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Original Article

miR-199a-5p regulates the expression of metastasis-associated genes in B16F10 melanoma cells

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Abstract: MicroRNAs are regulatory factors that play important roles in tumor development, invasion and metastasis. Previously, we showed that *miR-199a* is abnormally expressed in clinical melanoma specimens and expression was closely associated with clinical features of metastasis. However, the exact molecular mechanisms by which *miR-199a-5p* influences melanoma invasion and metastasis remains unclear. In this study, we investigated gene expression changes of metastasis-associated genes in B16F10 melanoma cells following targeted silencing or overexpression of *miR-199a-5p*, using mouse tumor metastasis PCR arrays. Comparison of gene expression changes in *miR-199a-5p*-silenced versus overexpressing cells identified a set of upregulated genes (> 2-fold) including *Cd44*, *Cdh1*, *Cxcr4*, *Etv4*, *Fxyd5*, *Rpsa*, *Mmp3*, *Myc*, *Rb1*, *Tcf20*, *Hprt1, Actb1* and downregulated genes (> 2-fold) including *Ctsk*, *Itga7* and *Tnfsf10*. Regulation of a subset of these genes (*Myc*, *Tnfsf10* and *Cd44*) following *miR-199a-5p* silencing or overexpression was validated by reverse transcription-polymerase chain reaction (RT-PCR) and western blot. In conclusion, our study demonstrates that *miR-199a-5p* regulates melanoma metastasis-related genes, and may provide a basis for the development of novel, molecularly targeted drugs.

Keywords: Melanoma, *miR-199a-5p*, metastasis PCR array

Introduction

Melanoma is a malignant tumor initiated from neural crest cells and often occurs in the skin, with a clear familial aggregation [1]. Melanomas are prone to invasion and metastasis and are associated with a high mortality rate. In the early stages of disease, melanoma patients exhibit mild symptoms, with regional lymph node metastasis and even distant metastasis often visible in confirmed cases of melanoma, associated with a poor prognosis. Therefore, a deeper understanding of the specific pathways underlying melanoma cell invasion and metastasis will likely have far-reaching significance for the treatment of patients with melanoma.

Recent studies have shown that microRNAs (miRNAs) are important regulatory factors involved in the development, invasion and metastasis of melanoma [2]. *MiR-18b* has been shown to target MDM2-TP53 signaling [3], *miR-7-5p* partially inhibits IRS-2 expression and AKT signaling [4]. And *miR-9* can be targeted to regulate NF-κB1 signaling, thereby inhibiting melanoma cell proliferation, invasion and metastasis [5]. Conversely, *miR-182* regulates MATF-M and FOXO3; [6] and *miR-21* can downregulate *PTEN* and *PDCD4* mRNA expression and BTG2 protein expression, promoting proliferation, invasion and metastasis of melanoma [7]. However, the specific molecular mechanisms by which miRNAs may affect tumor invasion and metastasis in melanoma remain unclear.

Our previous miRNAs microarray analyses of clinical melanoma specimens revealed abnormal expression of *miR-199a*, and this was closely positively related to clinical features of melanoma metastasis. In the present study, a mouse tumor metastasis PCR array was used to investigate expression changes of 84 known tumor metastasis-associated genes, following targeted inhibition or overexpression of *miR-199a-5p* in B16F10 melanoma cells. These results were subsequently validated by RT-PCR and western blot analysis. Understanding the specific molecular mechanisms by which *miR-199a-5p* regulates melanoma invasion and metastasis will provide new strategies for the development of novel, molecularly targeted drugs with the ultimate goal of improving patient survival.

Materials and methods

Cell culture

The highly metastatic, B16F10 mouse melanoma cell line was purchased from Nanjing KeyGen Biotech Limited Company (China). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin, and incubated in a 5% CO_2 humidified incubator at 37°C.

Antibodies and reagents

Myc, Tnfsf10 and *Cd44* antibodies were purchased from Abgent Company (San Diego, CA, USA). The mmu-miR-199a-5p mimics and mmu-microRNA-199a-5p inhibitor plasmid was purchased from RiboBio Biological Technology Company (China). The SuperArray PCR master mix microarray kit and Mouse Metastasis gene chip (PAMM-028A) were purchased from SuperArray (USA) and SABiosciences (USA), respectively.

Grouping

Experimental treatment groups were as follows: i) Blank group: non-treated B16F10 cells; ii) miR-199a-5p silenced group: liposome-mediated transfection of B16F10 cells with mmu-microRNA-199a-5p inhibitor, iii) miR-199a-5p overexpression group: liposome-mediated transfection of B16F10 cells with mmu-miR-199a-5p mimics and plasmid.

Transfection

B16F10 cells were seeded at a density of 1×10^5 cells/well (final volume 2 ml) in 6-well plates. Near-confluent (80-90%) or sub-confluent (30-50%) cells were subsequently transfected with either mmu-microRNA-199a-5p inhibitor (5 μl) or mmu-miR-199a-5p mimics (5 μl), respectively.

RT-PCR

Cells were harvested 48 h post-transfection and RNA was extracted using TRIzol solution in accordance with the manufacturer's instructions. RNA purity and concentration were measured using a NanoDrop ND-1000(USA). Complementary DNA samples were prepared using a reverse transcription kit in accordance with the manufacturer's instructions (K1662, Fermentas, Canada). In brief, total RNA (1 μg), oligo$(dT)_{18}$ (1 μl) and diethylpyrocarbonate water (up to 12 μl) were combined in a PCR tube, incubated at 65°C for 5 min and then placed on ice for 2 min. A reaction mixture containing 5× Reaction Buffer (4 μl), RNase inhibitor (20 U/μl, 1 μl), dNTP Mix (10 mM, 2 μl) and M-MuLV Reverse Transcriptase (200 U/μl, 1 μl) in a total volume of 20 μl was then added to each tube, and reactions were incubated at 42°C for 60 min and 70°C for 5 min. PCR reactions were assembled in a final volume of 20 μl including cDNA (1 μl), Forward Primer (0.5 μM), Reverse Primer (0.5 μM) and PCRMix (10 μl). Primer sequences are as follows: *Myc* (NM_010849.4) 353 bp: forward: 5' AAGGGAAGACGATGACGG 3', reverse: 5' TGAGCGGGTAGGGAAAGA 3'; *Tnfsf10* (NM_009425.2) 257 bp: forward: 5' GGGCATTCATTTCTCAAC 3', reverse: 5' AGTCCGTACTCGGCATCT 3'. *Cd44* (NM_001039150.1), 763 bp: forward: 5' CCTTGGCCACCACTCCTAAT 3', reverse: 5' GTGGTCACTCCACTGTCCTG 3'. All primers were synthesized by Sangon Biotech Company (Shanghai, China) and primer of GADPH was bought from Fermentas (Canada). PCR was performed using the following cycling conditions: 5 min at 95°C (one cycle), then 30 s at 94°C, 30 s at 53°C, 30 s at 72°C (30 cycles) and a final extension of 7 min at 72°C. PCR reactions (5 μl) were analyzed on a 1.5% agarose gel containing ethidium bromide for 1 h, and *GAPDH* was used as an internal reference gene. DNA was visualized under UV light (254 nm) and images were captured.

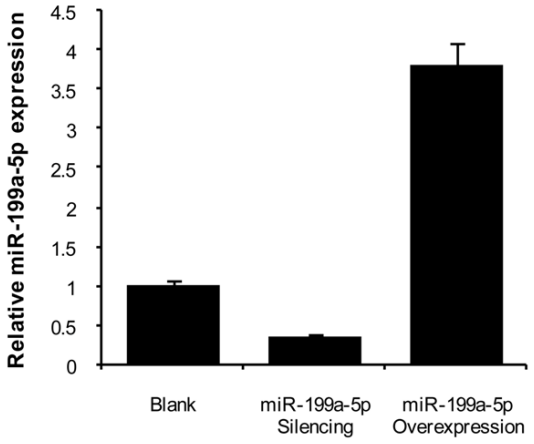


Figure 1. Targeted inhibition or overexpression of *miR-199a-5p* in B16F10 cells. Relative *miR-199a-5p* expression levels in different groups.

Real-time PCR validation of miR-199a-5p expression

RNA and cDNA were prepared as described above. Following reverse transcription, reactions were prepared using SYBR Premix Ex Taq™ (DRR041A, Takara, Japan) in accordance with the manufacturer's instructions. PCR reactions were performed in a 20 μl reaction volume containing 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) and ddH_2O (6.8 μl). Real-time PCR was performed using the following cycling conditions: 95°C for 10 s (one cycle), then 95°C for 5 s, 60°C for 30 s, 95°C for 15 s, 60°C for 15 s and 95°C for 15 s (40 cycles). The relative expression of *miR-199a-5p* (F) was calculated as follows: $F = 2^{-\Delta\Delta ct}$ (where Δct1 = sample group average target gene ct value-average internal reference gene value; Δct2 = Blank group average target gene ct value - average internal reference gene value; ΔΔct = Δct1-Δct2).

Real-time PCR microarray screening

Total RNA was extracted from B16F10 tumor cells following defined treatments using TRIzol solution and RNA concentration and purity were assessed by UV spectrophotometry. Metastasis-associated genes were identified using real-time mouse tumor metastasis PCR arrays (performed by Kangchen Biotech, Shanghai, China). Differentially expressed genes ≥ 2-fold ($P < 0.05$) were considered significant.

Western blot

B16F10 cells were collected 48 h post-transfection and total protein was extracted as previously described. The concentration of protein extracts was measured according to the Bradford method, and extracts (30 μg/lane) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were then blocked in 5% skim milk/PBS-Tween for 1.5 h and incubated with primary antibodies murine anti-Myc, Tnfsf10 and Cd44 (1:1000) and murine anti-α-tublin (1:10 000) at 4°C overnight. After washing with PBS-Tween (3×), membranes were incubated with murine, horseradish peroxidase (HRP)-conjugated secondary antibody (1:10 000) for 1 h at room temperature, washed with PBS-Tween (3×), and signal was developed using enhanced chemiluminescence (ECL) substrate.

Statistical analysis

Data were analyzed using SPSS 13.0 (SPSS, Chicago, IL, USA), and are represented as the mean ± standard deviation (SD). A paired t-test was used to examine differences between each group. A P value < 0.05 was considered statistically significant.

Results

Silencing or overexpression of miR-199a-5p in B16F10 cells

To investigate *miR-199a-5p*-induced gene expression changes in B16F10 cells, we performed both loss-of-function and gain-of-function analyses following transient transfection of cells with either mmu-microRNA-199a-5p inhibitor or mmu-miR-199a-5p mimics plasmid, respectively. After 24 h, analysis of *miR-199a-5p* expression by real-time PCR revealed downregulation of *miR-199a-5p* in mmu-microRNA-199a-5p inhibitor transfected cells relative to controls (0.354-fold ± 0.025). In contrast, *miR-199a-5p* expression was upregulated (3.815-fold ± 0.282) in mmu-hsa-miR-199a-5p mimics transfected cells compared with controls (**Figure 1**). These results confirm the successful targeted silencing and overexpressing of *miR-199a-5p* in B16F10 cells.

Table 1. Different expressions of tumor metastatic-related genes

Gene Name	miR-199a-5p Silencing/Blank	miR-199a-5p Overexpression/Blank	miR-199a-5p Silencing/Overexpression
Cd44	—	—	2.28
Cdh1	—	-2.77	3.33
Cxcr4	12.24	3.18	3.85
Ctbp1	2.34	—	—
Ctsk	—	2.12	-2.04
Csf1	—	2.67	—
Ela2	—	2.69	—
Etv4	—	-3.38	3.99
Fxyd5	—	-2.2	2.67
Rpsa	2.21	—	2.48
Mycl1	3.44	—	—
Mmp3	10.03	3.31	3.03
Mmp13	3.21	2.6	—
Myc	—	-3.25	3.02
Nme2	—	-2.33	—
P2ry5	—	3.04	—
Kiss1r	2.06	2.92	—
Hgf	8.22	5.4	—
Hras1	—	2.11	—
Htatip2	3.2	3.28	—
Il18	6.29	7.59	—
Il8rb	13.12	22.22	—
Cd82	2.15	—	—
Kiss1	2.3	3.37	—
Rb1	—	-3.47	3.07
Tcf20	—	—	2.13
Timp4	2.05	2.71	—
Hprt1	—	-2.34	2.93
Actb1	—	—	2.11
Tnfsf10	—	7.47	-5.5
Itga7	—	4.88	-2.64

Note: Blank space instead of differences in gene expression within 2 times, not practical significance.

Tumor metastatic PCR arrays of B16F10 cells following modulation of miR-199a-5p expression

To investigate the effects of *miR-199a-5p* on melanoma cell invasion and metastasis, we used tumor metastasis PCR arrays to detect expression changes in 84 known tumor metastasis-associated genes in control, *miR-199a-5p*-silencing and *miR-199a-5p*-overexpressing B16F10 cells (**Table 1**). Real-time PCR analyses revealed that the majority of genes exhibited Ct values lower than 30, corresponding with higher gene expression (since genes with a Ct value > 35 are regarded as low/not expressed). The percentages of Ct values corresponding to < 25, 25-30, 30-35 and Absent Calls were as follows: Blank group, 67% (49), 16% (12), 11% (8), 5% (4); *miR-199a-5p*-silenced group, 60% (50), 14% (12), 16% (13), 10% (8) and the *miR-199a-5p*-overexpressing group, 57% (46), 20% (16), 11% (14), 10% (8) (**Figure 2A-C**).

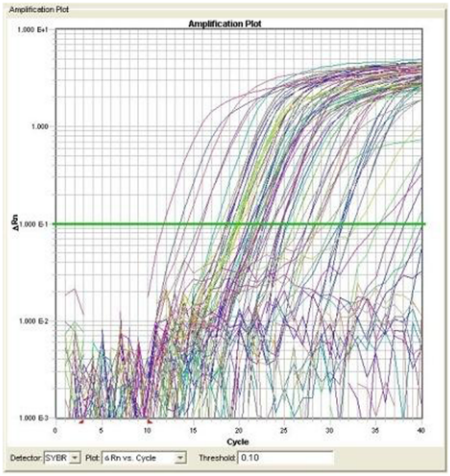
To further investigate gene expression changes related to *miR-199a-5p* expression, we next investigated genes differentially expressed in *miR-199a-5p*-silenced cells compared with controls. We identified upregulation of 14 genes (> 2-fold) including *Cxcr4*, *Ctbp1*, *Rpsa*, *Mycl1*, *Mmp3*, *Mmp13*, *Kiss1r*, *Hgf*, *Htatip2*, *Il18*, *Il8rb*, *Cd82*, *Kiss1* and *Timp4*, and there has no downregulated gene. Overexpression of *miR-199a-5p* led to upregulation of 17 genes (> 2-fold) including *Cxcr4*, *Ctsk*, *Csf1*, *Ela2*, *Mmp3*, *Mmp13*, *P2ry5*, *Kiss1r*, *Hgf*, *Hras1*, *Htatip2*, *Il18*, *Il8rb*, *Kiss1*, *Timp4*, *Itga7*, *Tnfsf10*, and downregulation of 7 genes, (> 2-fold) including *Cdh1*, *Etv4*, *Fxyd5*, *Myc*, *Nme2*, *Rb1* and *Hprt1*. Comparison of genes differentially expressed in *miR-199a-5p*-silenced cells compared with overexpressing cells revealed upregulation (> 2-fold) of 12 genes including *Cd44*, *Cdh1*, *Cxcr4*, *Etv4*, *Fxyd5*, *Rpsa*, *Mmp3*, *Myc*, *Rb1*, *Tcf20*, *Hprt1* and *Actb1* and downregulation of 3 genes including *Ctsk*, *Itga7* and *Tnfsf10* (**Table 1**).

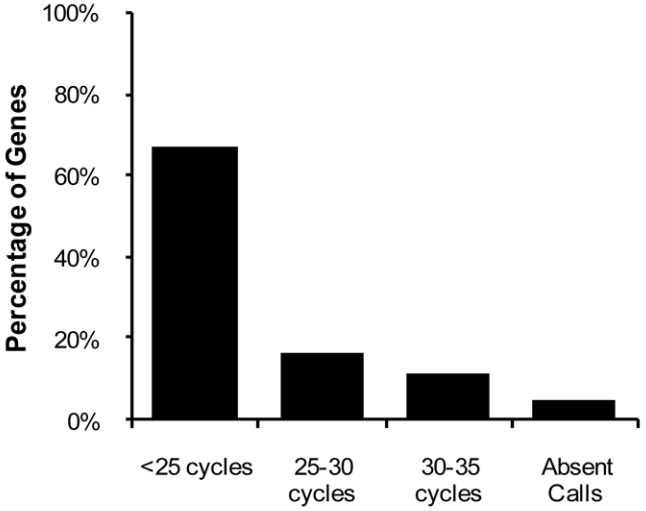
Expression of Myc, Tnfsf10 and CD44 following miR-199a-5p silencing and overexpression

To validate the accuracy of our PCR microarray results, we assessed the expression of two candidate targets, *Myc* and *Tnfsf10*, by RT-PCR and Western blot. According to the PCR microarray, comparison of gene expression between *miR-199a-5p*-silenced versus overexpressing cells revealed a 3.02-fold upregulation of *Myc* and 5.5-fold downregulation of *Tnfsf10*. RT-PCR and Gel-pro Analyzer image analysis revealed significant upregulation of *Myc* in *miR-199a-5p*-silenced cells compared with overexpressing cells IOD (integrated optical density) value of *Myc* (353 bp) to *GAPDH* (495 bp) of (0.72 ± 0.05) *vs* (0.53 ± 0.04), respectively; $p < 0.05$), consistent with western blot results. Conversely, *Tnfsf10* expression was significantly downregulated in *miR-199a-5p*-silenced cells compared

A

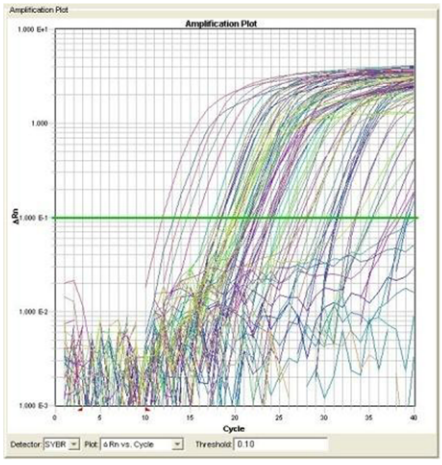
Blank

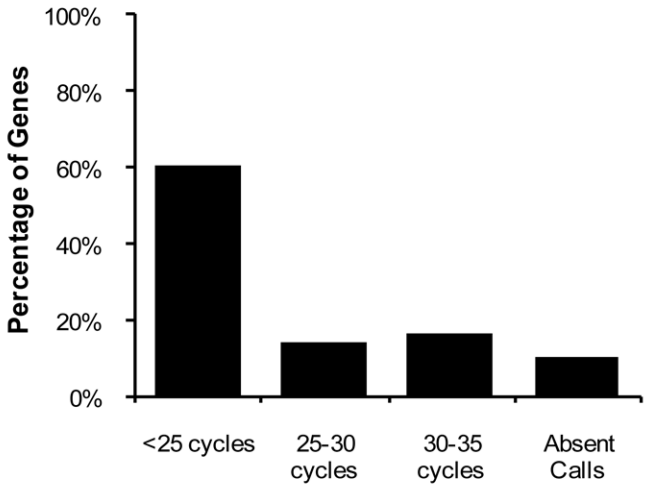




B

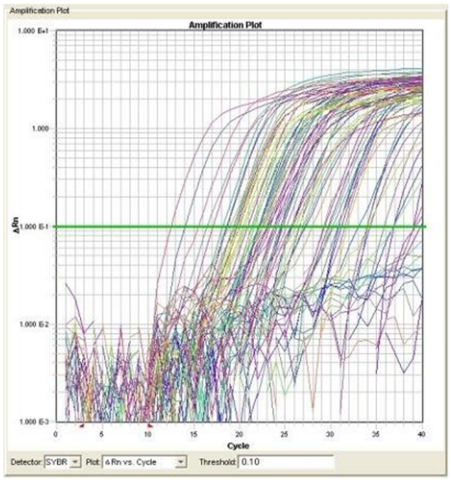
miR-199a-5p Silencing





C

miR-199a-5p Overexpression



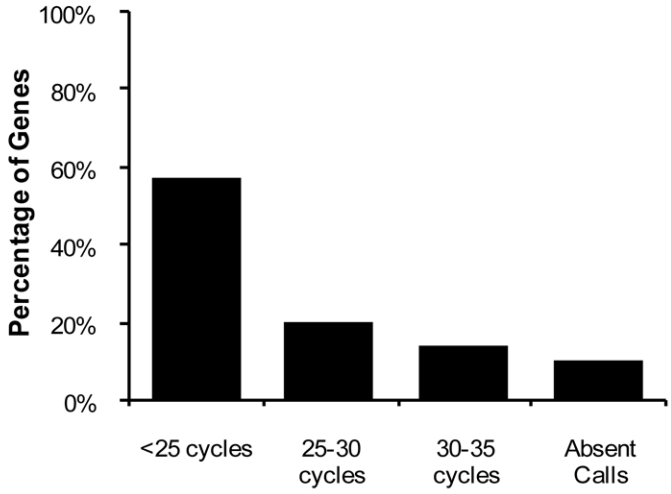


Figure 2. Tumor metastatic PCR arrays of WM451 cells following targeted inhibition or overexpression of *miR-199a-5p*. A. Amplification curves and percentage of tumor metastatic genes in the negative control (blank) cells. B. Amplification curves and percentage of tumor metastatic genes in *miR-199a-5p*-silenced cells. C. Amplification curves and percentage of tumor metastatic genes in *miR-199a-5p*-overexpressing cells.

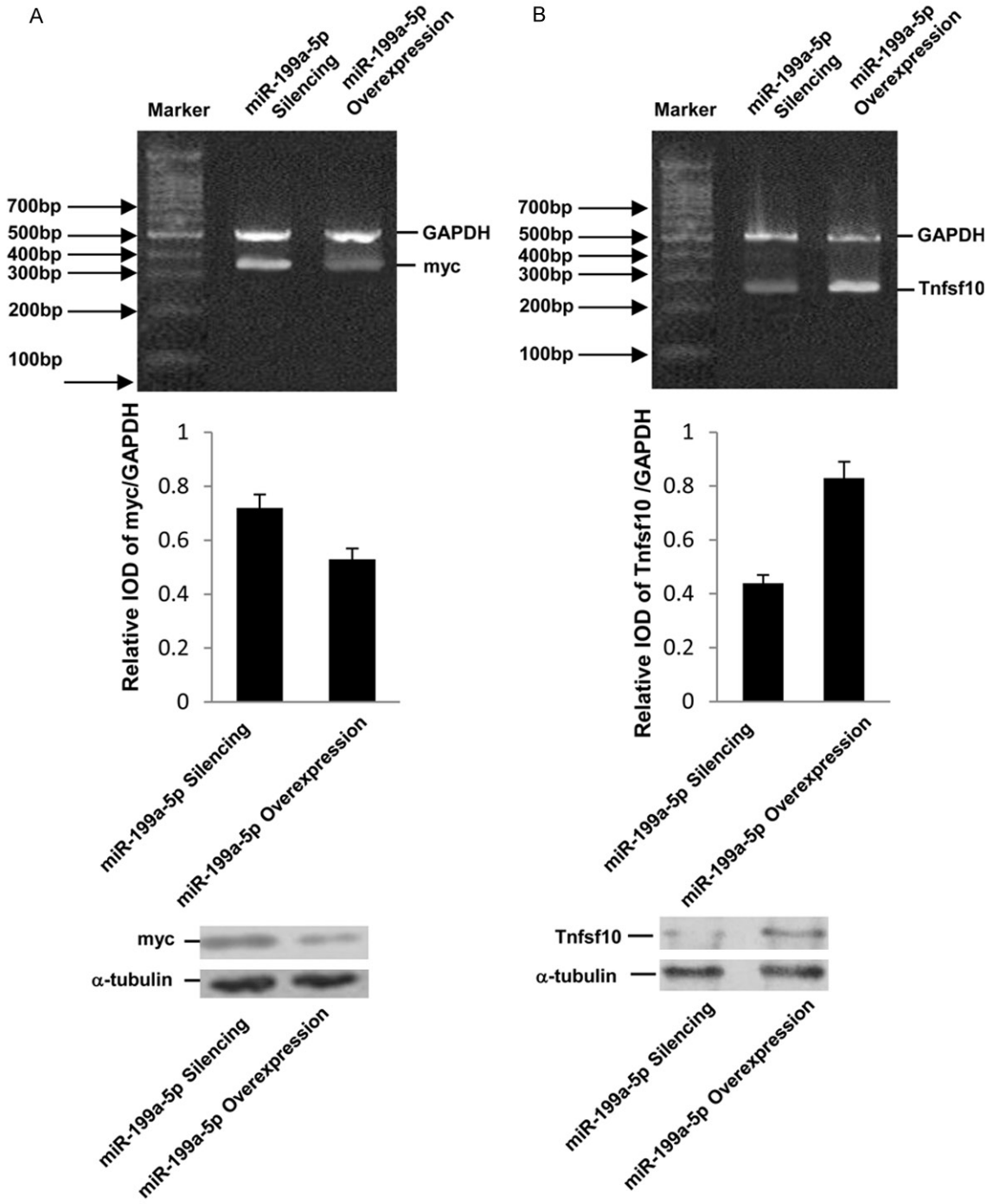


Figure 3. Expression of *Myc* and *Tnfsf10* following targeted inhibition or overexpression of *miR-199a-5p*. A. Expression of *Myc* was detected by RT-PCR and western blot. B. Expression of *Tnfsf10* was detected by RT-PCR and Western blot.

with overexpressing cells (IOD value of *Tnfsf10* (257 bp) to *GAPDH* of (0.44 ± 0.03) *vs* (0.83 ± 0.06); $p < 0.05$). Furthermore, *Tnfsf10* protein levels were also dramatically decreased in *miR-*

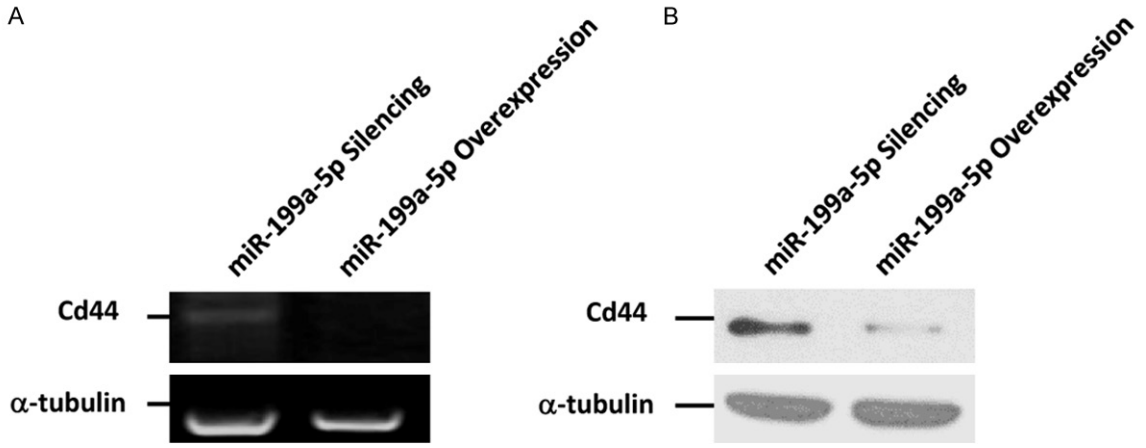


Figure 4. *miR-199a-5p* targets Cd44. A. Expression of Cd44 was detected by RT-PCR. B. Expression of Cd44 was detected by Western blot.

199a-5p-silenced cells (**Figure 3**). Our PCR array analysis also identified upregulation of *Cd44* (2.28-fold), which was previously reported to be a target of *miR-199a-5p* [8], in *miR-199a-5p*-silenced cells compared with overexpressing cells. This upregulation was subsequently validated by RT-PCR and western blot (**Figure 4**), further confirming the accuracy and reliability of our PCR microarray results. Thus, *miR-199a-5p* exerts an effect on melanoma cell invasion and metastasis by regulating the expression of multiple genes.

Discussion

miR-199a-5p with a low expression in breast cancer can be targeted by DRAM1 and Beclin1 to inhibit radiation-induced autophagy in breast cancer cells [9, 10]. Low expression of *miR-199a-5p* has previously been observed in hepatic carcinoma [11]. Cisplatin-induced downregulation of *miR-199a-5p* is capable of activating autophagy of hepatoma cells, leading to drug resistance [12]. Low expression of *miR-199a* is also observed in ovarian cancer, targeting IKKβ and thereby affecting NF-κB activity. Furthermore, reactive oxygen species may inhibit *miR-199a* and *miR-125b*, leading to induction of ERBB2 and ERBB3 expression in ovarian cancer [13-15]. The *miR-199a* has also been reported as a tumor suppressor gene in renal cancer and testicular cancer in addition to other tumors [16, 17]. In contrast, *miR-199a* may also act as an oncogene when highly expressed in gastric cancer. *miR-199a* is not only capable of targeting mitogen-activated protein kinase 11 [18], but also Smad4, negatively regulating tumor growth factor β signaling pathway [19]. Thus, *miR-199a-5p* exhibits multiple biological functions and tissue specificity.

The *miR-199a* is highly expressed in melanoma exhibiting high invasive ability [20]. The expression of *miR-199a* is increased in clinical specimens of older patients (> 60 years) with melanoma [21]. *miR-199a-5p*, *miR-199a-3p* and *miR-1908* may target ApoE to drive LRP1/LRP8-dependent tumor metastasis and angiogenesis [22]. Previously, we observed that *miR-199a-5p* expression was remarkably correlated to the high metastatic ability of melanoma, and may represent one of several key miRNAs in melanoma metastasis [1].

To investigate the transcriptomic effect of *miR-199a-5p* in melanoma, we used murine tumor metastasis PCR microarray chips containing metastasis-associated genes involved in cell adhesion, extracellular matrix components, cell cycle, cell growth and proliferation, apoptosis, transcription and other biological processes. A subset of these differentially expressed genes was then validated using RT-PCR and western blot, verifying the accuracy of the PCR microarray results and validity of this screening approach. While we did not find *miR-199a-5p* binding target sequences in *Myc* and *Tnfsf10* 3'UTRs using TargetScan prediction software, it is possible that *miR-199a-5p* indirectly influences tumor invasion and metastasis-related genes, by adjusting network-related transcription factors and binding proteins. Future studies exploring the signaling pathways through which *miR-199a-5p* influences metastases-

related genes in mouse melanoma cells are therefore necessary.

Effective control of tumor metastasis requires a deeper understanding of genes driving critical processes of invasion and metastasis in melanoma cells. To this end, miRNA interference technology has been shown to inhibit the progression of melanoma invasion [23]. Therefore, the study of *miR-199a-5p* and its network-based regulation of invasion and metastasis may provide a new strategy for the development of molecularly targeted therapy, with the goal of improving treatment outcome in melanoma patients.

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Disclosure of conflict of interest

None.

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