MiR-203 suppresses tumor growth and invasion and down-regulates MiR-21 expression through repressing Ran in esophageal cancer

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1. Introduction

Esophageal cancer is sixth leading cause of cancer death worldwide with a variable geographic distribution. Two main histological types have been identified, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), regarding to different etiologic and pathologic characteristics. EAC is common in western countries while ESCC is frequent in east Asia, especially in China whose incidence in the high-risk northern and central China exceeds 100 cases per 100,000 people per year [1]. Despite improvements in the diagnosis and treatment of this cancer, the overall survival for advanced and metastatic esophageal cancer is still poor, with a 5-year survival rate of less than 20% after surgery in China [1].

Thus, there is a great need to disclose molecular mechanisms of pathogenesis of esophageal cancer to identify more tumor specific biomarkers and therapeutic targets for early diagnosis and treatment. The pathogenesis of esophageal cancer still remains unclear so far. It has been shown that both genetic and epigenetic alterations may be involved in the progress of this cancer. Genetic abnormalities such as gene amplification and mutation, chromosomal loss and gain or loss of heterozygosity (LOH) are important etiological factor involved in tumorigenesis of esophageal cancer [2]. Recently, epigenetic disruption such as aberrant CpG island methylation has been demonstrated as a key event in the development of esophageal cancer via regulating the expression of tumor-related genes and microRNAs (miRNAs) [3].

MiRNAs are an endogenous class of 20–25 nucleotide-long single-stranded non-coding RNAs that regulate gene expression at the posttranscriptional level by binding to the 3′-untranslated region (3′-UTR) of mRNA which subsequently leads to mRNA degradation and translation repression. Previous studies have demonstrated that a single miRNA may target multiple genes due to the imperfect complementarity with target mRNA, while the expression of a single gene may be modulated by different miRNAs [4]. The remarkable performance of individual miRNAs to modulate multiple transcripts allows miRNAs to control a variety of physiological processes such as cell proliferation, differentiation and apoptosis. Thus, alterations in miRNA expression are thought to play critical roles in cancer initiation and progression through regulating the expression of various oncogenes and tumor suppressive genes [5].

Over the past few years, aberrant miRNA expression profiles were reported in esophageal cancer tissues compared with matched non-malignant tissues [3,6]. MiR-21 was observed to be frequently up-regulated in ESCC cell lines and tumor tissues [7].
2. Materials and methods

2.1. Plasmid constructs

Hsa-miR-203 (miR-203) precursor was purchased from Oncor (Cologne, Germany) and inserted into pcDNA3.1-ZEO/miR vector (named pcDNA/miR-203) according to the manufacturer’s instructions (Invitrogen, USA). Scramble miRNA was cloned into the same vector and used as negative control (named pcDNA/miR-NC). The sequence of control miRNA is 5′-AGCTGAGAACCCTAAGAAT-3′. To construct luciferase reporter vector, the human small GTPase Ran 3′-UTR containing putative binding sites for miR-203 was amplified by PCR and cloned in pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA) into restriction sites Sac-1 and Suf-1. The construct was named pmirGLO-Ran. The primers used were as follows: 5′-CTAAGGAGAATGAGTTGTATT-3′ (Forward primer for Ran 3′UTR), 5′-GATAATTGAAAATACCTTATTTC-3′ (Reverse primer for Ran 3′UTR). As a control, pmirGLO-Ran-Mut vector was constructed by site-directed mutagenesis. Viable cells were counted by trypan blue exclusion method with a hemacytometer. The expression of miR-203 was confirmed by real-time PCR. Real-time PCR was used to detect the expression of mature miR-203, miR-21 and the expression of primary transcript of small GTPase Ran. For gene expression analysis, 1 μg total RNA was transcribed in a final volume of 20 μl to synthesize first-strand cDNA using ImProm-II® reverse transcription system (Promega, USA) according to the instructions of the manufacturer. The transcript levels were detected by real-time PCR using Brilliant II SYBR® green qPCR master mix (Stratagene, USA) to monitor amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize expression.

2.2. Cell proliferation, colony formation and apoptosis analysis

2.3. Clinical samples

Twelve pairs of primary esophageal squamous cell carcinoma tissues and corresponding adjacent normal esophageal tissues were obtained from patients in Jieyang City People’s Hospital with informed consent and agreement. All tissue samples were collected from untreated patients undergoing surgery and were immediately frozen in liquid nitrogen and stored at –80 °C until the extraction of total RNA.

2.4. Quantitative real-time PCR (qRT-PCR)

Real-time PCR was used to detect the expression of mature miR-203, miR-21 and the expression of primary transcript of small GTPase Ran. Total RNA was extracted from cultured cells or frozen tissues using Trizol reagent (Invitrogen, USA) following the protocols of the manufacturer. For miRNA expression analysis, 1 μg of total RNA was applied for reverse transcription to synthesize miRNA cDNA using NCored™ Vilo™ miRNA cDNA synthesis kit (Invitrogen, USA). qRT-PCR analysis was performed using EXPRESS SYBR® GreenER™ miRNA qRT-PCR kit (Invitrogen, USA) based on manufacturer’s instructions. 20 μl of the reverse transcription mix was amplified by PCR with denaturation at 95 °C for 2 min and 40 cycles at 95 °C for 10 s and 60 °C for 1 min. U6 was used as an endogenous control for normalization. Each sample was analyzed in triplicate.

Primer sequences used were as follows: 5′-GCCGTAAATGTTGACACCGTAGC-3′ (RT primer for miR-203), 5′-GCTAGCTTATGACACGTGTA-3′ (RT primer for miR-21), 5′-GTCGCGACGGACGATATACAA-3′ (RT primer for U6). For gene expression analysis, 1 μg total RNA was reverse transcribed in a final volume of 20 μl to synthesize first-strand cDNA using ImProm-II® reverse transcription system (Promega, USA) following the protocols of the manufacturer. The transcript levels were detected by real-time PCR using Brilliant II SYBR® green qPCR master mix (Stratagene, USA) to monitor amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to normalize expression. 25 μl PCR reactions in triplicate were carried out by an initial denaturation at 95 °C for 10 min followed by 40 cycles, each consisting of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C and then 1 cycle for melting curve consists 1 min at 95 °C, 30 s at 55 °C, 30 s at 95 °C. Primer sequences used were as follows: 5′-TGCTGCTTCTAGAAAAGCTCAT-3′ (Forward primer for Ran), 5′-ACGCCTGACATCA-CAGGAC-3′ (Reverse primer for Ran), 5′-AGAGGGCTGGGTCATTTG-3′ (Forward primer for GAPDH), 5′-AGGCCCTGCACACGCTTC-3′ (Reverse primer for GAPDH). The 2−ΔΔCt method for relative quantification of gene expression was used to determine miRNAs and Ran mRNA expression levels. Real-time PCR primers were designed using Primer Express software (version 5.0, Applied Biosystems).

2.5. Cell proliferation, colony formation and apoptosis analysis

2.6. Transwell migration and invasion assays

2.7. Lentivirus production and transduction

Lentiviral particles expressing wild-type human small GTPase Ran were produced with ViraPower Lentiviral Packaging Kit (Invitrogen, USA). The coding region of human small GTPase Ran was amplified by PCR and subcloned into EcoRI and XhoI sites of pLent6.3/CMV/VS-MSVG/locZ lentiviral vector (Invitrogen, USA) to generate pLCM-Ran construct. The primers used are 5′-CCGATTGCCCGCCATGTTGCCGGGGGAG-3′ (Forward primer) and 5′-CCCTCGAGTCACAGGTCATCATCC-3′ (Reverse primer). The EmGFP-expressing lentiviral vector derived from pLent6.3/ CMV/VS-MSVG/locZ lentiviral vector was co-transfected with pLent6.3/CMV/VS-MSVG/locZ lentiviral vector. The infection efficiency of the viral particles was determined by flow cytometry (Beckman, USA). For apoptosis analysis, miR-203 and control-transfected cells were processed by using PE annexin V apoptosis detection kit (BD, USA) in compliance with the manufacturer’s protocol and subjected to flow cytometric analysis (Beckman, USA).
were collected and centrifuged at 3000 rpm for 10 min at 4 °C to remove the debris. Viral particles were stored at −80 °C after filtration with 0.45 μm filters (Millipore, USA). The titers of lentiviral stocks were determined by flow cytometry.

The human full-length GTPase Ran was eventually transduced into miR-203 transfected-Ec-109 cells by lentiviral infection (named miR-203 + pLClM-Ran). Lentiviral vector was introduced into miR-203 transfected-Ec-109 (named miR-203 + pCLM) and miR-NC cells (named miR-NC + pCLM) which were used as negative controls. The expression of wild-type human Ran in miR-203 transfected-Ec-109 cells was visualized by Western-blot. Transduction efficiency was optimized and assessed by flow cytometry for GFP-positive cells.

2.8. Soft-agar colony assay

Anchorage-independent cell growth was assayed in 6-well plates. 1 × 10⁰ Ec-109 cells were seeded in 2 ml of standard growth medium containing 0.3% low-melting-temperature soft-agar (Lonza, USA) and plated onto solidified 0.7% soft-agar base layer dissolved in complete culture medium in 6-well plates. Cultures were fed once a week with 0.5 ml of complete medium. Triplicate samples were prepared for each transfectant and the experiment was repeated twice. Colonies becoming visible microscopically after 4 weeks were photographed with an Olympus phase-contrast microscope.

2.9. In vivo tumorigenesis assay

Female athymic BALB/c nude mice (4–6 weeks) were purchased from Laboratory Animal Center of the Army Research Center (Beijing, China). All animals were housed at pathogen-free condition. Animal experiments were performed in accordance with current guidelines for the Care and Use of Laboratory Animals of Army Research Center. All experimental procedures were conducted with the guidelines provided by NIH (National Cancer Institute, USA). Website: http://nihfrederick.cancer.gov/Laspi/Arcic/Frederick/GuidelinesFlmLa.jsp). For xenografts, nude mice were divided into 4 groups (n = 8 for each) and were subcutaneously (s.c.) inoculated into the right flanks with 2 × 10⁶ or 1 × 10⁶ of miR-203-transfected or scramble control-transfected cells, respectively. Mice (n = 8) injected with 2 × 10⁶ of Ec-109 cells were used as positive control. For inoculation of Ran-restored cells, mice (n = 8 for each) were injected s.c. with 2 × 10⁶ of Ec-109, miR-203 + pCLM-Ran, miR-203 + pCLM and miR-NC + pCLM cells, respectively. Tumor sizes were measured every 5 days using caliper. Tumor volumes (cm³) were calculated by using the following standard formula: length × width² / 2. Mice were sacrificed and photographed at 5 weeks postinjection.

2.10. Western blotting analysis

Cultured cells were harvested and lysed with RIPA lysis buffer (Beyotime, China) for 10 min on ice. After centrifugation at 12,000g for 10 min, the concentration of proteins was measured using Bradford’s reagent (Bio-Rad laboratories, USA). The protein samples were denatured by boiling for 10 min and loaded onto SDS–PAGE (12%) gel for electrophoresis. The proteins were transferred onto PVDF membrane (Millipore, USA) which was then incubated in the blocking solution (3% non-fat dried milk) at room temperature for 1 h. The anti-Ran antibody (Novus, USA) was used and smaller colonies in soft agar compared with control transfectants (Fig. 1C). For tumor formation assay in nude mice, animals (n = 8) were initially inoculated s.c. with 2.0 × 10⁶ of miR-203 or scramble control-transfected cells, respectively. Animals (n = 8) injected s.c. with 2.0 × 10⁶ of Ec-109 cells were used as positive control. Tumor growth was evaluated for 35 days after injection. Obvious tumor formation was detected in all of 8 nude mice inoculated with 2.0 × 10⁶ of Ec-109 cells or scramble control-transfected cells. In contrast, no significant tumor formation was observed in nude mice inoculated with 2.0 × 10⁶ of miR-203-transfected cells (Fig. 1D).

We thereafter decided to examine tumor formation by increasing the numbers of injected cells. 1 × 10⁷ of miR-203 or scramble control-transfected cells were subsequently inoculated in nude mice (n = 8 for each). After 35 days, tumor growth was observed in 3 of 8 nude mice inoculated with miR-203-transfected cells. In contrast, all 8 control mice developed tumors. However, evaluation of tumor volumes and tumor weights at the sacrifice time eventually showed that tumors induced by miR-203-transfected cells were much smaller and lighter than those induced by control cells (Fig. 1E–G). Furthermore, a higher expression of miR-203 was detected in tumors induced by miR-203-transfected cells relative to the control (Supplementary Fig. 4). Inhibitory effects of miR-203 on the migration and invasion as well as tumorigenicity of esophageal cancer cells suggest that miR-203 plays a tumor-suppressing role in ESCCs.

3. Results

3.1. Overexpression of miR-203 induces cell apoptosis, suppresses cell proliferation, migration and invasion and inhibits tumorigenicity

The expression of miR-203 has been reported to be significantly down-regulated in ESCC [3]. However, the involvement of miR-203 in the pathogenesis of esophageal cancer is still far from being fully elucidated. In current studies, to investigate the potential impacts of miR-203 on the proliferation, apoptosis, migration, invasion and tumorigenicity of esophageal cancer cells, miR-203 precursor and scramble miRNA was cloned and exogenously expressed in esophageal cancer cells. Stably transfected cells were selected by blasticidin. The ectopic expression of mature miR-203 in stable transfectants was confirmed by qRT-PCR (Supplementary Fig. 1).

In order to investigate the impact of miR-203 on cell proliferation and cell cycle progress, trypan blue exclusion assay was carried out to measure cell growth and cell cycle analysis was conducted with flow cytometry. The data showed that overexpression of miR-203 suppressed the proliferation of Ec-109 cells (Supplementary Fig. 2A), modulated cell cycle by inducing G1 arrest and decreased the percentages of Ec-109 cells in S phase compared to control (Supplementary Fig. 2B). Our observations are consistent with previous report [19]. Resistance to apoptosis is a critical hallmark of tumor cells for limitless proliferation. Flow-cytometric analysis showed that the proportion of early apoptotic cells was significantly increased in miR-203-transfected Ec-109 cells compared with control cells. The early apoptosis rate reached 25.8% in miR-203-transfected cells compared to 15.4% in control cells, indicating that ectopic expression of miR-203 could trigger apoptosis enhancement in Ec-109 cells (Supplementary Fig. 3).

Transwell migration and matrigel invasion assays were performed to assess the effects of miR-203 on cell migration and invasion. As shown in Fig. 1A and B, compared to the scramble control, exogenous expression of miR-203 could effectively repress the migration ability and invasion capacity of Ec-109 cells by about 32.4% (p < 0.01) and about 76.8% (p < 0.01), respectively, indicating suppressive effects of miR-203 on the migration and invasion of ESCCs.

We next examined the effects of miR-203 on the tumorigenicity of esophageal cancer cells. Anchorage-independent growth assay demonstrated that miR-203-transfected cells generated much fewer and smaller colonies in soft agar compared with control transfectants (Fig. 1C). For tumor formation assay in nude mice, animals (n = 8) were initially inoculated s.c. with 2.0 × 10⁶ of miR-203 or scramble control-transfected cells, respectively. Animals (n = 8) injected s.c. with 2.0 × 10⁶ of Ec-109 cells were used as positive control. Tumor growth was evaluated for 35 days after injection. Obvious tumor formation was detected in all of 8 nude mice inoculated with 2.0 × 10⁶ of Ec-109 cells or scramble control-transfected cells. In contrast, no significant tumor formation was observed in nude mice inoculated with 2.0 × 10⁶ of miR-203-transfected cells (Fig. 1D).

In order to disclose the molecular mechanism through which miR-203 realizes its tumor-suppressive functions, computational prediction using open access websites including TargetScan (http://www.targetscan.org), PicTar (http://pictar.mdc-berlin.de)
and MiRanda (http://www.microrna.org) and the target prediction methods previously reported [20,21] were adopted to identify functionally relevant targets of miR-203. Small GTPase Ran was found to be the target gene of miR-203, which contains putative target sequence. Compared to control, the decreased expression of this protein in miR-203-transfected cells was confirmed by both Western-blot (Fig. 2A) and real-time PCR (Fig. 2B).

We further tested whether Ran protein is a direct target of miR-203 by luciferase assay. 3'-UTR region of Ran-encoding gene containing putative binding site of miR-203 and its site-directed substitution mutant (Fig. 2C) were cloned into luciferase reporter vector (named PmiR-GLO-Ran and PmiR-GLO-Ran-mut, respectively) and co-transfected with miR-203, respectively. Cells co-transfected with empty vector (PmiR-GLO-vector) and...
miR-203 were used as negative control (pmiR-GLO-NC). The relative luciferase activity was significantly down-regulated by about 43.5% in the reporter construct containing wild type 3'-UTR compared to the mutant and empty vector control (Fig. 2D). These results suggest that small GTPase Ran is a direct downstream target gene of miR-203 and miR-203 suppresses the expression of Ran through directly binding to its 3'-UTR.

3.3. MiR-21 is down-regulated by miR-203 through targeting small GTPase Ran

MiR-21 has been identified as the most commonly overexpressed oncogenic miRNA in solid tumors including ESCC [22,23]. In order to verify whether ectopic expression of miR-203 could influence the expression of miR-21, we compared the expression of miR-21 in miR-203- and vector control-transfected Ec-109 cells by real-time PCR. The results showed that the expression of miR-21 was significantly reduced in miR-203-transfected Ec-109 cells to about 45.8% of the control (p < 0.01) (Fig. 3A), suggesting that miR-203 could be a potential negative regulator for miR-21 expression.

Following this confirmation, the expression level of miR-21 was analyzed by real-time PCR in siRNA-425-Ran- and scramble control siRNA-transfected cells. The results showed that the expressions of miR-21 was remarkably down-regulated in Ran specific siRNA-transfected Ec-109 cells compared to control-transfected cells (p < 0.01) (Fig. 3D). These data suggest that the decreased expression of miR-21 correlates with the specific Ran silencing by siRNA suppression and miR-203 may down-regulate miR-21 expression through repressing Ran. However, the analysis also showed that the expression of Ran and miR-21 was lower in tumors induced by miR-203-transfected cells than in those induced by scramble control miRNA-transfected cells (Supplementary Fig. 5). Data obtained suggest that miR-203 may be one of the critical events that modulate the expression of oncogenic protein small GTPase Ran and oncomir miR-21 in esophageal cancer.

3.4. Restoration of Ran counteracts the tumor suppressive effects of miR-203

We identified here that miR-203 exerted tumor suppressive effects in ESCCs and small GTPase Ran was its direct target gene. To investigate whether the regulatory effects of miR-203 on the apoptosis, proliferation, migration, invasion and tumor formation of Ec-109 cells are mediated by Ran, we cloned ORF of wild-type human Ran without its 3'-UTR into lentiviral expression vector to avoid miRNA interfering. Ran was exogenously expressed in miR-203-transfected Ec-109 cells by lentivirus infection (named miR-203 + pLCM-Ran). Empty lentiviral vector was introduced in miR-203-transfected Ec-109 cells (named miR-203 + pLCM) and miR-NC cells (named miR-NC + pLCM) which were used as negative

Fig. 2. MiR-203 targets small GTPase Ran gene. (A) The expression of small GTPase Ran in scramble miRNA and miR-203-transfected Ec-109 cells was analyzed by Western blot. GAPDH was used as an internal control. (B) The expression of Ran was quantitatively detected at transcriptional level by real-time PCR. Experiments were carried out in triplicate and all data were shown as means ± SD, *p < 0.01. (C) Putative miR-203-binding sequence within the 3'-UTR of Ran mRNA. Mutations in the complementary site for the seed region of miR-203 in 3'-UTR of Ran gene were indicated. (D) The reporter plasmids containing wild type of 3'-UTR of Ran gene (pmiR-GLO-Ran) or mutant (pmiR-GLO-Ran-Mut) were co-transfected into Ec-109 cells with miR-203 expressing plasmid to evaluate luciferase activity. Empty plasmid without 3'-UTR of Ran gene was used as control (pmiR-GLO-NC). The normalized luciferase activity in control-transfectant was set as relative luciferase activity. Data were presented as means ± SD (n = 3), *p < 0.05.
control. Ran restoration in miR-203-transfected Ec-109 cells was subsequently confirmed by Western blot (Supplementary Fig. 6). Our analysis further found that Ran restoration not only increased the proliferation (Supplementary Fig. 7) and the apoptosis resistance (Supplementary Fig. 8) of miR-203 transfected cells but also partially increased their capacity of migration (Fig. 4A) and invasion (Fig. 4B) when compared with negative control, indicating that the suppressive effects of miR-203 on cell migration and invasion could be partially rescued by Ran restoration.

We showed above that miR-21 expression could be down-regulated by miR-203, we thereafter examined the effect of Ran restoration in miR-203-transfected cells on the expression of miR-21. Our results showed that restoring expression of Ran increased miR-21 expression by about 2 folds \( (p < 0.01) \) (Fig. 4C), indicating that Ran restoration counteracts the inhibitory effects of miR-203 on miR-21 expression.

MiR-21 expression was up-regulated by Ran restoration, we therefore investigated its implication in anti-tumor effects of miR-203 using xenograft experiments. 2\( \times 10^6 \) miR-203 + pLCM-Ran cells were inoculated in nude mice \( (n = 8) \). Ec-109 and miR-NC + pLCM cells were used as positive controls \( (8 \text{ mice for each}) \). MiR-203 + pLCM cells were used as negative control \( (n = 8) \). Our experiments showed that Ec-109 and miR-NC + pLCM cells induced tumor formation in all nude mice and no obvious tumor formation was observed in mice inoculated with MiR-203 + pLCM cells. Interestingly, miR-203 + pLCM-Ran cells induced tumor formation in 2 of 8 inoculated mice (Fig. 4D), indicating that restoration of Ran in miR-203-transfected cells could partially rescue anti-tumor effects of miR-203. However, evaluation of tumor volumes and measurement of tumor weights at the sacrifice time demonstrated that tumors induced by Ran restoration were much smaller and lighter than those induced by controls (Fig. 4E–G). Data obtained suggest that miR-203 inhibits Ec-109 cell tumor formation by suppressing Ran and Ran restoration partially counteracts anti-tumor functions of miR-203.

3.5. MiR-203 expression inversely correlates with the expression of Ran and miR-21 in ESCC

The expression of miR-203, Ran and miR-21 was quantitatively analyzed by real-time PCR in 12 pairs of primary ESCC and corresponding adjacent normal esophageal tissues. Results showed that the overall average expression level of miR-203 was lower in tumor tissues than in adjacent normal tissues. In contrast, the overall average expression levels of Ran and miR-21 were higher in tumor tissues relative to normal tissues (Fig. 5), indicating that miR-203 expression has an inverse correlation with the expression of Ran and miR-21 in ESCC when compared to adjacent normal tissues, which is consistent with our above-mentioned data that ectopic expression of miR-203 down-regulates miR-21 expression through targeting Ran.

4. Discussion

Deregulation of oncogene and tumor-suppressor gene expression has been identified as one of the critical causes of tumorigenesis. The recent discovery of miRNAs provides additional new insights into the molecular mechanism of gene expression control. It has been reported that miRNA could regulate gene expression by inducing target mRNA cleavage or translational repression through binding to its 3’-UTR. Aberrant miRNA expression was recently
found in cancer and recognized as a hallmark of cancer [4,5]. Studies on miRNA expression profiling and their functions in cancer have indeed provided evidence of the implication of miRNA with tumor development and progression [4,5]. However, up to date, only a small proportion of identified miRNAs have been investigated to elucidate their precise biological roles in esophageal cancer.

Down-regulation of miR-203 has been reported in a number of different types of cancers including ESCC [24–26]. The aim of current work is to elucidate the biological functions of miR-203 in

Fig. 4. The effects of Ran restoration in miR-203-transfected esophageal cancer cells. (A) Representative results of cell migration across membranes without matrigel and the percentage of cells across membranes was indicated by histogram. Data were shown as means ± SD (n = 3),  "p < 0.01. (B) Representative results of cell invasion across membranes with matrigel and the percentage of cells across membranes was indicated by histogram. Data were expressed as means ± SD (n = 3),  "p < 0.01. (C) The expression of miR-21 was evaluated by real-time PCR. Data were presented as means ± SD (n = 3),  "p < 0.01. (D) Xenograft growth in nude mice. 2.0 × 10⁶ cells were injected s.c. into one flank of nude mice. Tumor formation was induced by Ran restoration in miR-203-Ec-109 not in lentiviral vector-transfected miR-203-Ec-109 cells. Ec-109 and MiR-NC + pLCM cells were used as positive control for tumor formation in xenograft mice. (E) Tumor volumes were measured at indicated time-points and shown as average of duplicate measurements with standard error bars (n = 8 each group). (F) Representative tumor tissues were extracted from mice. (G) Average weight of tumors in nude mice was calculated and shown as means ± SD,  "p < 0.05.
ESCC. Our results demonstrated that ectopic expression of miR-203 in ESCC cells could significantly induce cell apoptosis, suppress cell proliferation, migration and invasion and inhibit their tumorigenicity. The data suggest that miR-203 plays tumor suppressor roles in esophageal cancer cells. Recently it has been shown that miR-203 suppresses the proliferative potential of esophageal cancer cells and acute lymphoblastic leukemia cells through targeting ANP63 and ABL1, respectively [19,27] and inhibits the migration of prostate cancer cells by targeting Lasp1, Ckap2, Wasf1 and Asap1 [28]. In our present reports, studies on molecular mechanism of miR-203 in tumor suppression revealed that small GTPase Ran is a direct target gene of miR-203 validated by its remarkable down-regulation in miR-203-expressed cancer cells and by luciferase assay. It is consistent with a recent report that Ran up-regulation is correlated with miR-203 down-regulation during the repair of damaged skin [29].

Ran is a small GTPase of the Ras superfamily and involved in regulation of multiple important biological processes, especially in nucleocytoplasmic transport, mitotic spindle assembly and nuclear envelope formation. The overexpression of Ran has been observed in diverse tumor types and its up-regulation is correlated with a poor prognosis of tumor patients, suggesting the importance of this small GTPase in tumor growth and progression [30–32]. To specifically address the implication of Ran in the tumor suppressive roles of miR-203, wild-type Ran was exogenously expressed in miR-203-transfected esophageal cancer cells. Our observations showed that the restoration of Ran partially increased apoptotic resistance and rescued the suppressive effects of miR-203 on cell proliferation, migration and invasion. However, our xenograft experiments showed that Ran restoration could partially enhance xenograft growth of miR-203-transfected cells in vivo, suggesting that down-regulation of Ran partially contributed to the tumor suppressive effects of miR-203.

Significant down-regulation of miR-21 was observed in miR-203-transfected ESCCs. MiR-21 is the only miRNA known to be frequently overexpressed in many types of human malignancy and actually considered as oncogenic miRNA that acts as oncogene [22]. Extensive studies showed that its overexpression is involved in all the known carcinogenic processes such as hyperproliferation, angiogenesis, invasion and metastasis and significantly associated with the poor prognosis of tumor patients [22,23]. In the present studies, overexpression of miR-203 led to the down-regulation of miR-21 expression. We further identified that Ran was able to regulate miR-21 expression, and miR-203 could down-regulate miR-21 expression through repressing Ran. In our experiments, Ran knockdown by Ran-specific siRNA reduced the expression of miR-21 and Ran restoration in miR-203-transfected cells increased miR-21 expression and therefore counteracted inhibitory effects of miR-203 on miR-21 expression. It should be mentioned that it may be a particular phenomenon observed in Ec-109 cells and be different in other cell lines, considering that the pattern of expression of miRNAs is tissue dependent. Furthermore, an inverse correlation of miR-203 expression with the expression of Ran and miR-21 was observed in ESCC tissues when compared to corresponding adjacent normal tissues, suggesting that misregulation of miR-203 may be a critical event responsible for the overexpression Ran and miR-21 in ESCC. This observation is consistent with our data that miR-203 down-regulates miR-21 expression through suppressing Ran. However, how Ran regulates miR-21 expression remains to be elucidated.

In conclusion, the data presented here suggest that miR-203 plays tumor suppressor roles in esophageal cancer cells and small GTPase Ran is a direct downstream target of miR-203 which can be involved in multiple tumor suppressive effects of miR-203. We established for the first time a link between miR-203, small GTPase Ran and miR-21, which has been identified as an oncogenic miRNA ubiquitously overexpressed in diverse types of malignancy. The identification and characterization of the tumor suppressive effects of miR-203 may provide a novel therapeutic approach for ESCC treatment.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary material

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