Original Paper

Differential Expression of MicroRNA-19b Promotes Proliferation of Cancer Stem Cells by Regulating the TSC1/mTOR Signaling Pathway in Multiple Myeloma

Ni Wang[a,b]   Xiaohua Liang[b]   Weijian Yu[b]   Shihang Zhou[b]   Meiyun Fang[a,c]

[a]Department of Hematology, the First Affiliated Hospital of Dalian Medical University, Dalian, [b]Blood Typing Research Laboratory, Dalian Blood Center, Dalian, [c]Department of Hematology, Affiliated Zhongshan Hospital of Dalian University, Dalian, China

Abstract

Background/Aims: MiR-19b has been reported to be involved in several malignancies, but its role in multiple myeloma (MM) is still unknown. The objective of this study was to explore the biological mechanism of miR-19b in the progression of MM. **Methods:** First, we performed real-time polymerase chain reaction (PCR) and Western blot to study the expression of miR-19b, tuberous sclerosis 1 (TSC1), and caspase-3 in different groups. MTT assay was performed to explore the effect of miR-19b on survival and apoptosis of cancer stem cells (CSCs). Computation analysis and luciferase assay were utilized to confirm the interaction between miR-19b and TSC1. **Results:** A total of 38 participants comprising 20 subjects with MM and 18 healthy subjects as normal controls were enrolled in our study. Real-time PCR showed dramatic upregulation of miR-19b, but TSC1 was evidently suppressed in the MM group. MiR-19b overexpression substantially promoted clonogenicity and cell viability, and further inhibited apoptosis of CSCs *in vitro*. Furthermore, miR-19b overexpression downregulated the expression of caspase-3, which induced apoptosis. Using in silico analysis, we identified that TSC1 might be a direct downstream target of miR-19b, and this was further confirmed by luciferase assay showing that miR-19b apparently reduced the luciferase activity of wild-type TSC1 3′-UTR, but not that of mutant TSC1 3′-UTR. There was also evident decrease in TSC1 mRNA and protein in CSCs following introduction of miR-19b. Interestingly, reintroduction of TSC1 abolished the miR-19b-induced proliferation promotion and apoptosis inhibition in CSCs. **Conclusion:** These findings collectively suggest that miR-19b promotes cell survival and suppresses apoptosis of MM CSCs via targeting TSC1 directly, indicating that miR-19b may serve as a potential and novel therapeutic target of MM based on miRNA expression.

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Meiyun Fang

Hematology Department, Affiliated Zhongshan Hospital of Dalian University,
No. 6, Jiefang Street, Zhongshan District, Dalian, 116001 (China)
E-Mail tsc1mf@163.com

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Introduction

Multiple myeloma (MM) is a malignant tumor of plasma cells, affecting approximately 488, 200 people globally as estimated in 2015 [1]. Current understanding of the pathogenesis of MM is insufficient to develop measures to prevent it [2]. Identified risk factors are age, male sex, smoldering MM, and monoclonal gammopathy of undetermined significance (MGUS), a family history of MM or its precursor states, and African ancestry [2]. For a long time, it has been plausible that a small group of cancer stem cells (CSCs), residing in bone marrow niches, lead to the occurrence of disease relapse and refractory clones [3]. Different groups have identified a variety of CSC phenotypes in MM, such as CD138+ cells, CD19-/CD45low-/CD38high/CD138+ cells, CD138-/CD19+ cells with cytoplasmic light chain-restricted cells, and CD138-/CD34- with memory B-cells (CD19+/CD27+) [4-7]. Some defects of CSCs are found in other pathological disorders, such as the tuberous sclerosis 1 (*Tsc1*) gene, the mouse double minute 2 homologue (*Mdm2*) gene, and deletion of *Bmi1* [8-10]. Tuberous sclerosis complex (TSC) is a genetic disease caused by mutation of either the *TSC1* or *TSC2* gene, which code for the proteins hamartin and tuberin respectively. These proteins are the main modulators of mammalian target of rapamycin complex 1 (mTORC1). In turn, the mTOR pathway plays important roles in the regulation of cell growth, proliferation, and survival [11]. The TSC1 and TSC2 complex acts as a GTPase-activating protein for Ras homologue enriched in brain (Rheb), one of the most important upstream regulators of mTORC1. A range of upstream signaling pathways like the PI3K–Akt pathway converge at TSC1 and TSC2, but they can also modulate the activity of mTORC1. Originally, it was described that deletion of PTEN, a negative modulator of the PI3K–Akt pathway, from hematopoietic stem cells (HSCs) in adult mice enhances HSC proliferation [11].

MicroRNAs (miRNAs), a class of small endogenous non-coding RNAs, suppress the translation of specific target mRNAs to modulate gene expression. MiRNAs are associated with modulation of an array of pathological and physiological processes, such as tumorigenesis and metastasis, and they seem to be promising therapeutic targets [12, 13]. Recently, miRNA profiles have been determined in primary patient samples and in human myeloma cell lines in a series of studies, and the pattern of miRNA expression has been related to patient survival and aberrations of specific genes [14]. Intriguingly, MM and MGUS have different expression profiles of some miRNAs, including the miR-17–92 cluster [15]. MiR-92a was the first potential biomarker found in the plasma of patients with MM, and was decreased in MM rather than in smoldering MM or MGUS [16]. A recent study has indicated that miR-16 and miR-25 are independent markers for prognosis of newly diagnosed MM [17].

Differential expression of TSC1 is also involved in the onset of MM and is associated with significant upregulation of miR-19b [18-20]. In this study, we performed in silico analysis and found miR-19b practically targeted TSC1. We predict that differential expression of miR-19b triggers dysregulated TSC1 and is associated with the pathology of MM based on the above evidence.

Materials and Methods

Enrollment of subjects

A total of 38 participants comprising 20 subjects with MM and 18 healthy subjects as normal controls were enrolled in our study from the Department of Hematology, the First Affiliated Hospital of Dalian Medical University, China. The study was approved by the Ethics Committee of Dalian Medical University. Written informed consent for participation in the study was obtained from all subjects after the potential risk had been duly explained to them. All protocols for this study complied with the Declaration of Helsinki.

Multiple myeloma stem cells collection

The Magnet-based Cancer Stem Cell Isolation Kit (ProMab Biotechnologies Inc., Richmond, CA) was used to isolate stem cells according to the manufacturer's instructions.

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RNA isolation and real-time polymerase chain reaction (PCR)

The RNeasy Mini Kit (Qiagen, Hilden, Germany) was used to isolate total RNA from the cells in accordance with the manufacturer's instructions. RNA quality and quantity were determined using the Nanodrop spectrophotometer (Thermo Scientific Inc., Wilmington, DE) and 1.5% (w/v) agarose gel following the standard protocol. Moloney Murine Leukemia Virus Reverse Transcriptase (Applied Biosystems, Foster City, CA) was used to reverse transcribe RNA to cDNA according to the manufacturer's instructions. The ABI 7900HT Sequence Detection System (Applied Biosystems) with SYBR-Green I (Qiagen) was used to perform real-time PCR. TSC1 primers used in the study were as follows: Sense 5′-CAACAGGCGTCTTGGTGTTG-3′; Reverse 5'-ACACACTGGCATGGAGATGG-3′. Cycle threshold (Ct) values were identified using SDS software (Applied Biosystems), the value of $2^{-\Delta\Delta Ct}$ was used to describe the relative gene expression levels of TSC1 mRNA and miR-19b.

Cell transfection

Cells were cultured to 80% confluency, and then co-transfected with wild-type or mutant-type TSC1 3'-UTR vector and miR-19b mimic by electroporation using the 4D-Nucleofector X Kit (Lonza, Cologne, Germany) according to the manufacturer's protocol.

Cell proliferation assay

We used the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich, CA) to assess cell proliferation as described previously [21]. All experiments were performed in triplicate independently.

Clonogenic assay

For colony formation assay, CSCs transfected with miR-19b mimic were maintained in 24-well plates at a density of 200 cells/well, and the medium was changed every 3days. After 10 days, the clones were washed with PBS, and stained with crystal violet for 5 min. Images were captured microscopically and the clones were counted.

Luciferase assay

Pfu DNA polymerase (Fermentas, Thermo Fisher Scientific, Waltham, MA) was used to amplify the coding sequence of TSC1 containing the binding site of miR-19b. The resulting PCR products were inserted into pcDNA3.1(+), and TSC1 expression vectors were generated. We used the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) to perform the site-directed mutagenesis of the 3′-UTR fragment of TSC1 combining with the miR-19b seed sequence according to the manufacturer's protocol. Luciferase assays were performed by co-transfecting MM stem cells with either reporter plasmid or Renilla luciferase vector (as control) and miR-19b mimic. MM stem cells were harvested 48h post-transfection, and the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used to measure the luciferase activity. Experiments were performed in triplicate independently.

Western blot analysis

TSC1 protein was estimated by Western blot analysis. MM stem cells and tissue samples were homogenized using non-denaturing lysis buffer (Invitrogen, Carlsbad, CA) containing 1g/mL each aprotinin and leupeptin, 1mM egtazic acid, 1mM EDTA, 0.3mM phenylmethylsulfonylfluoride, 150mM NaCl, 0.2mM sodium orthovanadate, 1% Triton X-100, 0.5% NP40, and 10mM Tris–HCl (pH 7.4). Lysates were centrifuged at 10, 000×g for 20min at 4°C. The protein concentration of the lysates was determined using a detergent-compatible protein assay kit (Pierce, Rockford, IL). Proteins in the supernatant were separated on 10%-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. TBST (100mM NaCl, 10mM Tris, 0.1% Tween 20 and 5% nonfat milk powder (w/v)) was used to block the membranes for 12h at 4°C or for 1h at room temperature. Membranes were then incubated with primary antibodies against TSC1 (1:5000; Abcam, Cambridge, UK), p-S6K (1:2000; Abcam), and β-actin (1:15000; Abcam) for 12h at 4°C. Bound antibodies were then visualized using horseradish peroxidase-conjugated secondary antibodies (1:15000; Abcam). An enhanced chemiluminescence-detection system (ECL Advanced kit; Amersham Biosciences, Little Chalfont, United Kingdom) was used to detect the bound

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antibodies. Finally, protein expression was quantified using Imaging Gauge software version 3.45. All experiments were performed in triplicate independently.

Apoptosis analysis

Cells were harvested 48h after transfection and the MM stem cells were washed with PBS. FITC-Annexin V/propidium iodide Apoptosis Detection Kit (BestBio, Shanghai, China) was used to stain the cells. Flow cytometry analysis (BD FACS Canto II, BD Biosciences, San Jose, CA) was performed immediately. Each analysis was carried out in triplicate at least.

Statistical analysis

All statistical analysis was performed using SPSS software version 19.0 (SPSS Inc., Chicago, IL) and a p value less than 0.05 was considered to indicate statistical significance. The χ^2 test or Student's t-test was used to compare variables between the experimental and control groups.

Results

miR-19b expression levels in MM samples

A total of 38 participants comprising 20 subjects with MM and 18 healthy subjects as normal controls were enrolled in our study. Using quantitative real-time PCR, we investigated the expression of miR-19b in samples from all participants. Real-time qPCR showed that miR-19b was dramatically upregulated in MM patients compared with the controls (Fig. 1), suggesting that overexpression of miR-19b induced MM tumorigenicity.

High miR-19b expression in MM CSCs line promotes cell proliferation in vitro

We estimated miR-19b expression levels in MM CSCs transfected with NC (negative control) or miR-19b mimic using real-time PCR. Briefly, electroporation was performed to transfect the MM CSCs with NC or miR-19b mimic when the cells had reached 80% confluency. miR-19b mimic significantly upregulated miR-19b levels compared with NC (Fig. 2A). Based on the evident increase in miR-19b levels in cells following the introduction of miR-19b mimic,
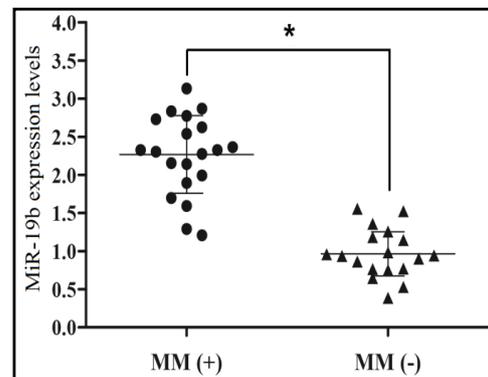

Fig. 1. Differential expression of miR-19b in MM samples and normal controls. MiR-19b was highly expressed in MM compared with MM (-) group (p<0.05).
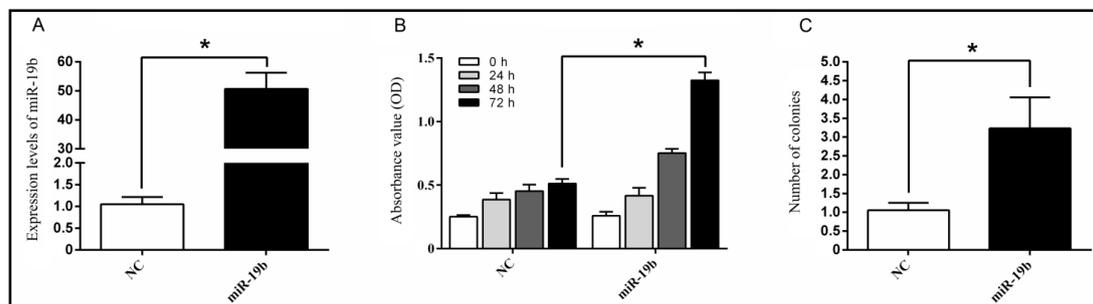

Fig. 2. Overexpression of miR-19b promotes cell proliferation in vitro. (A) MiR-19bexpression in MM CSCs was upregulated after transfection with miR-19b mimic compared with NC transfected CSCs (p<0.05). (B) Increased viability of CSCs transfected with miR-19b mimic compared with CSCs transfected with NC (p<0.05). (C) Clonogenic ability of MM CSCs transfected with miR-19b mimic was enhanced compared with NC-transfected CSCs (p<0.05).

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we further explored the biological function of miR-19b in MM. First, we performed MTT assay to examine the effect of miR-19b on proliferation (Fig. 2B). Cells transfected with miR-19b mimic induced remarkable promotion of MM CSC survival, indicating that miR-19b overexpression enhanced proliferation of CSCs. Next, we performed a colony formation assay to confirm the above hypothesis. The assay displayed the same trend in which upregulated miR-19b remarkably promoted clonogenic ability of CSCs in comparison with NC (Fig. 2C). These data confirmed that miR-19b substantially promoted clonogenicity and cell viability.

High miR-19b expression in MM CSCs inhibits apoptosis in vitro

Annexin V/propidiumiodide (PI) staining was performed to explore the effect of miR-19b on apoptosis of MM CSCs. There was an evident decrease in the apoptotic proportion of cells transfected with miR-19b mimic compared to those transfected with NC (Fig. 3A), revealing that miR-19b inhibited apoptosis of CSCs *in vitro*. Caspase-3 has been universally acknowledged for inducing apoptosis. Western blot analysis revealed much lower expression levels of activated caspase-3 in the cells transfected with miR-19b compared with the NC cells (Fig. 3B). Furthermore, we quantified the ratio of activated caspase-3 to β-actin and the ratio of activated caspase-3 to total caspase-3 as described previously [21]. Both ratios were decreased in miR-19b transfected cells compared with NC cells (Fig. 3C and 3D). This confirms that miR-19b significantly decreased activated caspase-3 production versus the NC cells with either β-actin or total caspse-3 as reference, suggesting that miR-19b inhibited apoptosis collectively via regulating activated caspase-3 expression levels.

Fig. 3. Inhibition of apoptosis by miR-19b overexpression. (A) Annexin V/PI staining in CSCs transfected with miR-19b mimic or NC; miR-19b markedly inhibited apoptosis of CSCs compared with NC (p<0.05). (B) Western blot analysis showed reduced pro-caspase-3 and activated caspase-3 levels in CSCs transfected with miR-19b mimic compared with NC-transfected CSCs (p<0.05). (C) Repressed activated caspase-3 levels in CSCs transfected with miR-19b mimic compared with NC-transfected CSCs, normalized to β-actin (p<0.05). (D) MiR-19b significantly repressed activated caspase-3 levels in comparison with NC, normalized to total caspase-3 (p<0.05).
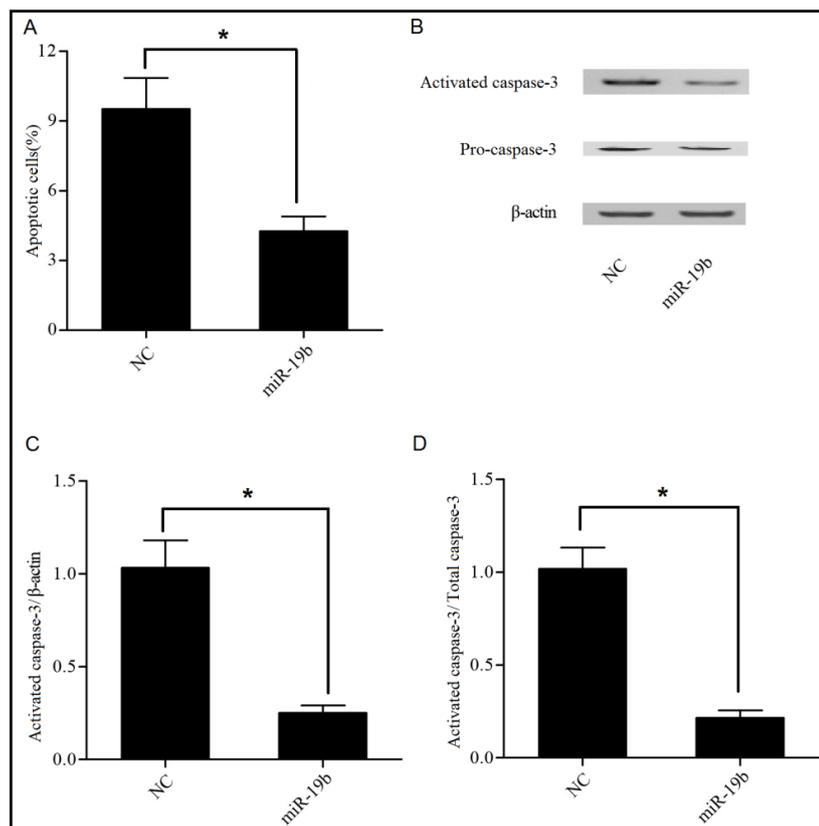
miR-19b suppresses TSC1 through targeting the 3′-UTR region of TSC1

To explore the mechanism by which miR-19b promoted proliferation and inhibited apoptosis of MM CSCs, bioinformatics databases, such as miRanda and TargetScan, were searched to identify potential targets of miR-19b. TSC1 was consequently identified as a target according to the search results of these two bioinformatics databases. The "seed sequence" in the 3′-UTR of TSC1 (Fig. 4A) was highly conserved across the species, indicating that the "seed sequence" may play an important role in human evolution. Furthermore, to confirm whether TSC1 was a direct downstream of miR-19b, we generated luciferase reporter vectors transfected with either wild-type or mutant 3′-UTR of TSC1. The Dual-Luciferase Reporter Assay System was then used to measure luciferase activity 48h after transfection. MiR-19b significantly reduced luciferase activity in the cells transfected with wild-type 3′-UTR of TSC1, while miR-19b had almost no effect on luciferase activity in cells transfected with mutant 3′-UTR of TSC1 (Fig. 4B). This suggested that TSC1 was a validated target of miR-19b, and that miR-19b suppressed TSC1 expression.

Fig. 4. TSC1 is a direct target of miR-19b. (A) MiR-19b directly targeted the highly conserved "seed sequence" in the TSC1 3′-UTR. (B) Luciferase activity in MM CSCs transfected with luciferase reporter vector containing WT (wild-type) or MUT (mutant) TSC1 3′-UTR, along with miR-19b mimic or NC. MiR-19b significantly downregulated luciferase activity of wild-type TSC1 3′-UTR compared with NC (p<0.05), but not that of mutant TSC1 3′-UTR.
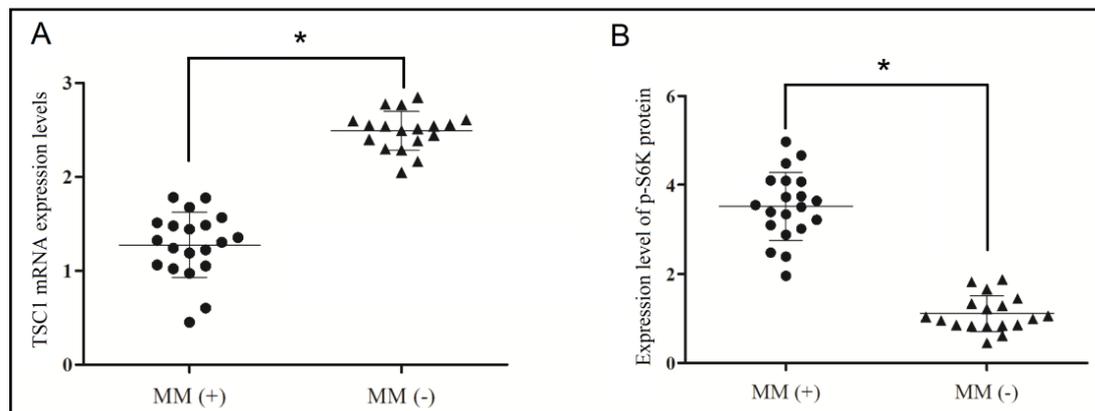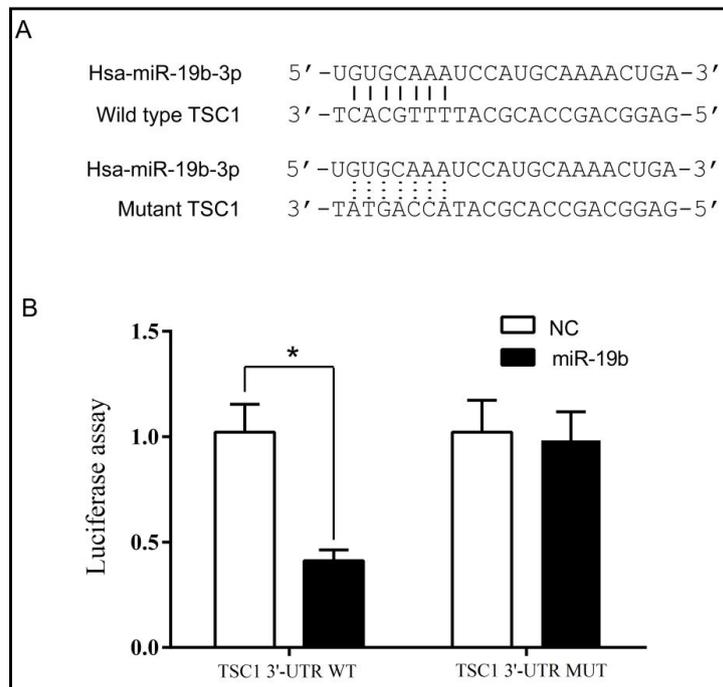

Fig. 5. Differential TSC1 mRNA and p-S6K protein expression in MM tissues and normal controls. (A) Lower TSC1 mRNA expression in MM samples compared with normal controls (p<0.05). (B) Higher p-S6K protein level in MM samples compared with normal controls (p<0.05).

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TSC1 and p-S6K expression in MM samples

We examined TSC1 and p-S6K expression in 38 samples (20 subjects with MM and 18 normal controls) using quantitative reverse transcription PCR and Western blot analysis. In MM samples, mRNA levels of TSC1 (Fig. 5A) were much lower, while protein levels of p-S6K (Fig. 5B) were much higher than those in the controls, indicating that overexpression of miR-19b induced tumorigenicity of MM by regulating expression of TSC1 and p-S6K.

Downregulation of TSC1 inhibits MM cell proliferation and induces apoptosis in vitro

The following results demonstrated that both TSC1 mRNA (Fig. 6A) and protein (Fig. 6B) expression levels in MM CSCs were blocked following transfection with miR-19b, showing that miR-19b reduced expression of TSC1. To explore the mechanism by which miR-19b promoted proliferation and inhibited apoptosis in MM CSCs, we performed MTT assay and cell apoptosis analysis. First, CSCs were transfected with NC plasmid or miR-19b mimic or with miR-19b mimics plus TSC1 as a rescue experiment. Cell proliferation was apparently
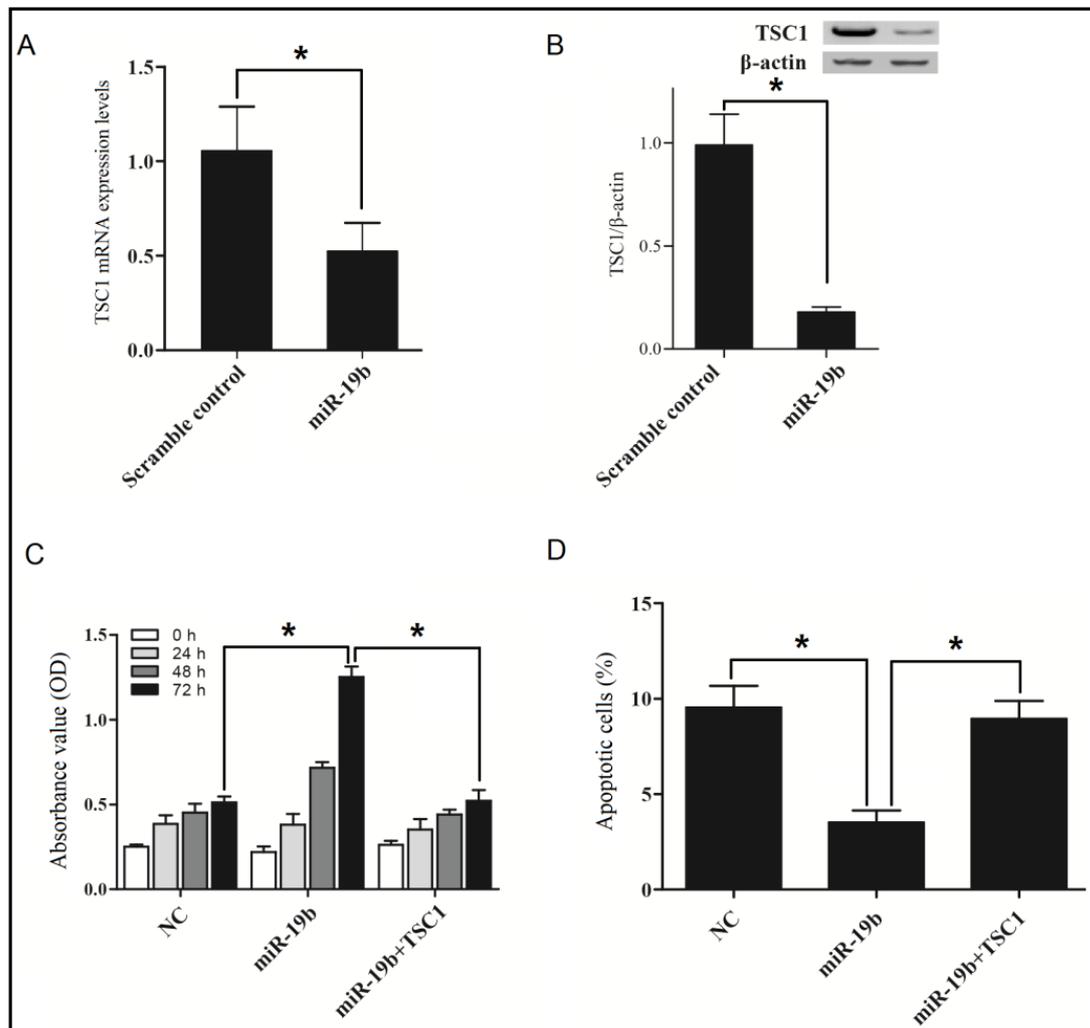

Fig. 6. (A) Decreased TSC1 mRNA levels in MM CSCs transfected with miR-19b mimic compared with NC (p<0.05). (B) Decreased TSC1 protein level in MM CSCs transfected with miR-19b mimic compared with NC-transfected CSCs, normalized to β-actin (p<0.05). (C) Viability of CSCs transfected with miR-19b mimic was promoted compared with NC-transfected CSCs (p<0.05), which was abolished by TSC1. (D) Apoptosis was suppressed in CSCs transfected with miR-19b mimic compared with NC-transfected CSCs (p<0.05); but this was blocked by complementing with TSC1.

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promoted in the cells transfected with miR-19b mimic compared with NC-transfected cells (Fig. 6C). Interestingly, introduction of TSC1 restored the effect of miR-19b transfection. MiR-19b mimic apparently suppressed apoptosis compared with NC plasmid (Fig. 6D). Meanwhile, inhibition of apoptosis induced by miR-19b was abrogated by overexpression of TSC1. These data validated the finding that miR-19b promoted cell proliferation and inhibited apoptosis by suppressing TSC1 expression.

Discussion

MM is essentially a malignancy characterized by proliferation of terminally differentiated plasma cells and clonal B cells. It remains incurable regardless of significant progress achieved in the biology and treatment of the condition [22]. The mechanism by which Givinostat (ITF2357), a histone deacetylase inhibitor, impacts the progression of MM may involve decreased expression of miR-19a and miR-19b, which belong to the miR-17–92 cluster localized within the *MIRHG1* locus (also called *C13orf25*) on chromosome 13q31.3. The miR-17–92 cluster has been associated with tumorigenesis in B-cell neoplasms, such as MM and other types of cancers. In fact, miR-17–92 cluster overexpression mediates hyperproliferation of B cells and autoimmune disorders, while miR-17–92 deletion results in a reduction in development and proliferation of B cells [23]. A more recent study reported a direct role of miR-19a and miR-19b in the pathogenesis of MM [24]. Moreover, MM tumor growth in nude mice can be inhibited by miR-19a and miR-19b antagonists [15]. This combined effect on the cytokine and its receptor produce the most effective suppression in IL-6 signaling. Besides the IL-6 axis, ITF2357 regulates multiple genes associated with MM development and apoptosis, such as miR-19a and miR-19b, *p21*, *APAF-1*, *FGFR3,* and *c-MYC* [15, 23]. MiR-19b has been reported to have a role in thrombosis, cardiovascular disorders and the aging process, and its dysregulation was observed in multiple cancers, such as cervical cancer, glioma, lung cancer, and breast cancer [25]. Some studies have indicated that miR-19b was increased in cancer cells, and it enhanced chemoresistance and proliferation, whereas other studies described its role in inhibition of migration and angiogenesis [26]. Multiple studies have shown that miR-19b acted as an oncogene in chemoresistance, migration, and cancer proliferation [27]. Nevertheless, Zhang *et al.* discovered that in breast cancer, miR-19b played a negative regulatory role in tissue factor expression, which is critical in tumor metastasis and angiogenesis [28]. Yin *et al.* discovered that miR-19b suppressed angiogenesis [29]. HIF1A was also increased following miR-19b knock-down [30]. In this study, we recruited 38 subjects, including 20 patients newly diagnosed with MM and 18 subjects without MM (normal controls). We performed real-time PCR to evaluate miR-19b expression and TSC1 in MM patients and normal controls. The results showed that miR-19b was significantly upregulated, while TSC1 was significantly downregulated in MM compared with the normal controls. We then performed RT-PCR and MTT assay to explore the effect of miR-19b on cell viability, and found that miR-19b overexpression promoted CSC proliferation.

The role of TSC1 and TSC2 in breast cancer has been investigated with an emphasis on the key role of TSC1 and TSC2 in the mTOR pathway and TSC [31]. As a heterodimer of the tumor inhibitor proteins TSC1 and TSC2, the TSC complex acts as an upstream negative modulator of mTORc1 activity [32]. If cytokines, growth factors or nutrients are present, signals mediated by receptors suppress TSC activity and active GTP-bound Rheb enhances the activity of mTORc1 by phosphorylation of mTOR at Ser2448 [33]. Recently, multiple reports have described a critical role for TSC1 in mitochondrial homeostasis, and T cell quiescence and survival [34]. Moreover, it has been demonstrated that TSC1 is critical in mast cell survival and function, antigen presentation and innate immune responses, B cell development, regulatory T cell function, anti-tumor immunity and iNKT cell anergy, terminal maturation, and effector lineage fate decisions of iNKT cells [35-39]. Others investigated the impact of TSC1 on memory CD8^{+} response and antigen-specific primary CD8^{+} response, proving that mTORc1 activity is important in effector/memory lineage CD8^{+} cell division.

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Preliminary findings from a previous study indicate that TSC1$^{flox/flox}$ (TSC1$^{f/f}$) CD-4Cre mice had fewer CD8^{+} cells that produce gamma interferon and fewer antigen-reactive CD8^{+} cells compared with wild-type controls in response to bacterial infection [40].

The validated specific miR-451 target of TSC1 was found to trigger activation of PIK3/Akt/mTOR signaling in MM CSCs. As a novel anti-myeloma agent, suppressor of miR-451 increased the effectiveness of myeloma treatment by reducing MDR1 mRNA expression, decreasing clonogenicity, and inducing apoptosis [19]. In this study, we found that the apoptotic proportion of CSCs was evidently decreased in cells transfected with miR-19b. This was further confirmed by Western blot analysis which revealed reduced caspase-3 levels in cells following introduction of miR-19b. Moreover, we searched bioinformatics databases to explore the potential targets of miR-19b, and we focused on TSC1 based on the search results. Furthermore, we generated luciferase reporter vectors containing either wild-type or mutant TSC1 3′-UTR, and found that miR-19b significantly suppressed wild-type TSC1 3′-UTR luciferase activity, but not that of mutant TSC1 3′-UTR. Finally, we used real-time PCR, Western blot, and MTT assay to reveal the mechanism by which miR-19b induced cell proliferation and inhibited apoptosis. We found that both TSC1 mRNA and protein levels were downregulated subsequent to transfection with miR-19b. Also, miR-19b mimic apparently promoted cell proliferation and suppressed apoptosis; however, the effects of miR-19b could be abolished by TSC1.

Conclusion

These findings collectively suggest that miR-19b induces cell survival and suppresses apoptosis of MM CSCs by directly targeting TSC1, indicating that miR-19b could serve as a novel potential therapeutic agent in the treatment of MM.

Disclosure Statement

The authors declare to have no competing interests.

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