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Let-7b and microRNA-199a inhibit the proliferation of B16F10 melanoma cells

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Abstract. Cutaneous melanoma is an aggressive form of human skin cancer characterized by high metastatic potential and poor prognosis. Biomarkers of metastatic risk are critically needed to instigate new auxiliary measures in high-risk patients. In clinical specimens of skin melanoma, we previously found that let-7b, microRNA-199a and microRNA-33 were significantly associated with metastatic melanoma, and thus may be the key to melanoma treatment. In this study, we examined the effect of overexpression and inhibition of let-7b and microRNA-199a. Plasmids overexpressing these genes were transfected into B16F10 melanoma cells, and let-7b and microRNA-199a expression were evaluated at the RNA, protein and cellular level. Cyclin D1 expression was significantly higher in cells transfected with let-7b plasmid and let-7b inhibitor compared with control cells ($P<0.05$). In turn, Met expression in the microRNA-199a plasmid group and microRNA-199a inhibitor group was significantly higher than in the control group ($P<0.05$). The proliferation rate of B16F10 cells transfected with let-7b or microRNA-199a was lower than that of the control group, particularly until the third day after transfection when the proliferation rate dropped to the lowest value ($P<0.05$). In addition, the apoptosis rates of the let-7b plasmid group and microRNA-199a plasmid group were significantly higher compared to that of the control group ($P<0.05$). These results suggest that let-7b and microRNA-199a may be negative regulators of B16F10 cell proliferation.

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Introduction

Melanoma, a malignancy that arises from melanocytes, accounts for approximately 10% of skin tumors. It has hereditary aspects (1), and in recent years its incidence has increased (2). The early symptoms are often difficult to detect, thus making treatment more challenging since the disease progresses rapidly and undergoes metastasis to result in poor prognosis. Clinical diagnosis is most frequently made once the cancer has undergone distant metastasis or regional lymph node metastasis. The mortality rate currently stands at 80%, while the 5-year survival rate is less than 5% and is significantly correlated with the number of metastases (3). Therefore, the interpretation of melanoma invasion and metastasis mechanisms to control tumor development is the key to lowering mortality.

microRNAs are small, non-coding RNAs that regulate gene expression by binding to mRNA 3'UTRs, which results in mRNA degradation or translation (4-8). microRNA regulates embryonic development, cell proliferation, apoptosis, cell differentiation, lipid metabolism and other important cellular functions, particularly in tumor development, where its function is similar to that of oncogenes or tumor suppressor genes (9-11). When oncogenes and tumor suppressor genes are disrupted, or the activity of telomerase persists, tumorigenesis occurs. The most notable feature of tumor cells is uncontrolled proliferation and non-differentiation. There are three checkpoints in the cell cycle, which control G_0/G_1, G_1/S and G_2/M. By regulating the cell cycle, it is possible to inhibit tumor growth and proliferation.

In clinical specimens of skin melanoma, we previously found that let-7b, microRNA-199a and microRNA-33 were significantly associated with metastatic melanoma (12). Notably, opposing findings have been described for ovarian carcinoma (13) and non-small cell lung cancer (14). Downregulated expression of let-7b and microRNA-199a was significantly correlated with poor prognosis in ovarian carcinoma (13). In this study, B16F10 melanoma cells were transfected with plasmids that targeted let-7b and microRNA-199a, in order to investigate the correlation between microRNA expression and melanoma metastasis.

Materials and methods

The study was approved by the The Third XiangYa Hospital of Central South University, Hunan, China.

Materials. PGCsi-U6/neo/GFP-hsa-let-7b plasmid and PGCsi-U6/neo/GFP-hsa-miR-199a plasmid were obtained from Ji Kai Chemical Technology Company (Shanghai, China). Hsa-let-7b miRNA inhibitor, hsa-microRNA-199a miRNA inhibitor and microRNA-Ribo™ Inhibitor Negative Control were purchased from Rui Bo Biological Technology Company (Guangzhou, China).

Grouping. Experiments were divided into the following groups: i) control group: B16F10 cells; ii) let-7b plasmid group: liposome-mediated targeting of let-7b overexpression plasmid; iii) microRNA-199a plasmid group: liposome-mediated targeting of microRNA-199a overexpression plasmid; iv) empty plasmid group: liposome-mediated targeting of empty plasmid; v) let-7b microRNA inhibitor group: liposome-mediated targeting of let-7b inhibitor fragment; vi) microRNA-199a inhibitor group: liposome-mediated targeting of microRNA-199a inhibitor fragment; vii) inhibitor control group: liposome-mediated targeting of negative control inhibitor fragment.

Transfection. B16F10 cells were seeded at a density of $5x10^4$ cells/ml in 6-well plates. Each well contained 2 ml RPMI-1640 complete medium. When cells had reached 80-90% confluency, plasmids were transfected, while inhibitor fragments were transfected into cells at 30-50% confluency. Cells were washed twice with PBS, and 1.5 ml serum-free RPMI-1640 medium was added to each well. Then 4.0 μg plasmid DNA or 5.0 μl inhibitor was added to 250 μl serum-free RPMI-1640 medium (liquid A), and 8 μl or 5 μl liposomes were added to 250 μl serum-free RPMI-1640 medium (liquid B). Then, liquids A and B were mixed, incubated for 15 min at room temperature, and added to 6-well plates at 500 μl/well. Approximately 4-6 h after transfection, RPMI-1640 complete medium was replaced.

Real-time PCR verification of let-7b and microRNA-199a expression. Cells were collected at 24-36 h after transfection and lysed using TRIzol solution to extract RNA. Following quantitation using an ultraviolet spectrophotometer, 1 μg genomic RNA was incubated in 12 μl buffer solution at 70˚C for 10 min. The reaction protocol was performed according to the manufacturer's instructions (reverse transcription kit, K1662, Fermentas, Canada), and DNA was amplified by PCR. Reactions consisted of the following constituents: RNA (1 μg), 62.5 nM RT primer mix (1 μl), DEPC water (added to make the total volume to 20 μl), 5X reaction buffer (4 μl), 20 μg/μl RNase inhibitor (1 μl), 10 mM dNTP mix (2 μl) and M-MuLv (1 μl), totaling 20 μl reaction volume. Reaction conditions consisted of 42˚C for 60 min, then 70˚C for 5 min. Following reverse transcription, reactions were prepared according to SYBR Premix Ex Taq™ (DRRO41A, Takara, Japan) instructions. The following reaction constituents were used: cDNA (2 μl), SYBR (10 μl), 10 μM forward primer (0.4 μl), 10 μM reverse primer (0.4 μl), 50X ROX (0.4 μl), ddH_2O (6.8 μl), totaling 20 μl. Reaction conditions were as follows: 95˚C for 10 sec, then 95˚C for 5 sec, 60˚C for 30 sec, 95˚C for 15 sec, 60˚C for 15 sec and 95˚C for 15 sec for a total of 40 cycles. Based on the results obtained, the relative expression value $F=2^{-\Delta\Delta ct}$ was calculated ($\Delta ct1$ = sample group average target gene ct value - average internal reference gene value; $\Delta ct2$ = control group average target gene ct value - average internal reference gene value; $\Delta\Delta ct = \Delta ct_1 - \Delta ct_2$).

Western blot verification of cyclin D1 and Met expression. Cells were harvested 72 h after transfection then lysed with sodium dodecyl sulfate loading buffer solution. The protein concentration of each sample was determined using the Bradford method. Thirty micrograms of protein supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked for 1.5 h and incubated with mouse anti-cyclin D1 antibody (1:1,000), mouse anti-Met antibody (1:1,000), or mouse anti-α-tubulin antibody (1:10,000). All membranes were incubated at 4˚C overnight. Membranes were washed three times with PBST then incubated for 1 h at room temperature in anti-mouse secondary antibody (1:10,000). ECL chemiluminescence was used to reveal proteins, and Bandscan software (Glyko, Novato, CA, USA) was used to scan the gray value of western blot bands to calculate the relative content of protein as follows: protein relative content = gray value of protein bands/gray value of α-tubulin bands.

MTT mapping cell growth curve. 96-well plates were inoculated with 10,000 B16F10 cells per well. After 24 h, cells were transfected with plasmids (five wells per group). MTT assays were performed 1 to 5 days after transfection as follows: 20 μl MTT solution was added to each well and cells were incubated for 4 h, then the supernatant was carefully discarded to terminate the reaction. Next, 150 μl DMSO was added to each well, and samples were oscillated for 10 min to fully dissolve any crystals. Absorption was measured at 490 nm.

Apoptotic rate detected by flow cytometry. Cells were harvested 24 h after transfection, then centrifuged at 800 rpm for 5 min and supernatant was removed. The cell suspension was then fixed in pre-cooled 10% (v/v) ethanol at 4˚C for 24 h. The cell density was adjusted to 10,000 cells/ml and fixed cells were treated with propidium iodide staining solution for 30 min in the dark. Apoptosis was measured using a flow cytometer.

Statistical analysis. Data were analyzed with SPSS 13.0 (SPSS, Chicago, IL, USA). Experimental data were presented as means ± SD and the paired t-test was used to show differences between each group. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Transfection of plasmids or inhibitors into B16F10 cells. At 24 h after transfection, B16F10 cells were examined by fluorescence microscopy to ensure successful uptake of plasmids or inhibitors. Green fluorescent cells were observed in the empty plasmid, let-7b plasmid and microRNA-199a plasmid groups (Fig. 1A and B), and red fluorescent cells were observed in

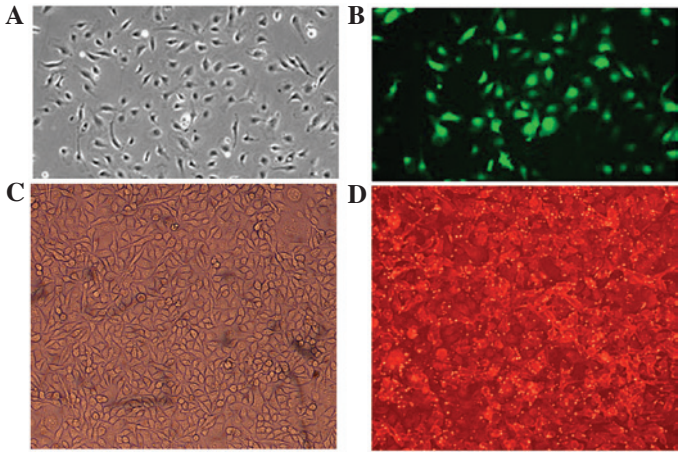

Figure 1. Plasmid groups and inhibitor groups of B16F10. (A) Plasmid group of B16F10 (visible light x100). (B) Plasmid group of B16F10 (fluorescence x100). (C) Inhibitor group of B16F10 (visible light x100). (D) Inhibitor group of B16F10 (fluorescence x100).

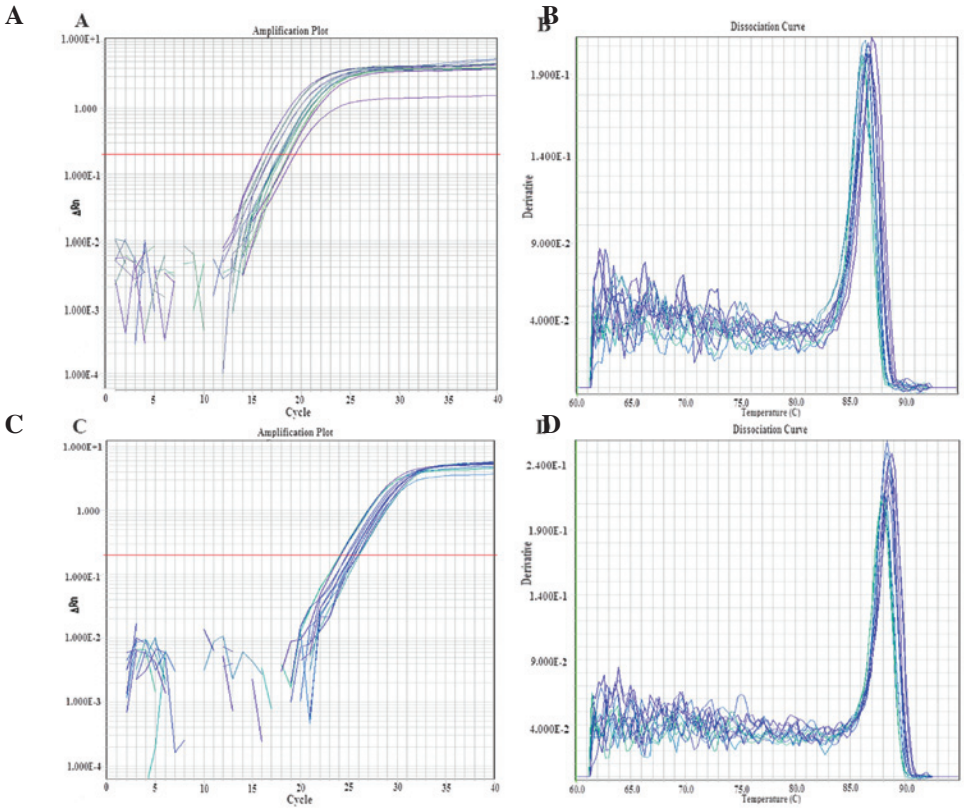

Figure 2. Let-7b and microRNA-199a real-time PCR. (A) Amplification curve of let-7b. (B) Dissolution curve of let-7b. (C) Amplification curve of microRNA-199a. (D) Dissolution curve of microRNA-199a.

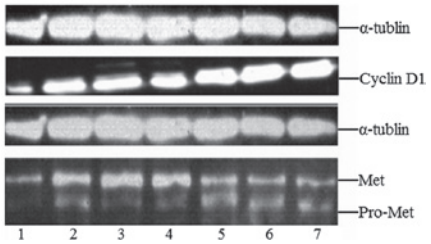

Figure 3. Expression of cyclin D1 and Met (1, control group; 2, empty plasmid group; 3, let-7b plasmid group; 4, microRNA-199a plasmid group; 5, inhibitor control group; 6, let-7b inhibitor group; 7, microRNA-199a inhibitor group).

the let-7b inhibitor, microRNA-199a inhibitor and inhibitor control groups (Fig. 1C and D). In turn, no green fluorescent cells were observed in the control group. Plasmids were tagged with green fluorescent protein (GFP), and inhibitor fragments were labeled with cyanine (Cy) 3.

Real-time PCR verification of let-7b and microRNA-199a gene expression

Let-7b gene expression. Compared with the control group, the relative gene expression of the let-7b plasmid group

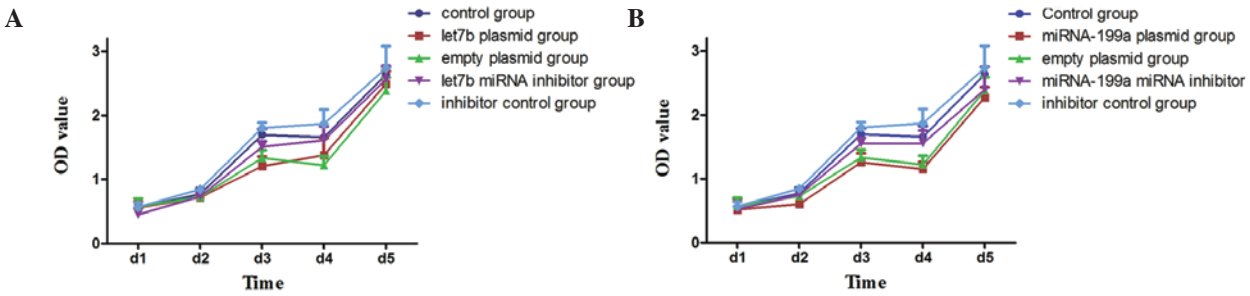

Figure 4. Effect of let-7b and microRNA-199a on B16F10 cell proliferation.

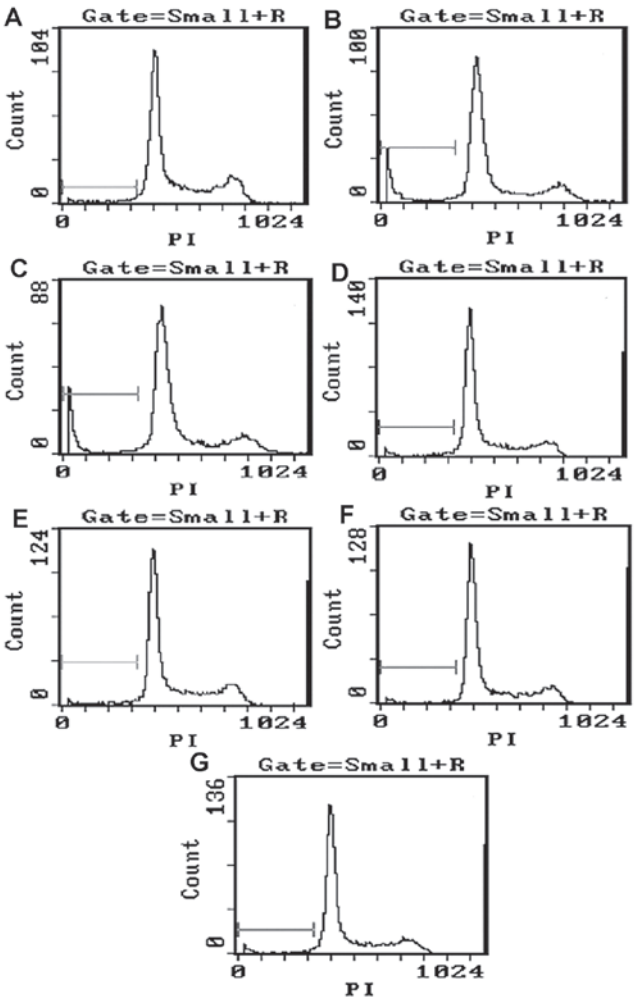

Figure 5. Apoptosis rate. (A) Control. (B) Let-7b plasmid. (C) microRNA-199a plasmid. (D) Empty plasmid. (E) Let-7b inhibitor. (F) microRNA-199a inhibitor. (G) Inhibitor control.

(3.8776±0.1372) was significantly higher (t=29.651, P<0.05), and the relative expression of the let-7b inhibitor group (0.2057±0.0263) was significantly lower (t=42.704, P<0.05). However, there were no significant differences between the empty plasmid group (1.1400±0.2769) and the inhibitor control group (0.9760±0.2300) (P>0.05; Fig. 2A and B).

microRNA-199a gene expression. Compared with the control group, the relative expression of the microRNA-199a plasmid group (2.8660±0.2821) was significantly higher (t=13.656, P<0.05), while the relative expression of the microRNA-199a inhibitor group (0.2656±0.0253) was significantly lower (t=41.028, P<0.05). However, there were no significant differences between the empty plasmid group (0.9809±0.1703) and the inhibitor control group (0.7512±0.0690) (P>0.05; Fig. 2C and D).

Western-blot detection of cyclin D1 and Met expression

Cyclin D1 protein expression. Compared with the control group (0.997±0.041), the relative expression of cyclin D1 in the let-7b plasmid group (2.023±0.315), the microRNA-199a plasmid group (1.763±0.172), the empty plasmid group (1.490±0.292), the let-7b inhibitor group (1.857±0.377), the microRNA-199a inhibitor group (1.590±0.286) and the inhibitor control group (1.443±0.099) was higher. However, in the let-7b plasmid and let-7b inhibitor groups the relative expression of cyclin D1 was significantly increased (t≥13.733, P<0.05), and between other groups there were no significant differences (P>0.05; Fig. 3).

Met protein expression. Compared with the control group (2.2±0.198), the expression of Met in the let-7b plasmid group (3.24±0.340), the microRNA-199a plasmid group (5.19±0.309), the empty plasmid group (2.85±0.047), the let-7b inhibitor group (2.49±0.068), the microRNA-199a inhibitor group (4.87±0.044) and the inhibitor control group (2.73±0.033) was higher. However, in the microRNA-199a plasmid and microRNA-199a inhibitor groups the relative expression of Met was significantly increased (t≥17.905, P<0.05), and between other groups, there were no statistically significant differences (P>0.05; Fig. 3).

Cell proliferation rate. B16F10 cells were treated with the different plasmids or inhibitors, and 1-5 days after transfection absorption values were measured, as described in Materials and methods (Fig. 4).

Compared with the control group, the proliferation rate of B16F10 cells transfected with let-7b or microRNA-199a was lower, and on the third day after transfection, the rate reached the lowest value (P<0.05). The rate was not significantly different in the empty plasmid, let-7b inhibitor, microRNA-199a inhibitor and inhibitor control groups (P>0.05).

Apoptotic rate detected by flow cytometry. Compared with the control group (5.77±1.74%), the apoptotic rate of the let-7b

plasmid group (11.8±1.19%) and the microRNA-199a plasmid group (11.3±1.59%) was significantly higher ($t \geq 36.867$, $P<0.05$). However, no significant differences were found in apoptotic rates between the empty plasmid group (6.75±1.59%), the let-7b inhibitor group (4.39±1.52%), the microRNA-199a inhibitor group (4.97±1.47%), the inhibitor control group (6.68±1.71%) and the control group ($P>0.05$; Fig. 5).

Discussion

Very little is known about microRNA expression patterns in melanoma. Our previous studies have shown that in metastatic skin melanoma 47 microRNAs showed a two-fold or greater reduction in expression, while 6 microRNAs exhibited a two-fold or greater increase in expression, including let-7b, microRNA-199a, microRNA-33, microRNA-193b, microRNA-22 and microRNA-222. Quantitative polymerase chain reaction (qPCR) of tumor samples revealed that let-7b, microRNA-199a, microRNA-33 and clinical transfer index (age, survival time, metastasis and death resulting from metastasis) are positively correlated (12). It has been reported that microRNA signatures differentiate melanoma subtypes; seven microRNAs (microRNA-142-3p, microRNA-486, microRNA-214, microRNA-218, microRNA-362, microRNA-650 and microRNA-31) significantly correlated with acral melanoma compared to non-acral melanoma (15). Furthermore, let-7b and microRNA-199a were upregulated in uveal (ocular) melanoma, showing highly significant associations with the high metastatic gene expression signature and loss of chromosome 3, which have been shown to be more accurate predictors of metastasis than any clinical or pathological factors, and have served as surrogate endpoints for the identification of biomarkers in uveal melanoma (16-18). Similar findings have been found in both skin melanoma and uveal (ocular) melanoma, but it is unclear as to whether this finding is coincidental or whether it is an important indicator of metastasis.

Let-7b and microRNA-199a are involved in ovarian carcinogenesis and are associated with the prognosis of serous ovarian carcinoma; lower let-7b and microRNA-199a expression is correlated with worse prognosis (13). Let-7b is significantly downregulated in acute lymphoblastic leukemia compared with acute myeloid leukemia (19).

microRNA regulates the expression of genes at the post-transcriptional level, which can directly regulate oncogene and/or tumor suppressor gene expression, and is also associated with the cyclin kinases to regulate the cell cycle. For example, let-7b negatively regulates the expression of Ras (20). *In vitro* overexpression of let-7b in melanoma cells downregulated the expression of cyclins D1, D3, A and cyclin-dependent kinase (Cdk) 4 (21). Let-7b overexpression leads to an increased fraction of cells in G2/M, direct downregulation of Cdc34, and stabilization of Wee-1 kinase in primary human fibroblasts (22). microRNAs may also desensitize stem cells to external signals, leading to cell cycle arrest in G1/S transition (23,24).

In this study, we investigated the effect of let-7b and microRNA-199a overexpression and inhibition on a melanoma cell line. We found that the expression of let-7b and microRNA-199a unexpectedly contrasted with the corresponding protein expression, suggesting that other mechanisms may be involved aside from microRNAs. microRNAs generally negatively control gene function; however, when an internal environmental factor changes they may switch to positively regulating gene function. Our study suggests that inhibitors of let-7b and microRNA-199a genes caused these microRNAs to perform opposing functions in melanoma cells. microRNAs can act upon different target mRNAs. For example, let-7b controls CDC25A, CCND1, PLXND1, basigin expression (25), and also controls tumor suppressor genes such as RB1 and DLC1. In B16F10 cells, let-7b and microRNA-199a are expressed at different levels than in normal cells, and have different target genes, regulating cyclin D1 or acting as the inhibitory factor of Met protein deactivation to elevate the expression of cyclin D1 or Met protein. The genes regulating the expression of proteins are different, but these genes may act as a network so that when the intracellular equilibrium is altered to affect the expression of a signaling protein this affects the expression of another factor, and thus indirectly controls protein expression. microRNA-193b represses cell proliferation and regulates cyclin D1 in melanoma (26), while microRNA-34b, microRNA-34c and microRNA-199a have been shown to negatively regulate MET expression (27). Notably, microRNA-199a is downstream of ERK2 (28). While we have not determined the microRNA target genes in this study, our results demonstrate the existence of networks between microRNAs.

This study also explored the effect of let-7b and microRNA-199a at the cellular level using B16F10 cells. Overexpression of let-7b and microRNA-199a significantly decreased cell proliferation and increased apoptosis, suggesting that let-7b and microRNA-199a may be negative regulators of B16F10 cells.

microRNAs are of high interest in cancer research but are difficult to investigate (29). In this study, we investigated the effect of let-7b and microRNA-199a on melanoma proliferation at the microRNA level, providing new scientific evidence and ideas for clinical diagnosis and treatment. However, the mechanism of microRNA regulation of mRNA and its clinical applicability requires further study.

Acknowledgements

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