Original Research Article

Effect of up-regulation of micro-ribonucleic acid-22 and NLRP3 inhibition on viability of malignant melanoma cells

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Abstract

Purpose: *To determine the effect of up-regulation of micro-ribonucleic acid-22 (miR-22) targeting nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3 (NLRP3) on the proliferation and invasiveness of malignant melanoma cells, as well as the underlying mechanisms.*

Methods: *Mouse-derived melanoma B16 cells were subjected to thawing, sub-culture and transfection. The cells were assigned to groups (A1 - A5) in line with different transfections viz: A1 (miR-22 mimic overexpression-transfected), A2 (miR-22 inhibitor-transfected), A3 (miR-22 mimic+siNLRP3-transfected), A4 (miR-22 mimic NC sequence-transfected), and A5 (un-transfected).*

Results: *The miR-22 expression was significantly up-regulated in A1, relative to A2, A3, A4, and A5 groups, but significantly lower, relative to A3, A4, and A5. The NLRP3 mRNA and protein levels were significantly lower in A1 than in other groups, but significantly up-regulated in A2, relative to A3, A4, and A5 (p < 0.05). Cell proliferation rate and colony formation rate were significantly lower in A1 group but were significantly up-regulated in A2, relative to A3, A4, and A5.*

Conclusion: *Targeting NLRP3 inhibition by up-regulating miR-22 expression level significantly reduces the proliferation, invasiveness, and matrix metalloproteinase levels of melanoma B16 cells, thereby reducing the occurrence and development of malignant melanoma. The results provide some useful reference data for the treatment of malignant melanoma at the gene level.*

Keywords: *Micro-ribonucleic acid-22, NLRP3 inflammasome, Malignant melanoma, Cell proliferation activity, Cell invasion ability, Cell transfection*

INTRODUCTION

Malignant melanoma refers to cancerous transformation of the corresponding pigmented nevus. Due to the morphological changes in nevus cells, the tumor formed is called melanoma [1,2]. The etiology of melanoma is very complex. The most common risk parameters are environmental factors (sunlight exposure and chemical stimulation), genetic factors and immunological factors [3]. Melanoma is a highly malignant tumor that occurs mostly in the skin, but also in the mucous membranes and internal organs, and it accounts for about 3% of all tumors [4,5]. There has been a rise in fatality associated with this tumor in the past 12 years.

Studies show that there are about 150,000 new melanoma cases and about 50,000 melanoma-related deaths worldwide every year [6-8]. Melanoma is very malignant, and it is liable to blood and lymphatic metastasis, and it has a high mortality. Timely surgical resection when there is no metastasis may prolong the survival time of patients by 3 - 5 years. Once malignant melanoma undergoes distant metastasis to multiple organs, it may likely lead to organ failure and death, and the prognosis is poor [9,10]. Therefore, early diagnosis and timely and reasonable treatment are essential.

Surgery remains the primary and definitive treatment for early-stage melanoma, but it is rarely curative for advanced-stage melanoma [11]. In recent years, the applications of immuno- and targeted treatments have prolonged patients' lives and revolutionized the management of this neoplasm [12]. Unfortunately, many subjects develop resistance to targeted medications after several months, and studies suggest that micro-ribonucleic acid plays a key role in the development of drug insensitivity [13,14]. The miRNAs are a class of evolutionarily conserved, non-coding small ribonucleic acids generally between 21 - 23 nucleotides in length, and they regulate gene expression at the translational level [15]. There are as many as 1000 different miRNAs in vertebrate genomes which regulate at least 30% of gene expressions, and they are highly conserved, tissue-specific and time-sequential. The nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain-containing 3 (NLRP3) inflammasome is a multi-protein structure that exists in cells *in vivo*, and it is a crucial part of innate immunity which regulates the activation of caspase-1 and promotes the release of pro-inflammatory factors, i.e., interleukins IL-1β/IL-18 [16-18]. Accumulated evidence suggests that the inflammasome plays a central role in regulating tumorigenesis, growth, invasion, and metastasis. It has been shown that IL-18, the effector product of the NLRP3 inflammasome, maintains the epithelial homeostasis of colons and plays a protective role in colitis-associated colon cancer [19]. Some studies have also shown that NLRP3 enhanced carcinogenesis by affecting host immune response, cancer cell multiplication and microenvironment of cancer [20,21]. At present, the regulatory effect of miR-22/NLRP3 axis on the biological function of tumor cells in colon, ovarian, gastric, and other malignant tumors, have been demonstrated. However, there is limited research on the relationship between miR-22/NLRP3 and malignant melanoma.

The mechanism that reveals how miR-22 targeting NLRP3 inhibits the expression of malignant melanoma is still not clear. Therefore, in this study, mouse-derived melanoma cells B16 cells were subjected to thawing, sub-culture, and transfection. Based on what was transfected, the cells were divided into 5 groups: A1 (overexpressed miR-22 mimic) A2 group (miR-22 inhibitor), A3 group (transfected with miR-22 mimic+siNLRP3), A4 (miR-22 mimic NC sequence transfection), and A5 group (without transfection). The five groups of cells were compared with respect to miR-22 messenger ribonucleic acid (mRNA) expression, NLRP3 mRNA and protein expressions, proliferation potential, clone formation rate, invasiveness, and levels of inflammatory factors, amongst other parameters. Arising therefrom, the effect of NLRP3 inhibition-targeted miR-22 up-regulation on proliferation and invasiveness of tumor cells was evaluated, and the related mechanism was also assessed.

EXPERIMENTAL

Melanoma cells

Mouse-derived melanoma cells B16 cells purchased from Shanghai Guandao Bio-engineering Co. Ltd were used as experimental cells. The B16 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (modified with ATCC) tinged with 10 % FBS. Thereafter, the cells were kept in liquid nitrogen.

Cell thawing

Centrifuge tubes, disposable pipettes, RPMI 1640 medium and phosphate buffer saline (PBS) were sterilized for 20 min on an ultra-clean bench. Then, the B16 cells were removed from liquid nitrogen and thawed for 60 sec in a water bath preheated to 40 ℃. The cells were pipetted into a 15-mL centrifuge tube, and 6 mL of PBS solution was added, followed by centrifugation at 1300 rpm for 6 min. The supernatant was discarded, and the sediment was suspended in 6 mL of RPMI 1640 medium. After thorough mixing, the cells were cultured in a cell culture dish in the presence of 5 % carbon dioxide (CO_2) at 37 ℃. The medium was changed after 24 h, and sub-culture was performed until the cell growth density reached about 98 %.

Cell subculture

Following a 20-min UV sterilization of a cleaned bench, the culture medium, trypsin, and PBS were pre-heated. The PBS in cell culture dish was replaced with 1.5 mL trypsin, and following

mixing, the cells were left at 25 ℃ for about 3 min, after which cell morphology was examined under a high-definition microscope. When the cells became rounded, 2 mL of RPMI 1640 medium was added, and the cells were centrifuged at 1300 rpm for 6 min. The supernatant was replaced with 6 mL PBS solution, and the cells were centrifuged again at 1300 rpm for 6 min, and the supernatant was again replaced with RPMI 1640 medium. The re-suspended cells were put into 5 new culture dishes and cultured at 37 ℃ in a 5 % CO_2 atmosphere.

Cell transfection

Before cell transfection, the B16 cells were seeded at concentrations of 10^5 to 10^6 cells/well in six-well plates. When 80 % confluence was attained, miR-22 mimic was transfected into the cells. The transfection was carried out in line with the steps indicated in the transfection kit (Guangzhou Ribo Bio Company Ltd.). Five groups of cells were used (A1 - A5). In A1, transfection with miR-22 mimic overexpression was done. Transfection in A2 group of cells was done with miR-22 inhibitor. In A3 group, transfection was with miR-22 mimic+siNLRP3. In A4 group, transfection was with miR-22 mimic NC sequence, while cells in A5 group were not transfected.

Determination of cell proliferation

The CCK-8 method was employed for determination of cell multiplication. Cells in log stage of growth were 0.25 %-trypsinized for 4 min, after which the cell morphology was examined under a high-definition microscope. When the cells became rounded, addition of RPMI 1640 medium was followed by 6-min cell centrifugation at 1300 rpm, re-suspension in RPMI 1640 medium, and counting under a microscope using a counting plate. For this purpose, a drop of cell culture medium was placed on the counting plate, after which the cells were counted under a high-definition microscope, and the cell concentration was calculated. Then, cell seeding (density = 1×10^6 cells/mL) in a 96-well plate was followed by culturing in 80 µL of RPMI 1640 medium for 24 h. After addition of 15 µL CCK-8 reagent, the cells were incubated at 37 ℃ for 2 h, and the absorbance of each well was read at 520 nm.

Cell clone formation experiment

High-definition microscopy was used for morphological examination of B16 cells after cell trypsinization as before. After the cells became rounded, RPMI 1640 medium was introduced, after which the cells were centrifuged for 6 min at 1300 rpm. Thereafter, re-suspension of the cells in RPMI 1640 medium was done. Then, the cells were counted under a microscope using a counting plate to get the number of cell clones (NC). Next, 500 cells were placed in a culture dish and cultured for 1 day. The cultured cells were fixed with 4 % paraformaldehyde for 4 h, stained with crystal violet for 20 min, dried and counted under a high-definition microscope. The cell clone formation rate (CR) was calculated as indicated in Eq 1.

$$CR = (NC/NI)100 \ldots\ldots\ldots\ldots (1)$$

where NI is the number of cells inoculated

Cell scratch test

When logarithmic growth stage cells grew to 98 % confluence, three parallel lines were drawn on the bottom of the plate with a pipette tip. This was followed by culturing in serum-free RPMI 1640 and photographing under a fluorescence microscope on days 1, 3 and 5. The degree of cell fusion was calculated using Image J software.

Cell invasion assay

Logarithmic growth phase cells were inoculated in Tanswell upper chamber and cultured in serum-free RPMI 1640. Then, addition of RPMI 1640 medium having 10 % FBS was done to the 24-well plate underneath. Following 24-h culturing, 4 % paraformaldehyde fixation was done, and the cells were crystal violet-stained for 15 min. After drying, the cells were counted under a high-definition microscope.

Evaluation of parameters/indices

Real-time quantitative polymerase chain reaction (qRT-PCR) was used to determine the expression levels of miR-22, NLRP3, caspase-1 mRNA, and matrix metalloproteinases (MMP2 and MMP9). Western blot assay was used to measure the protein expression levels of NLRP3, caspase-3, and matrix metalloproteinases (MMP2 and MMP9), while levels of inflammatory factors (IL-6, IL-8, IL-10, and IL-1β) were measured with ELISA.

Statistical analysis

The SPSS 19.0 software was applied for data processing. Measurement data are presented as mean ± standard deviation (SD), and enumeration data are presented as %. Pairwise

comparisons were performed using one-way ANOVA. Differences were assumed to be statistically significant at $p < 0.05$.

RESULTS

Expression levels of miR-22 in cells in the 5 groups

Figure 1 a show miR-22 levels in the five groups of cells. There was a significant up-regulation of miR-22 in A1 than in A2, A3, A4, and A5, but miR-22 was significantly lower in A2 than in A3 - A5 ($p < 0.05$). However, miR-22 level was comparable in groups A3, A4, and A5 ($p > 0.05$). Figure 1 b is the miR-22 PCR band map showing that A1 had the highest brightness, while A2 band was the lowest. Band brightness was similar in A3, A4, and A5 groups.
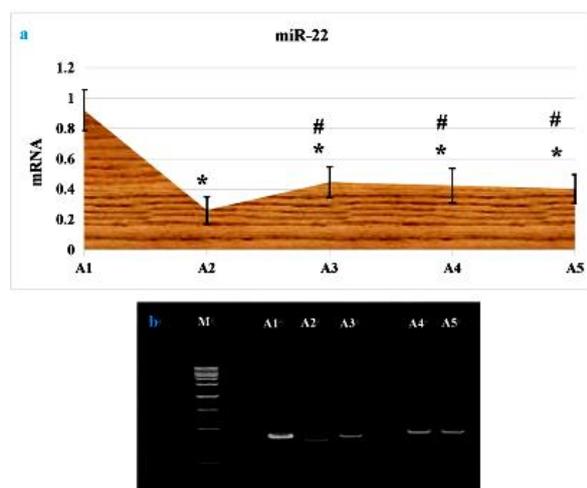

Figure 1: Expression levels of miR-22 in the 5 groups of cells. (a) miR-22 expression levels; (b) miR-22 PCR band map. *$P < 0.05$, vs. A1; #$p < 0.05$, vs. A2

mRNA and protein levels of NLRP3 in the five groups

Figures 2 a and b show the protein and mRNA levels of NLRP3, respectively, in all 5 groups, with A1 cells having significantly lower levels than A2, A3, A4, and A5 cells. However, NLRP3 mRNA and protein levels were highest in A2 ($p < 0.05$). Pairwise differences in mRNA and protein expressions of NLRP3 amongst A3, A4, and A5 groups were not significantly different. Band maps of PCR and western blot of NLRP3 are presented in Figures 2 c and d, respectively. The brightness of the bands of cells was highest in A1 group, while A2 group had the darkest band. The bands of cells in A3, A4, and A5 groups showed similar brightness.
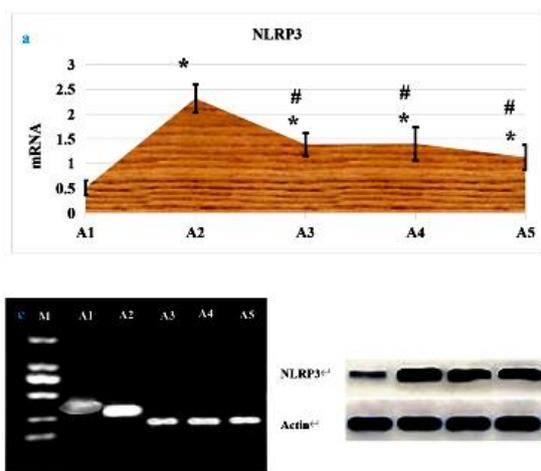

Figure 2: NLRP3 protein and mRNA levels in cells in the 5 groups. The NLRP3 mRNA expression, NLRP3 protein expression, PCR band diagram, and western blot band diagram are depicted in a, b, c, and d, respectively. *$P < 0.05$, vs. A1 group; #$P < 0.05$, vs. A2
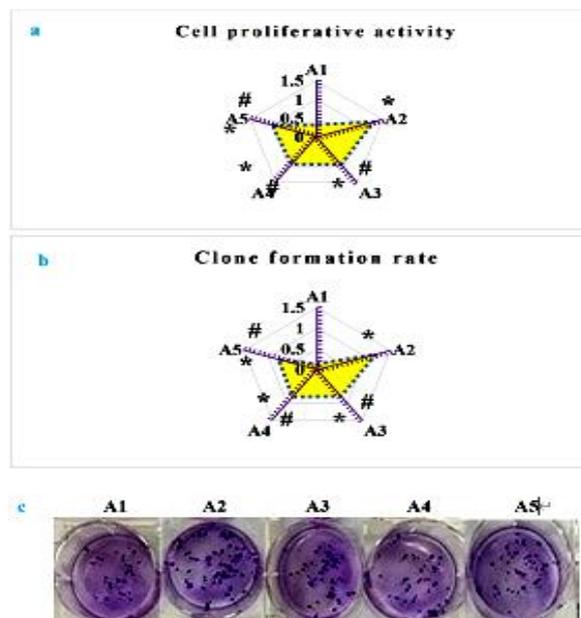

Figure 3: Comparison of cell proliferation and clone formation rate among the 5 groups. a, b, and c are cell proliferation, clone formation rate, and cell clone formation rate, respectively. *$P < 0.05$, vs A1 group; #$p < 0.05$, vs A2 group

Cell proliferation potential and clone formation rate

Figures 3 a and b show the cell proliferation and clone formation rate, respectively, of the five groups of cells. The cell proliferation and clone formation rate of cells were lowest in A1, but they were significantly up-regulated in A2, relative to A3, A4, and A5. The differences in cell proliferation and clone formation rate among A3 - A5 were not significant. Figure 3 c shows the

results of cell clone formation experiment which indicate that the number of clones formed in A1 group was the lowest, while A2 had the highest number of clones.

Cell invasiveness

A comparison of the invasion distance of cells in the five groups is presented in Figure 4 a. The cell invasion distance was significantly shorter in A1 group than in A2 - A5 groups, while invasion distance was longest in A2 group ($p < 0.05$). Figure 4 b shows the results of cell invasion experiment in each group. The number of cells that invaded in A2 group was the largest, while A1 had the least number of cells that invaded.
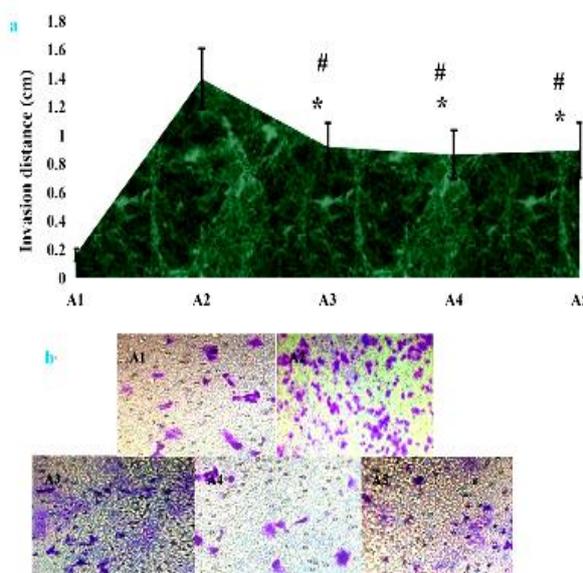

Figure 4: Cell invasiveness in each group. (a) Cell invasion distance; (b) the result of cell invasion experiments. *$P < 0.05$, vs. A1 group; #$p < 0.05$, vs. A2 group

mRNA expression levels of MMP2 and MMP9

Figures 5 a and b display comparison of mRNA expression levels of MMP2 and MMP9 amongst the five groups. The expression levels of MMP2 and MMP9 were significantly lower in A1 than in A2, A3, A4, and A5 groups, while MMP2 and MMP9 levels were significantly higher in A2 than in A3, A4, and A5. However, MMP2 and MMP9 levels were comparable in A3, A4, and A5 groups. Figures 5 c and d show MMP2 and MMP9 band maps, respectively. The MMP2 and MMP9 bands of cells in the A1 group were the darkest, while those of A2 group cells were the brightest.
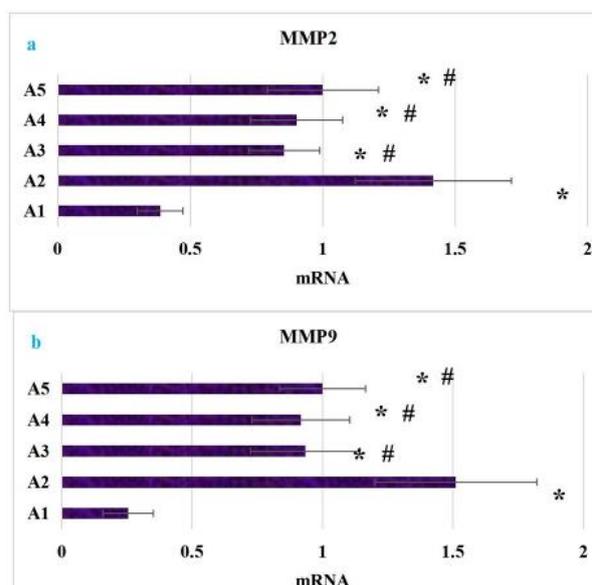

Figure 5: Serum bone gla protein levels of rats. (a, b, c, and d, are the expression level of MMP2 mRNA, expression level of MMP9 mRNA, band map of MMP2, and band map of MMP9, respectively). *$P < 0.05$, vs. A1; #$p < 0.05$, vs. A2

Levels of inflammatory factors

As presented in Figure 6, A1 cells had the lowest expressions of IL-6, IL-18, and IL-1β, while IL-10 expression was significantly highest in A1 ($p < 0.05$). The expression levels of IL-6, IL-18, and IL-1β were up-regulated in A2, relative to A3, A4, and A5 groups, but the level of IL-10 was significantly lower ($p < 0.05$). The levels of the four cell inflammatory factors were comparable in A3 - A5 groups.
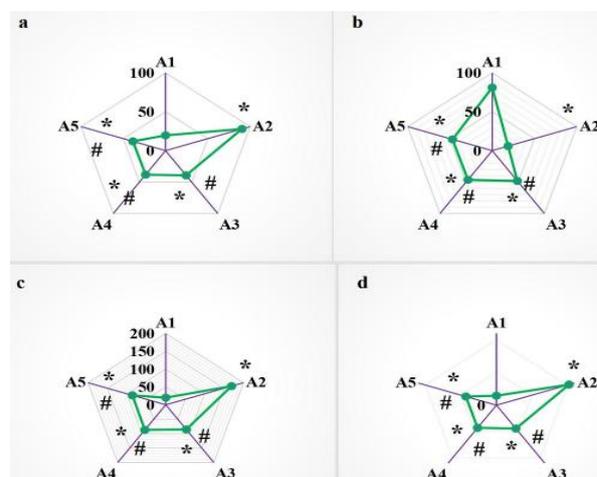

Figure 6: Expressions of inflammation-associated factors in the 5 groups. (a, b, c, and d indicate IL-6, IL-10, IL-18, and IL-1β, respectively). *$P < 0.05$, vs. A1; #$p < 0.05$, vs. A2

The expression levels of caspase-3 mRNA and protein

There were higher levels of caspase-3 mRNA and protein in A1 than in A2, A3, A4, and A5, but caspase-3 mRNA and protein were significantly lower in A2 than in A3 -A5 groups. However, A3, A4, and A5 groups had comparable caspase-3 mRNA and protein levels.
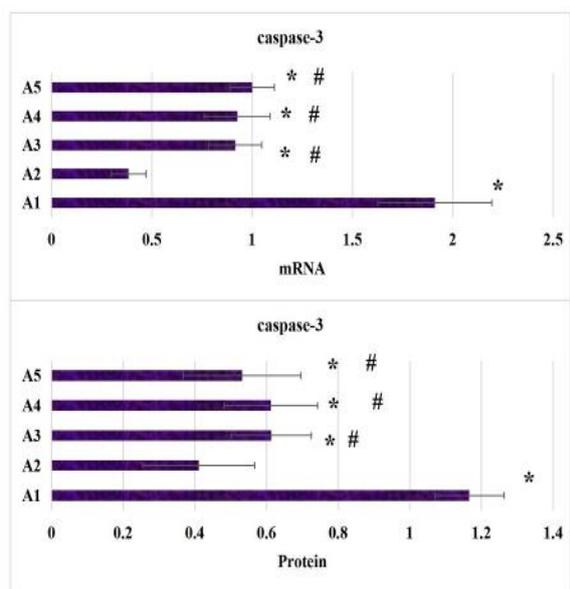

Figure 7: Expression levels of inflammatory factors in the 5 groups. Expression levels of caspase-3 mRNA (a) and caspase-3 protein (b). *$P < 0.05$, vs A1 group; #$p < 0.05$, vs A2 group

DISCUSSION

Malignant melanoma is a highly cancerous disease which has no specific treatment method. Surgery is only effective for treating early-stage melanoma. If the disease develops to the middle and advanced stages, the prognosis and five-year survival rate of patients become greatly reduced [22]. The miRNAs are a class of endogenous non-coding single-stranded RNAs involved in many cellular processes such as organ formation, information transmission, and cell growth and division. Studies have revealed that at least half of identified miRNAs are associated with the occurrence and development of tumors [23]. In this study, mouse-derived melanoma cells B16 cells were successfully transfected with either miR-22 mimic overexpression, miR-22 inhibitor, miR-22 mimic + siNLRP3, or miR-22 mimic NC sequence, with un-transfected cells serving as control. These data suggest that the transfection with miR-22 cells was successful, making the expression level of miR-22 in cells up-regulated or down-regulated. Analysis showed significantly lower NLRP3 mRNA and protein levels in A1 group, but the expressions were significantly up-regulated in A2 group, relative to A3, A4, and A5 groups. The target gene of miR-22 is NLRP3. Transfection with miR-22 mimic resulted in significant decreases in NLRP3 protein and mRNA, while transfection with miR-22 inhibitor resulted in significant increases in mRNA and protein expression levels of NLRP3. Thus, miR-22, by targeting and inhibiting NLRP3, inhibited the growth of malignant melanoma cells [24].

Cell proliferation potential and clone formation rate of cells were least in A1, but they were significantly higher in A2 than in A3, A4 and A5 groups. These results indicate that up-regulation of miR-22 expression level effectively inhibited proliferative activity of melanoma B16 cells. This is similar to the finding of Houshmandfar *et al* [25]. In cell clone formation experiment, the number of cell clones in A1 group was significantly low, while that in A2 group was higher, in line with the above data. The cell invasion assay showed the shortest cell invasion distance in A1 group, while cell invasion distance was longest in A2 group. This indicated that up-regulation of miR-22 expression level effectively inhibited the invasiveness of melanoma B16 cells. The mRNA expression levels of MMP2 and MMP9 in A1 group were significantly lower than those in other four groups, but they were significantly up-regulated in A2 group, relative to other three groups. Both MMP2 and MMP9 are matrix metalloproteinases, and they are involved in embryonic development, tissue regeneration and organ reconstruction [26]. These results indicate that up-regulating miR-22 expression level inhibited matrix metalloproteinases in melanoma B16 cells and regulated cellular events.

There were significantly lower levels of IL-6, IL-18, and IL-1β in A1 group than in other groups, while that of IL-10 was significantly higher. Thus, up-regulation of miR-22 expression targeting NLRP3 inhibition regulated levels of associated inflammatory factors and slowed down cell inflammation-induced damage. Caspase-3 is a protease and one of the most critical enzymes in the apoptosis pathway. It is closely associated with the occurrence of cancer, aging, and cardiovascular diseases. In this study, caspase-3 mRNA and protein levels in A1 group were significantly higher than those in other groups, but they were significantly down-regulated in A2 cells, relative to the other three groups. This indicates that up-regulation of miR-22 increased caspase-3 level, thereby regulating cell apoptosis in melanoma.

CONCLUSION

This study has demonstrated that targeting NLRP3 inhibition by up-regulating the expression level of miR-22 significantly reduces the multiplication, invasiveness, and matrix metalloproteinase activities of melanoma B16 cells, with adverse impact on the occurrence and development of malignant melanoma. However, although the expression levels of inflammatory factors and mRNA and protein levels of caspase-3 were assayed, their correlation with up-regulation of miR-22 expression levels targeting NLRP3 inhibition was not further investigated. The experiments in this study were performed only at the cellular level. No *in vivo* (animal experiments) or clinical experiments were carried out for more in-depth investigations. However, the results provide some useful reference data for the treatment of malignant melanoma at the gene level.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Wen Luo designed the study, supervised the data collection, and analyzed the data. Jieli Tian interpreted the data and prepared the manuscript for publication. Li Liu supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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