned into the pGL3 vector (Promega Corp., Madison, WI, USA), respectively. Eca-109 and KYSE150 cells were co-transfected with 150 ng miR-133b or miR-negative control plasmids and 50 ng pGL3-CUL4B wild-type or mutant reporter plasmids using Lipofectamine 2000. A total of 48 h following transfection, the Dual-luciferase assay kit (Promega, Madison, WI, USA) was used to determine the luciferase activity. Renilla luciferase activity was normalized to the Firefly luciferase activity. Each experiment was performed in triplicate.

Biinormatic analysis. The target gene of miR-133b was identified and compared using the online target prediction algorithms miRanda (http://www.microrna.org/), miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/predictedmirnagene.html) and PicTar (http://www pictar.org/).

Statistical analysis. All statistical analyses were performed using SPSS software 15.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard error of the mean from ≥3 independent experiments. Statistical significance was evaluated using a Student's t-test (two-tailed), one-way analysis of variance followed by a Tukey's post hoc test and the Mann-Whitney test. Pearson's correlation analysis was used to measure the correlation between CUL4B and miR-133b expression. P<0.05 was determined to indicate a statistically significant difference.

Results

miR-133b expression is downregulated in human ESCC tissues and cell lines. The biological function of miR-133b in the pathogenesis of ESCC was investigated by examining miR-133b expression in 47 paired ESCC and adjacent non-tumor tissues by RT-qPCR. miR-133b expression was significantly downregulated in ESCC tissues compared with adjacent normal tissues (P<0.01; Fig. 1A). Similarly, miR-133b expression in the six ESCC cell lines TE-1 (P<0.01), TE-8 (P<0.05), KYSE150 (P<0.05), KYSE450 (P<0.05), Eca-109 (P<0.01), and EC9706 (P<0.05), was significantly lower than that in the normal esophageal epithelial cell lines Het-1A and HEEC (Fig. 1B). miR-133b expression was also associated with tumor stage (P<0.05), tumor size (P<0.05) and differentiation status (P<0.05); however, it was not associated with other clinicopathological factors of patients with ESCC (Table I).

CUL4B is a correlated target gene of miR-133b in ESCC cells. The target gene of miR-133b in ESCC was identified using the online target prediction algorithms. Based on the Gene Expression Omnibus database analysis (17), it was revealed that human CUL4B, an important ubiquitination molecule associated with apoptosis, contained the conserved putative miR-133b target site (Fig. 2A). Dual-luciferase reporter analysis indicated that co-expression of miR-133b significantly inhibited luciferase activity in KYSE150 (P<0.01) and Eca-109 (P<0.05) cells containing the CUL4B-3'UTR reporter plasmid, compared with those containing the mutant plasmid (Fig. 2B and C). Endogenous expression of CUL4B was significantly inhibited following transfection of miR-133b in KYSE150 and Eca-109 cells compared with the control (P<0.05; Fig. 2D). Furthermore, CUL4B expression was significantly upregulated in ESCC tissues compared with adjacent non-tumor tissues (P<0.05; Fig. 2E) and there was a negative correlation between miR-133b and CUL4B mRNA levels in ESCC tissues (Fig. 2F). These results indicate that CUL4B is a direct target of miR-133b and suggest that miR-133b may exert its effect by inhibiting CUL4B expression.

miR-133b inhibits cell proliferation and promotes apoptosis by targeting CUL4B in ESCC. The biological function of miR-133b/CUL4B in ESCC was analyzed by transfecting miR-133b and/or CUL4B into KYSE150 and Eca-109 cells (Fig. 3A). The role of miR-133b/CUL4B on the proliferation of ESCC cells was investigated using a CCK-8 assay and the results indicated that overexpression of miR-133b significantly decreased the proliferation of KYSE150 and Eca-109 cells compared with cells transfected with the negative control (P<0.05; Fig. 3B). However, reintroduction of CUL4B into miR-133b-transfected KYSE150 or Eca-109 cells significantly reversed the effects of miR-133b on ESCC cell proliferation (P<0.05; Fig. 3B). Additionally, overexpression of miR-133b significantly increased apoptosis in KYSE150 cells, which was significantly reversed when CUL4B was reintroduced into miR-133b-transfected KYSE150 cells (all P<0.05; Fig. 3C and D). In Eca-109 cells, miR-133b overexpression also significantly increased levels of apoptosis and the reintroduction of CUL4B inhibited the effects of apoptosis (P<0.05; Fig. 3E and F).

miR-133b inhibits the AKT/GSK3β/β-catenin pathway by downregulating CUL4B in ESCC. It has been demonstrated that CUL4B activates AKT/GSK3β/β-catenin signaling and may promote proliferation and invasion in malignant neoplasms (18). Therefore, it was hypothesized that miR-133b/CUL4B inhibits ESCC cell proliferation and promotes apoptosis by inhibiting AKT/GSK3β/β-catenin signaling pathway. After transfection of miR-133b or miR-NC plasmid, CUL4B, GSK-3β, p-GSK-3β(Tyr216) and β-catenin levels were measured using western blot analysis. The results indicated that the expression of GSK3β (P<0.01) and p-GSK3β (P<0.05) were significantly increased in KYSE150 cells transfected with miR-133b compared with those transfected with the negative control (Fig. 4A and B). By contrast, the expression of the CUL4B (P<0.01) and downstream proliferation/apoptosis-associated target protein β-catenin (P<0.05) were significantly decreased. Similarly, levels of GSK3β and p-GSK3β were significantly increased (P<0.05), but the levels of CUL4B and β-catenin were
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significantly decreased (P<0.05) in Eca-109 cells transfected with miR-133b compared with cells transfected with negative control (Fig. 4C and D). Taken together, these results indicate that miR-133b/CUL4B may affect ESCC cell proliferation and apoptosis by regulating the AKT/GSK3β/β-catenin pathway.

Discussion

miR-133b is a muscle-specific molecular marker, which serves a role in skeletal muscle development, myoblast differentiation and myogenic-associated disease (19,20). It has been demonstrated that miR-133b serves a suppressive role during tumor
growth, invasion, metastasis and apoptosis. miR-133b inhibits gastric cancer cell metastasis in vitro and in vivo by directly suppressing the expression of zinc finger protein Gli1 (21). miR-133b promotes the apoptosis and inhibits the proliferation of osteosarcoma cells by directly targeting B cell lymphoma-2 like protein (22). Downregulation of miR-133b in colorectal cancer tissues compared with adjacent non-tumorous tissues is associated with the poor survival of patients (23). Furthermore, it has been demonstrated that miR-133b inhibits the invasiveness of esophageal types of cancer by inhibiting FSCN1 expression (14). However, the molecular mechanisms of miR-133b in ESCC cell apoptosis and proliferation remain unknown.

The results of the present study demonstrated that miR-133b significantly decreased tumor cell proliferation and promoted apoptosis in vitro. These results indicate that miR-133b may be used as a novel method of treating patients with ESCC. miR-133b levels were also highly associated with tumor stage and differentiation status; differentiation-associated miR-133b levels may be used to predict tumor progression in patients with ESCC that have undergone surgery; however, further studies are required to validate this.

It was also demonstrated that miR-133b/CUL4B served a role in ESCC cell growth and apoptosis. CUL4B is a member of the cullin family and forms a complex that functions as an E3 ubiquitin ligase and catalyzes the polyubiquitination of specific protein substrates in the cell (24,25). Previous studies have demonstrated that CUL4B expression is significantly upregulated in various types of human cancer, promoting cell proliferation, invasion and tumorigenesis (26-28). For example, CUL4B promotes the proliferation and inhibits the apoptosis of osteosarcoma and glioblastoma cells (26,27). In addition, CUL4B is a novel prognostic marker correlating with colon cancer pathogenesis and progression (28). The mechanism
Figure 3. miR-133b inhibits cell proliferation and promotes apoptosis by targeting CUL4B in ESCC. (A) KYSE150 and Eca-109 cells were co-transfected with miR-133b/miR-NC and CUL4B/empty vectors for 72 h and the expression of CUL4B was evaluated by western blotting. (B) Overexpression of miR-133b inhibited the proliferation of ESCC cells and these effects on cell proliferation were significantly reversed by transfection with CUL4B. (C) Flow cytometric analysis of apoptosis in KYSE150 cells. (D) Overexpression of miR-133b in KYSE150 cells significantly increased cell apoptosis and these effects on apoptosis were significantly reversed by transfection with CUL4B. (E) Flow cytometric analysis of apoptosis in KYSE150 cells. (F) Overexpression of miR-133b in Eca-109 cells increased cell apoptosis and the effects of apoptosis were significantly reversed by transfection with CUL4B. Data are expressed as the mean ± standard error of the mean. Each sample was analyzed in triplicate. *P<0.05, **P<0.01. CULB4B, cullin 4B; miR-133b, microRNA-133b; miR-NC, microRNA negative control; ESCC, esophageal squamous cell carcinoma; PI, propidium iodide; FITC, fluorescein isothiocyanate.

Figure 4. CUL4B activates the AKT/GSK3β/β-catenin pathway in ESCC. (A) Levels of CUL4B, GSK-3β, p-GSK-3β and β-catenin expression were analyzed by western blot analysis in KYSE150 cells transfected with miR-NC or miR-133b. (B) Quantification of western blot analysis. (C) Levels of CUL4B, GSK-3β, p-GSK-3β and β-catenin expression were analyzed by western blot analysis in Eca-109 cells transfected with mi-NC or miR-133b. (D) Quantification of western blot analysis. Data are expressed as the mean ± standard error of the mean. Each sample was analyzed in triplicate. *P<0.05, **P<0.01 vs. miR-NC. CUL4B, cullin 4B; miR-133b, microRNA-133b; miR-NC, microRNA negative control; ESCC, esophageal squamous cell carcinoma; Akt, protein kinase b; p-, phosphorylated; GSK-3β, glycogen synthase kinase 3β; NC, negative control.
underlying CUL4B function in cancer progression remains unclear; however, CUL4B may serve a role in epigenetic changes, including heterochromatin formation, histone modification, parental imprinting or X-chromosome inactivation (29,30). CUL4B also promotes cell cycle progression and tumorigenesis via degradation of numerous cyclin-dependent kinase inhibitors or p53 protein (31,32). In addition, CUL4B serves an important role in stabilizing β-catenin against proteasomal degradation in multiple signaling pathways. The results of the current study indicate that CUL4B activates the AKT/GSK3β/β-catenin pathway and may affect ESCC cell proliferation and apoptosis by regulating this pathway. These results provide important insights into the CUL4B pathway and shed light on the functional importance of proliferation and apoptosis in ESCC.

In conclusion, the current study demonstrated that miR-133b is downregulated in ESCC, and its expression is associated with advanced tumor stage and the differentiation status of patients with ESCC. Additionally, it was determined that miR-133b serves a crucial role in inducing proliferation and apoptosis by directly targeting CUL4B and may therefore be a novel therapeutic target to treat patients with ESCC.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

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

Authors' contribution

HH designed methods and experiments, performed the laboratory experiments and analyzed the data. YX and ZG co-designed the cell proliferation and apoptosis experiments and collaborated on associated data collection and their interpretation. XC and SJ collaborated on bioinformatics analysis and statistical analysis. ZX co-designed experiments, discussed analyses, interpretation, presentation and wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was performed in accordance with the Declaration of Helsinki and approved by the Human Ethics Committee of Jinling Hospital. Written informed consent was obtained from each study participant.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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