

Original Article

MicroRNA-138 suppresses cell proliferation of human malignant melanoma cells by targeting hTERT

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Received August 15, 2016; Accepted October 19, 2016; Epub April 15, 2017; Published April 30, 2017

Abstract: MicroRNA-138 (miR-138) has been proven to be a tumor suppressor gene in various types of tumors. However, the expression and the role of miR-138 in human malignant melanoma (MM) are still poorly understood. The aim of the present study was to investigate the functional significance of miR-138 and to identify its target genes in MM cells. We applied the quantitative real-time PCR (qRT-PCR) to detect the miR-138 levels in MM tissues (n=20) and cell lines, the overall survival rate of MM patients was determined by Kaplan-Meier survival curve. The cell proliferation, cell apoptosis and cycle were analyzed by cell counting Kit-8 (CCK-8) assay and by flow cytometry. Bioinformatical predication, luciferase reporter assay and western blot were used to identify the target gene of miR-138. We observed that miR-138 expression level was significantly decreased in MM tissues compared to their matched adjacent normal tissues. Kaplan-Meier survival analysis showed that the patients with low miR-138 expression presented significantly shorter survival time compared to those with high miR-138 expression. Moreover, overexpression of miR-138 significantly inhibited MM cell growth and prompted cell apoptosis and cycle arrest *in vitro*. We further identified human telomerase reverse transcriptase (hTERT) as a direct target gene of miR-138, and demonstrated that the protein level of hTERT was negatively mediated by miR-138 in MM cells. Meanwhile, overexpression of hTERT rescued the suppressive effects of miR-138 on MM cell proliferation. These findings suggested that miR-138 acts as a tumor suppressor in MM by targeting hTERT. MiR-138/hTERT may be a novel therapeutic target in MM.

Keywords: MicroRNA-138, malignant melanoma, cell apoptosis, cycle arrest, human telomerase reverse transcriptase

Introduction

Malignant melanoma (MM) is one of the most common skin cancers in humans [1]. The incidence of MM continues to rise more rapidly than that of all other malignancies except for lung cancer [2]. MM develops as a result of an accumulation of various endogenous and exogenous causes. Gene alterations play an important role in MM, and alterations in a large number of oncogenes and tumor suppressor genes have already been reported in MM [3]. Therefore, better understanding of the molecular mechanisms of MM tumorigenesis will help to explore novel therapeutic agents and prognostic markers in the treatment of patients with MM.

Human telomerase reverse transcriptase (hTERT), which confers the catalytic activity of

telomerase, is the restricting factor for telomerase activity [4]. As normal proliferating cells enter into the process of apoptosis or necrosis, the expression of hTERT expression gradually decreases and cells cease proliferation [5]. However, elevated hTERT expression and the activation of telomerase can result in unlimited proliferative ability in tumor cells [6]. Investigations have shown that hTERT has a significant role in cancer tumorigenesis, growth, migration and invasion [7]. Therefore, hTERT is considered as an attractive therapeutic target for cancer treatment, and understanding the regulatory mechanisms controlling hTERT expression is of great interest.

MicroRNAs (miRNAs) are a class of endogenously conserved small RNAs that regulate target gene expression by affecting mRNA translation and stability or by modulating the

promoter activity of target genes [8, 9]. miRNAs may control the majority of human genes, which are involved in a wide variety of biological and pathogenic processes, including cancers [10-13]. Dysregulation of miRNAs in cancer cells influences the function of oncogenes and tumor suppressors [12, 14]. Overexpression or downregulation of specific miRNAs has recently been reported in a variety of human malignancies. Among these miRNAs, miR-138 generally acts as a tumor suppressor in different cancer types, such as ovarian cancer [15], glioblastoma [16], squamous cell carcinoma [17], head and neck squamous cell carcinoma [18], as well as MM [19]. The overexpression of miR-138 inhibits cell proliferation and colony formation [20]. Downregulation of miR-138 in neuroblastoma and thyroid carcinoma is associated with the hTERT, which promotes malignant cell growth of many tumors [21, 22]. Current study found that miR-346 and miR-138 competitively bind to a common region in the 3'UTR of hTERT mRNA and have opposite effects on the expression and function of hTERT in human cervical cancer cells [23]. However, the expression of miR-138 and its role in human MM are still poorly understood.

In this study, we aim to determine whether miR-138 is differentially expressed in MM and to analyze its role in cancer cell growth. We found that the expression of miR-138 was significantly lower in MM tissues, and miR-138 overexpression in MM cells inhibited cell growth by inducing cell cycle arrest and apoptosis. It was also identified that hTERT is a direct and functional target of miR-138 in MM. Taken together, these results suggest that modulation of the mechanism responsible for miR-138 in MM could be used as a critical therapeutic strategy for MM intervention and warrants further investigation.

Materials and methods

Ethics statement

Investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines and has been approved by the authors' institutional review board.

Tissue specimens and cell lines

Tumor samples and their adjacent healthy tissue counterparts were obtained from 20 melano-

noma patients who underwent surgery between April 2013 and April 2014 in Zhongshan Hospital, Fudan University. The written informed consents had been signed by all patients in advance. The tissue samples were frozen in liquid nitrogen and stored at -80°C for RNA isolation. None patients had received any radiotherapy or chemotherapy prior to surgery. The metastatic melanoma cell lines (WM266-4, A375 and SK-MEL-2) were purchased from the American Type Culture Collection (ATCC). A2058 cells were a gift from Prof. Peter Klincken (Western Australian Institute for Medical Research). The cell line A375 was derived from a primary melanoma [24], and the WM266-4, A2058 and SK-MEL-2 cell lines were derived from melanoma lymph node metastases. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) at 37°C in 5% CO₂ and used within 20 passages of initial stock.

RNA extraction and quantitative real-time PCR

The miRNAs were isolated from melanoma tissues or cell lines using a RNeasy/miRNeasy Mini kit (Qiagen, Limburg, The Netherlands) according to the manufacturer's instructions. Total RNA was extracted using the TRIzol Reagent (Life Technologies, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cDNAs were synthesized using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania), and real-time quantitative PCR (RT-qPCR) was carried out using the SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a 7900 Real-Time PCR System (Applied Biosystems). Expression of U6 RNA was used to normalize the expression of miR-138. The specific primers for miR-138 were shown as follow: 5'-AGCTGGTGTGTAATCAGGCCG-3' (forward), 5'-TGGTGTCGTGGAGTCG-3' (reverse). The specific primers for U6 were shown as follow: 5'-CTCGCTTCGGCAGCACACA-3' (forward), 5'-AACGCTTCACGAATTTGCGT-3' (reverse). The relative expression level was quantified using the 2^{-ΔΔCt} method.

Oligonucleotide transfection

The miR-138 mimics or inhibitor and corresponding negative control (NC) were designed and synthesised by RiboBio (Guangzhou, China). The human MM cell lines WM266-4 or A2058 were transfected with miR-138 mimics,

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miR-138 inhibitor or the corresponding negative controls at a final concentration of 40 μ M using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. About 48 h after transfection, the transfection efficiency was assessed. The cells could be used for subsequent analysis when the transfection efficiency was above 80%.

Cell counting Kit-8 assay

The proliferation of cells was determined by the Cell counting Kit-8 (CCK-8) assay according to the manufacturer's instructions [25]. After transfected miR-138 into WM266-4 or A2058, cells were seeded in 96-well plates, each well of which contained 100 μ L PRMI-1640 medium supplemented with 10% FBS at 5×10^4 cells/well. After 48 h incubation, CCK-8 reagent (10 μ L) was added to each well of a 96-well flat bottomed microplate containing 100 μ L of culture medium to a final concentration of 10 μ L/100 μ L, and incubated for an additional 1 hours at 37°C. The absorbance rate was measured at 450 nm by auto Microplate Reader (Bio-Rad, USA). All experiments were performed in quintuplicate on three separate occasions.

Apoptosis assay

Forty-eight hours after transfection, cells were harvested, washed once with binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl_2) and stained with 5 μ L Annexin V-FITC (Bectone Dickinson, Mountain View, CA, United States) for 15 min and 10 μ L propidium iodide (PI, 20 μ L g/ml) (Bectone Dickinson, Mountain View, CA, United States) for 10 min. The cells were analyzed by flow cytometry using an EPICS XL-MCL FACS can (Bectone Dickinson, Mountain View, CA, United States). The data was analyzed with the Multi Cycle Software for Windows (Phoenix Flow Systems, San Diego, CA, United States). The experiments for the apoptosis assay were performed at least three times.

Cell-cycle analysis

The WM266-4 or A2058 cells were seeded into 6-well plates at a concentration of 0.5×10^5 cells/well the day before transfection. After the transfection with miR-138 mimics or mimics NC, the cells were incubated for 72 h. For flow cytometric analysis (FACSC alibur, BD Biosci-

ences), the cells were prepared by using the BD Cycletest TM Plus-DNA reagent kit (BD Biosciences) according to manufacturer's instructions. The G0/G1 and G2/M ratios were calculated by using analysis software (Cell Quest, BD Biosciences).

Plasmid and luciferase reporter assay

The full-length of the 3'-UTR of hTERT were generated by PCR amplification from the cDNA generated from WM266-4 cells using the primers: 5'-ATCTCGAGTGGCCACCCGCCACAG-3' (forward), 5'-ATCTCGAGCTGAGTGAGTGTG-3' (reverse). Using the XhoI-XbaI sites incorporated into the primers, they were subcloned into pcDNA3.0⁺ (Invitrogen) to generate pcDNA-hTERT 3'-UTR-full-length. MiR target site prediction for hTERT was performed using Targetscan Release 6.2, PicTar5 and miRanda 3.0 programs. The wildtype hTERT3'-UTR was constructed by PCR and inserted into the psiCHECKTM-2 vector (Promega, Madison, WI, USA). The mutant hTERT3'-UTR was generated by using Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), in accordance with the manufacturer's protocol, and then inserted into the psiCHECKTM-2 vector (**Figure 3A**). The WM266-4 cells were cultured to approximately 70% confluence, and co-transfected with wildtype hTERT3'-UTR or mutant hTERT3'-UTR plasmid (100 ng), and miR-138 mimics/inhibitor or corresponding NC oligos (50 nM), using Lipofectamine 2000. The relative firefly luciferase activity normalized with Renilla luciferase was measured 48 h after transfection by using the Dual-Light luminescent reporter gene assay (Applied Biosystems).

Western blot analysis

Forty-eight hours after transfection, total protein extracts were prepared using RIPA buffer with protease inhibitor Cocktail (Pierce, Rockford, IL, USA). Protein concentrations were examined using the BCA Protein Assay Kit (Pierce). Total proteins (20 μ g) were separated on 10% SDS PAGE and then transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk at 4°C overnight, the membranes were incubated with primary antibodies against hTERT (1:500, mouse monoclonal anti-hTERT, Tianjin Saier Biotech) at 4°C overnight, β -actin

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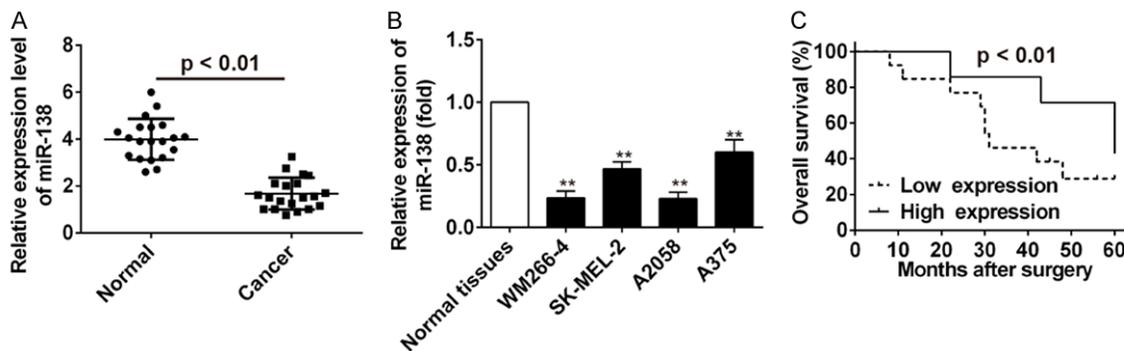


Figure 1. Mir-138 expression was downregulated in human malignant melanoma (MM) tissues and cell lines. A. The mRNA levels of miR-138 were quantified by qRT-PCR analysis in human MM tissues. B. The mRNA levels of miR-138 were quantified by qRT-PCR analysis in human MM lines. C. Kaplan-Meier survival analysis was performed using patient overall survival (n=20). All values are mean \pm SD. **vs control, $P < 0.01$.

(1:1000, Sigma, St. Louis, MO) was used as an internal control for protein loading. Horseradish peroxidase-conjugated (HRP) antibodies were used as the secondary antibodies. The bands were scanned using the ChemiDocXRS+ Imaging System (Bio-Rad) and quantified using Quantity One v4.6.2 software (Bio-Rad).

Statistical analysis

Each experiment was repeated at least 3 times. Numerical data are presented as the mean \pm SEM. The difference between means was analysed with Student's t test. All statistical analyses were conducted using SPSS 14.0 software (Chicago, IL). The overall survival was calculated using the Kaplan-Meier method. p value of < 0.05 was considered significant and < 0.01 was considered very significant.

Results

miR-138 expression was downregulated in human melanoma tissues and cell lines

The expression level of miR-138 was quantified by qRT-PCR analysis in human MM tissues. Results showed that compared with the adjacent normal tissues, the expression level of miR-138 was significantly downregulated in human MM tissues ($P < 0.01$) (Figure 1A). In order to further verify this differential expression of miR-138, we detected the miR-138 expression in four kinds of human melanoma cell lines (WM266-4, A375, SK-MEL-2 and A2058). Consistent with the results in melanoma tissues, miR-138 was downregulated in all melanoma cells compared with the normal tis-

sues ($P < 0.01$) (Figure 1B). To assess the clinical significance of decreased miR-138 in melanoma patients, Kaplan-Meier survival analysis was performed using patient overall survival. The results demonstrated that patients with low miR-138 expression presented significantly shorter survival time compared to those with high miR-138 expression ($P < 0.01$) (Figure 1C). These data revealed that low expression of miR-138 was an independent prognostic marker of melanoma patients.

Functional effects of miR-138 upregulation on melanoma cell proliferation, apoptosis and cell cycle

Because our data suggest that miR-138 expression is significantly downregulated in melanomas when compared with normal tissues, we further analysed the role of miR-138 in proliferation and apoptosis of melanoma cells *in vitro*. Therefore, the WM266-4 or A2058 melanoma cell lines (showing low endogenous miR-138 expression) were transiently transfected with miR-138 mimics or mimics NC. Quantitative RT-qPCR revealed a significant upregulation of miR-138 in cells transfected with miR-138 mimics as compared with mimics NC in both melanoma cell lines ($P < 0.01$) (Figure 2A). Subsequently, the effect of miR-138 upregulation on proliferation and apoptosis of WM266-4 or A2058 cells were investigated. In both cell lines, miR-138 upregulation resulted in increased proliferation as assessed by CCK-8 assay after 1-5 d (Figure 2B and 2C). Flow cytometry indicated that WM266-4 or A2058 cells with ectopic overexpression of miR-138

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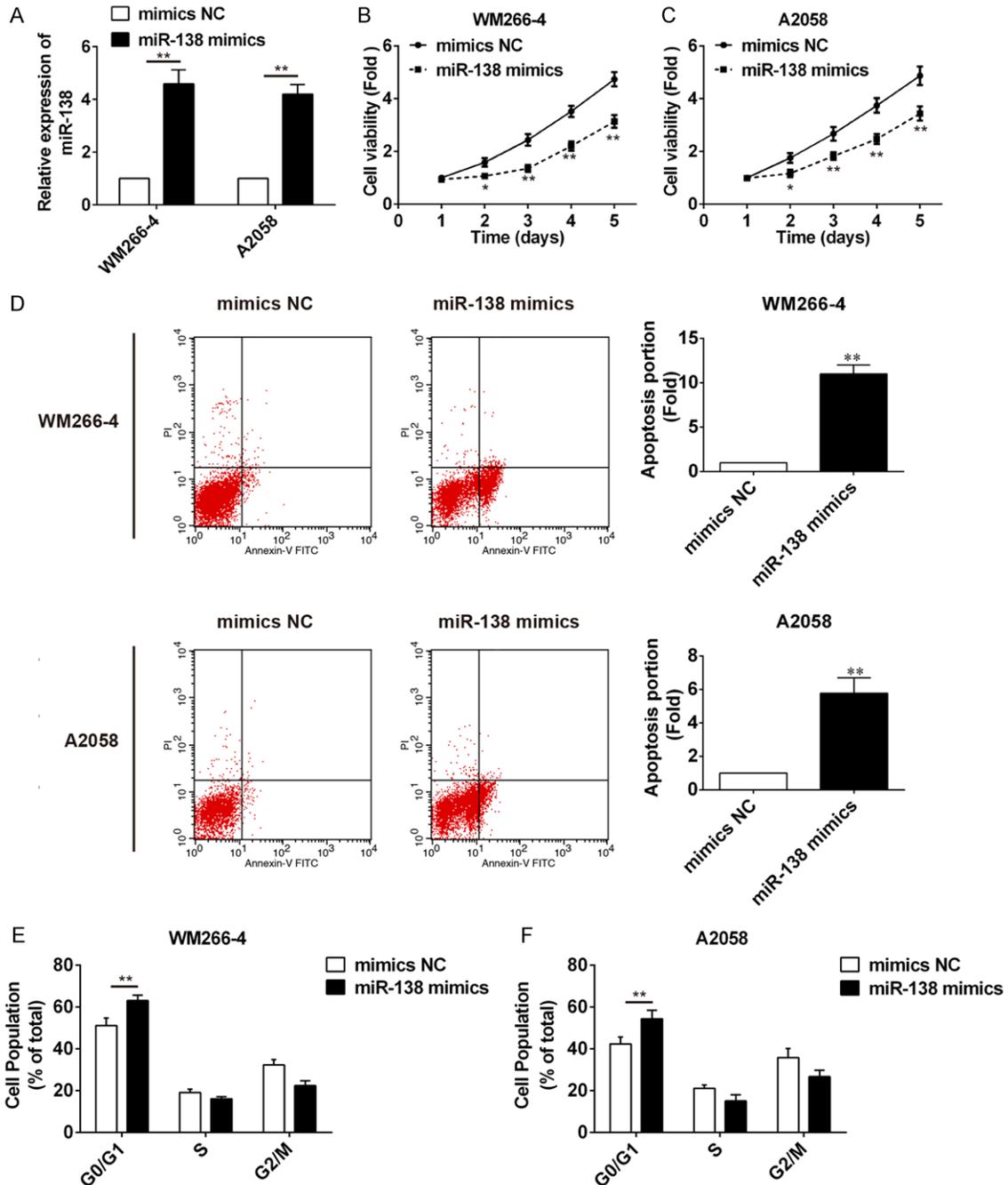


Figure 2. Functional effects of miR-138 upregulation on melanoma cell growth. A. The human MM cell lines WM266-4 or A2058 (showing low endogenous miR-138 expression) were transfected with miR-138 mimics at a final concentration of 40 μ M, miR-138 expression level were quantified by qRT-PCR analysis, mimics NC was used as negative control (NC). B and C. The WM266-4 or A2058 cells were transfected with miR-138 mimics at a final concentration of 40 μ M and the Cell Counting Kit-8 (CCK-8) assay was performed to detect the cell proliferation at 1-5 d post-transfection, mimics NC was used as NC. D. Apoptosis of WM266-4 or A2058 cells were detected by flow cytometric analysis after transfection with miR-138 mimics or mimics NC. E and F. After the transfection with miR-138 mimics or mimics NC, the WM266-4 or A2058 cells cycle was measured by flow cytometric analysis. All values are mean \pm SD. **vs control, P<0.01.

showed a significant increase in cell apoptosis portion compared with the control (**Figure 2D**).

Meanwhile, we also analyzed the cell cycle distribution in WM266-4 or A2058 cells after

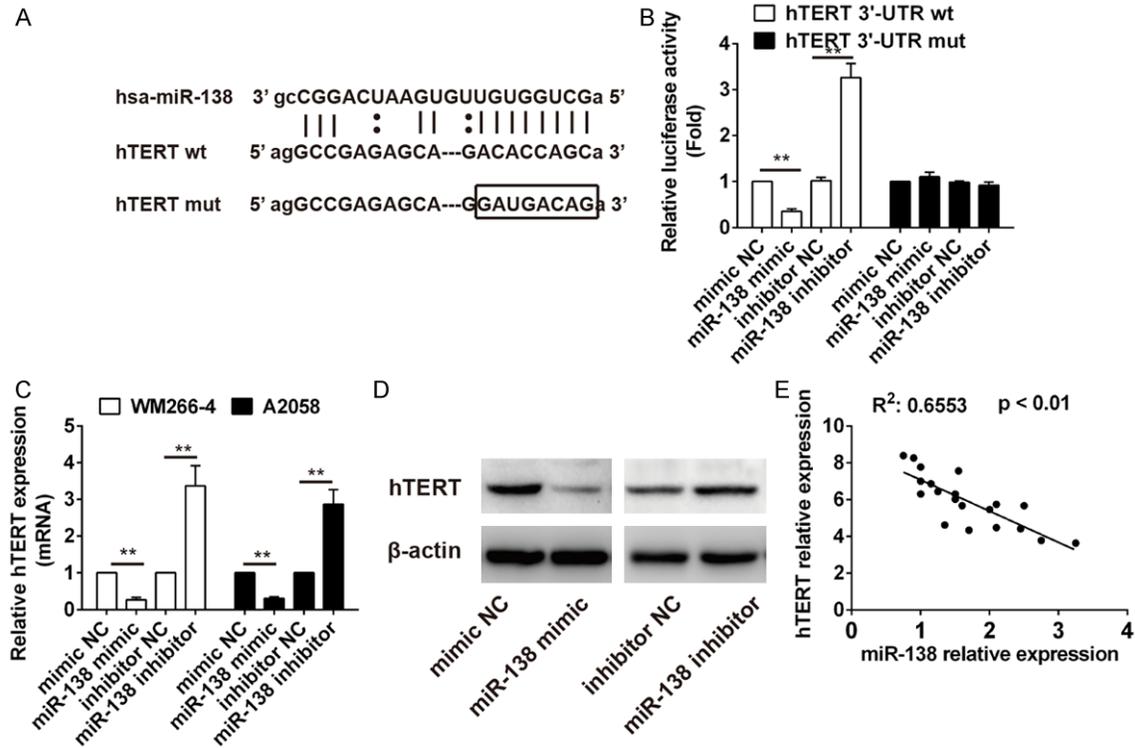


Figure 3. hTERT is the direct target of miR-138. A. The hTERT3'-UTR region containing the wild type or mutant binding site for miR-138. B. The relative luciferase activity of hTERT wild type or mutant 3'UTR in WM266-4 cells following transfection with the miR-138 mimic/inhibitor or corresponding mimic NC. C. hTERT mRNA expression in WM266-4 cells and A2058 cells following transfection with miR-138 mimic/inhibitor or corresponding mimic NC. D. Western blot was conducted to detect the protein level of hTERT in WM266-4 cells transfected with miR-138 mimic/inhibitor or corresponding mimic NC, β-actin was used as an internal control. E. miR-138 expression and hTERT level in MM tissues showed an inverse correlated trend from 20 MM patients ($R^2=0.6553$). All values are mean ± SD. **vs control, $P<0.01$.

transfection with miR-138 mimics or mimics NC. Flow cytometric analysis revealed that the G0/G1 ratio was increased and the G2/M ratio was decreased by the induction of miR-138 into the WM266-4 or A2058 cells (**Figure 2E** and **2F**), which suggested that miR-138 overexpression resulted in cell cycle arrested in the G0/G1 phase.

miR-138 reduces hTERT expression by directly targeting its 3'-UTR

Next, we performed bioinformatic analysis to predicate the putative targets of miR-138, and found that hTERT might be a target gene of miR-138 and the target site located in the 3'-UTR (**Figure 3A**). To verify this bioinformatic predication, the wild type or mutant type of hTERT3'-UTR was constructed and inserted into the psiCHECKTM-2 vector. Luciferase reporter assay was then performed in WM266-4 cell, results showed that the luciferase activity was signifi-

cantly downregulated only in WM266-4 cells co-transfected with miR-138 mimic and wild type of hTERT3'UTR, but miR-138 inhibitor significantly enhanced the luciferase activity compared with the inhibitor NC ($P<0.01$) (**Figure 3B**). In addition, miR-138 did not inhibit the luciferase activity of the reporter vector containing 3'-UTR of hTERT with mutations in the miR-138-binding site (**Figure 3B**). These data indicate that miR-138 was able to interact directly with the 3'-UTR of hTERT mRNA. To further confirm this result, we examined the effects of miR-138 on hTERT expression in WM266-4 or A2058 cells. The protein level of hTERT was then examined in WM266-4 or A2058 cells transfected with miR-138 mimic or inhibitor. The results demonstrated that the protein level of hTERT was significantly reduced in miR-138-overexpressing WM266-4 or A2058 cells, but markedly increased after knockdown of miR-138, compared to the control group

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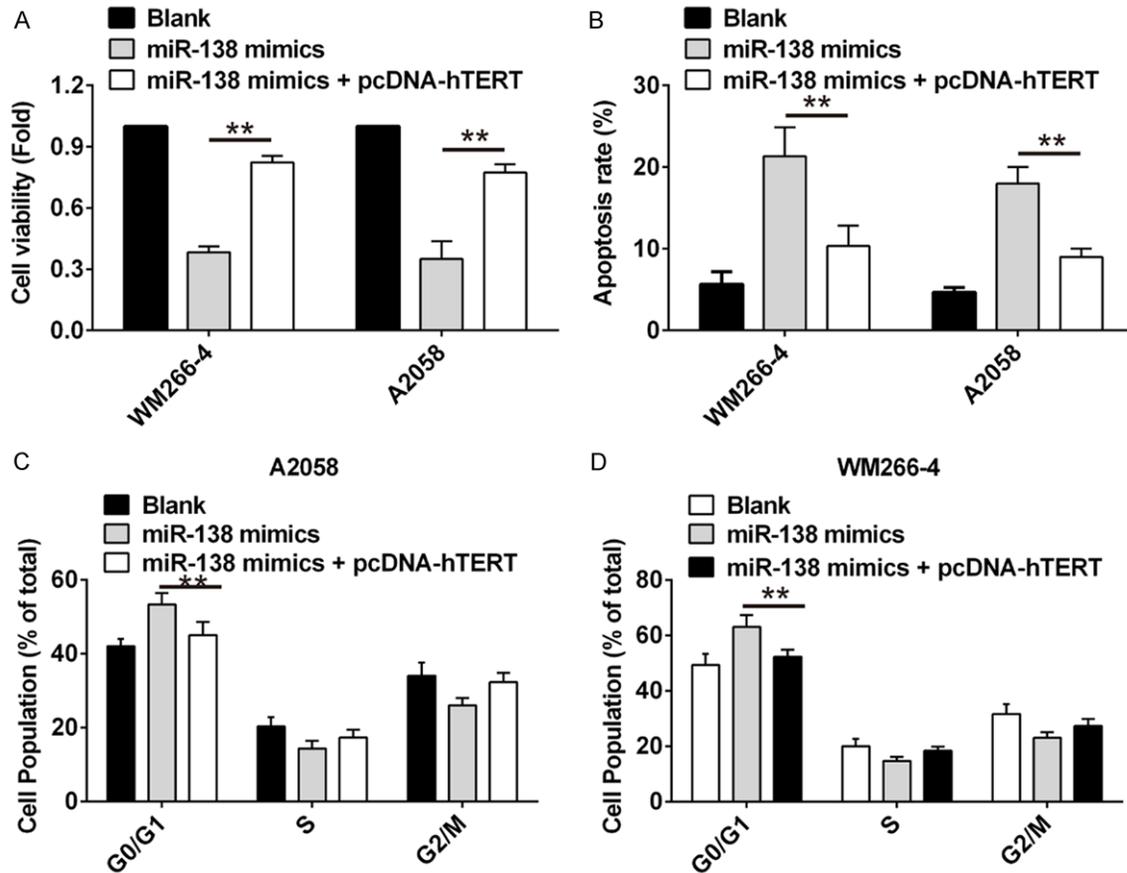


Figure 4. Overexpression of hTERT rescued the suppressive effects of miR-138 upregulation on MM cell proliferation. A. Cell counting Kit-8 (CCK-8) assay was conducted to examine cell proliferation in WM266-4 cells transfected with miR-138 mimic (miR-138), or co-transfected with miR-138 mimic and hTERT overexpression plasmid (miR-138mimics + pcDNA-hTERT). B. Apoptosis of WM266-4 cells was detected by flow cytometric analysis after transfection with miR-138 mimics or miR-138mimics + pcDNA-hTERT. C and D. After the transfection with miR-138 mimics or miR-138 mimics + pcDNA-hTERT, the A2058 or WM266-4 cells cycle was measured by flow cytometric analysis. All values are mean ± SD. **vs control, P<0.01.

(Figure 3C and 3D). Subsequently, we explored the association between miR-138 and hTERT in melanoma patients, their expressions in melanoma patient tissues were quantified by qRT-PCR. Our results revealed that hTERT level in the cancer tissues was inversely correlated with miR-138 expression in 20 melanoma patients ($R^2=0.6553$, $P<0.01$) (Figure 3E).

Overexpression of hTERT rescued the suppressive effects of miR-138 upregulation on MM cell proliferation

Based on the above data, we evaluated whether ectopic expression of hTERT could rescue the suppressive effect of miR-138, WM266-4 and A2058 cells were transfected with miR-138 mimics (miR-138 mimics group) or were

co-transfected with miR-138 mimics and pcDNA-hTERT plasmid (miR-138 mimics + pcDNA-hTERT group). WM266-4 and A2058 cells did not receive transfection (blank group). Subsequently, cell proliferation in each group was investigated by CCK-8 assay, our result showed that upregulation of miR-138 (miR-138 mimics group) was significantly suppressed cell proliferation, but overexpression of hTERT led to a marked increase in cell proliferation after co-transfection with miR-138 mimics and pcDNA-hTERT plasmid (Figure 4A). These data indicated that upregulation of hTERT significantly rescued miR-138-induced cell proliferation inhibition. To further validate these results, cell apoptosis and cycle were measured by flow cytometric analysis. As expected, apoptosis rate was markedly decreased in miR-138-

mimics + pcDNA-hTERT group compared with miR-138 mimics group (**Figure 4B**) ($P < 0.01$). Moreover, our results also revealed that overexpression of hTERT inhibited miR-138-induced cell-cycle arrest in WM266-4 and A2058 cells (**Figure 4C** and **4D**). Taken together, these results indicate that miR-138 regulated MM cell growth, at least in part, by downregulating hTERT.

Discussion

MM is a common skin tumor that derives from excessive hyperplasia of abnormal melanocytes. Clinical manifestations include bleeding, itching, tenderness, and ulcer [26]. Because of its high malignancy, incidence, and mortality, MM has drawn more and more attention. Previous studies have demonstrated that MM patients have a very poor prognosis, with median survival of 6-10 months and a 5-year survival rate of $< 5\%$ [27]. It is urgent to find new strategies and novel indicators to improve the diagnosis, treatment, and prognosis of MM patients.

miRNAs are major players in post-transcriptional regulation of tissue- and disease-specific gene expression. The importance of miRNAs as master gene regulators, as potential diagnostic markers for many different diseases and as targets for future therapeutic applications form the basis for investigating into the relevance of miRNAs in MM and other cancers [28]. In the present study, we found that miR-138 was obviously downregulated in human MM tissues and cell lines, and patients with low miR-138 expression presented significantly shorter survival time compared to those with high miR-138 expression. Moreover, upregulation of miR-138 was significantly suppressed cell growth in MM cells by inducing cell cycle arrest and apoptosis. Meanwhile, we identified a novel regulatory mechanism of hTERT gene expression mediated by miR-138 in MM. These findings provided new insights into melanoma research and therapeutic strategies for MM.

miR-138 has been proven to be a tumor suppressor gene in various types of tumors. Long et al. reported that downregulation of miR-138 in human colorectal cancer tissues was associated with lymph node metastasis, distant metastasis, and predicted poor prognosis. Ectopic expression of miR-138 can inhibit

colorectal cancer migration and invasion *in vitro* and *in vivo* [29]. In ovarian cancer, miR-138 can suppress cell invasion and metastasis by targeting SRY-box 4 (SOX4) and hypoxia inducible factor 1, alpha subunit (HIF-1 α) [15]. A recent study revealed that miR-138 suppresses proliferation, invasion and glycolysis in MM cells by targeting HIF-1 α [19]. Consistent with these results, we found that miR-138 was obviously downregulated in human MM tissues and cell lines. Moreover, our data showed that patients with low miR-138 expression presented significantly shorter overall survival compared to those with high miR-138 expression. These findings suggested that miR-138 may play important roles in the progression of MM. Subsequently, we performed the *in vitro* gain-of-function and loss-of-function experiments to elucidate the role of miR-138 in MM cells. We found that overexpression of miR-138 significantly inhibits cell proliferation abilities and induces cells apoptosis and cycle arrest in the G0/G1 phase *in vitro*. These data further confirmed that miR-138 acts as a tumor suppressor in human MM. Our report is consistent with the previous studies showing the tumor suppressor role of miR-138 in other types of tumors [15, 18]. The further steps of this study need to investigate the association between miR-138 expression and clinicopathological parameters of MM patients.

hTERT plays a critical role in tumorigenesis and immortalized cells through its telomere-dependent and-independent activity [30, 31]. The reactivation of hTERT expression is a critical step in carcinogenesis, which is required to maintain the rapid proliferation of cancer cells, and hTERT expression is strictly regulated in varying environments in human cells [30]. Mitomo et al. have shown that the overexpression of miR-138 induced a reduction in hTERT protein expression in human anaplastic thyroid carcinoma [22]. A recent study revealed that miR-346-mediated upregulation and miR-138-mediated downregulation competitively coordinate the regulation of hTERT expression by binding to a common site in the hTERT 3'-UTR, which promotes the growth of human cervical cancer cells [23]. In this study, we verified that miR-138 significantly inhibited hTERT expression level by targeting the 3'-UTR of hTERT, and hTERT level in the cancer tissues was inversely correlated with miR-138 expression in 20 melanoma patients. To further con-

firm the association between miR-138 and hTERT in MM cells, WM266-4 and A2058 cells were co-transfected with miR-138 mimics and pcDNA-hTERT plasmid, and cell proliferation or cell apoptosis and cycle were investigated by CCK-8 assay or flow cytometric analysis. Our results showed that upregulation of hTERT significantly rescued miR-138-induced cell proliferation inhibition, apoptosis and cell-cycle arrest.

These data suggested that miR-138 inhibited cell growth and induces cell apoptosis and cycle arrest in MM cells by directly targeting the 3'-UTR of hTERT.

In summary, our study revealed that miR-138 was downregulated in human MM tissues and cell lines, and low expression of miR-138 was an independent prognostic marker of melanoma patients. Furthermore, overexpression miR-138 inhibits cell proliferation and promotes cell apoptosis and cycle arrest in MM cells. Most importantly, we validated hTERT as a functional target of miR-129 *in vitro*. Our findings suggest that miR-138 may function as a novel tumor suppressor in MM and could be a potential therapeutic target for this disease.

Disclosure of conflict of interest

None.

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References

- [1] Miller AJ and Mihm MC. Melanoma. *N Engl J Med* 2006; 355: 51-65.
- [2] Pacheco I, Buzea C and Tron V. Towards new therapeutic approaches for malignant melanoma. *Expert Rev Mol Med* 2011; 13: e33.
- [3] Bai J, Zhang Z, Li X and Liu H. MicroRNA-365 inhibits growth, invasion and metastasis of malignant melanoma by targeting NRP1 expression. *Int J Clin Exp Pathol* 2015; 8: 4913-4922.
- [4] Poole JC, Andrews LG and Tollefsbol TO. Activity, function, and gene regulation of the catalytic subunit of telomerase (hTERT). *Gene* 2001; 269: 1-12.
- [5] Nandakumar J and Cech TR. Finding the end: recruitment of telomerase to telomeres. *Nat Rev Mol Cell Biol* 2013; 14: 69-82.
- [6] Cifuentes-Rojas C and Shippen DE. Telomerase Regulation. *Mutat Res* 2012; 730: 20-27.
- [7] Zhang Y, Chen X, Xu X, Wang X, Wang X, Yuan G, Sun D, Ka W, He D, Wen Z and Yao W. Knockdown of hTERT Alters Biophysical Properties of K562 Cells Resulting in Decreased Migration Rate *In Vitro*. *Cell Biochem Biophys* 2011; 61: 595-603.
- [8] Ameres SL and Zamore PD. Diversifying microRNA sequence and function. *Nat Rev Mol Cell Biol* 2013; 14: 475-488.
- [9] Bartel DP. MicroRNA Target Recognition and Regulatory Functions. *Cell* 2009; 136: 215-233.
- [10] Friedman RC, Farh KK, Burge CB and Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* 2009; 19: 92-105.
- [11] Kosik KS. MicroRNAs and Cellular Phenotypy. *Cell* 2010; 143: 21-26.
- [12] Di Leva G, Garofalo M and Croce CM. microRNAs in cancer. *Ann Rev Pathol* 2014; 9: 287-314.
- [13] Leung AK and Sharp PA. MicroRNA Functions in Stress Responses. *Mol Cell* 2010; 40: 205-215.
- [14] Farazi TA, Hoell JI, Morozov P and Tuschl T. microRNAs in Human Cancer. *Adv Exp Med Biol* 2013; 774: 1-20.
- [15] Yeh YM, Chuang CM, Chao KC and Wang LH. MicroRNA-138 suppresses ovarian cancer cell invasion and metastasis by targeting SOX4 and HIF-1 α . *Int J Cancer* 2013; 133: 867-878.
- [16] Qiu S, Huang D, Yin D, Li F, Li X, Kung HF and Peng Y. Suppression of tumorigenicity by MicroRNA-138 through inhibition of EZH2-CDK4/6-pRb-E2F1 signal loop in glioblastoma multiforme. *Biochimica Biophysica Acta* 2013; 1832: 1697-1707.
- [17] Liu X, Wang C, Chen Z, Jin Y, Wang Y, Kolokythas A, Dai Y and Zhou X. MicroRNA-138 suppresses epithelial-mesenchymal transition in squamous cell carcinoma cell lines. *Biochem J* 2011; 440: 23-31.
- [18] Liu X, Jiang L, Wang A, Yu J, Shi F and Zhou X. MicroRNA-138 suppresses invasion and promotes apoptosis in head and neck squamous cell carcinoma cell lines. *Cancer Lett* 2009; 286: 217-222.
- [19] Chen Y, Cao KE, Wang S, Chen J, He B, He GU, Chen Y, Peng B, Zhou J. MicroRNA-138 suppresses proliferation, invasion and glycolysis in malignant melanoma cells by targeting HIF-1 α . *Exp Ther Med* 2016; 11: 2513-2518.
- [20] Liu X, Lv XB, Wang XP, Sang Y, Xu S, Hu K, Wu M, Liang Y, Liu P, Tang J, Lu WH, Feng QS, Chen

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- LZ, Qian CN, Bei JX, Kang T and Zeng YX. MiR-138 suppressed nasopharyngeal carcinoma growth and tumorigenesis by targeting the CCND1 oncogene. *Cell Cycle* 2012; 11: 2495-2506.
- [21] Chakrabarti M, Banik NL and Ray SK. miR-138 overexpression is more powerful than hTERT knockdown to potentiate apigenin for apoptosis in neuroblastoma in vitro and in vivo. *Exp Cell Res* 2013; 319: 1575-1585.
- [22] Mitomo S, Maesawa C, Ogasawara S, Iwaya T, Shibazaki M, Yashima-Abo A, Kotani K, Oikawa H, Sakurai E, Izutsu N, Kato K, Komatsu H, Ikeda K, Wakabayashi G and Masuda T. Down-regulation of miR-138 is associated with overexpression of human telomerase reverse transcriptase protein in human anaplastic thyroid carcinoma cell lines. *Cancer Sci* 2008; 99: 280-286.
- [23] Song G, Wang R, Guo J, Liu X, Wang F, Qi Y, Wan H, Liu M, Li X and Tang H. miR-346 and miR-138 competitively regulate hTERT in GRSF1- and AGO2-dependent manners, respectively. *Sci Rep* 2015; 5: 15793.
- [24] Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H and Parks WP. In Vitro Cultivation of Human Tumors: Establishment of Cell Lines Derived From a Series of Solid Tumors. *J Natl Cancer Inst* 1973; 51: 1417-1423.
- [25] He Y, Meng XM, Huang C, Wu BM, Zhang L, Lv XW and Li J. Long noncoding RNAs: Novel insights into hepatocellular carcinoma. *Cancer Lett* 2014; 344: 20-27.
- [26] Luk NM, Ho LC, Choi CL, Wong KH, Yu KH and Yeung WK. Clinicopathological features and prognostic factors of cutaneous melanoma among Hong Kong Chinese. *Clin Exp Dermatol* 2004; 29: 600-604.
- [27] Tsao H, Atkins MB and Sober AJ. Management of Cutaneous Melanoma. *N Engl J Med* 2004; 351: 998-1012.
- [28] Iorio MV and Croce CM. MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med* 2012; 4: 143-159.
- [29] Long L, Huang G, Zhu H, Guo Y, Liu Y and Huo J. Down-regulation of miR-138 promotes colorectal cancer metastasis via directly targeting TWIST2. *J Transl Med* 2013; 11: 275-275.
- [30] Hanahan D and Weinberg Robert A. Hallmarks of Cancer: The Next Generation. *Cell* 2011; 144: 646-674.
- [31] Zhou J, Ding D, Wang M and Cong YS. Telomerase reverse transcriptase in the regulation of gene expression. *BMB Rep* 2014; 47: 8-14.