Down-regulation of SERPINA3 by miR-613 inhibits melanoma cell proliferation and invasion

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Abstract: MicroRNAs play critical roles in the development of many kinds of cancers. Serpin Peptidase Inhibitor, clade A member 3 (SERPINA3) was found to be abnormally overexpressed in melanoma tissue biopsies and the high expression of SERPINA3 was associated with poor patient survival. This study aimed to reveal the role of miR-613 in melanoma cell proliferation and its potential mechanisms. The expression of miR-613 was detected in both melanoma and normal tissues as well as the melanoma cell lines. In A-375 cells transfected with miR-613 mimics, western blot, colony formation assay and invasion assay were performed. SERPINA3, a directly target of miR-613, was verified by luciferase reporter assay and was upregulation to study its function in cell proliferation and invasion. Results showed that MiR-613 was decreased in melanoma tissues and cell lines, in A-375 with the lowest expression. Upregulation miR-613 suppressed A-375 cell proliferation and colony formation. MiR-613 decreased SERPINA3 expression via binding to the 3’ untranslated regions (3’-UTRs), while the mutant SERPINA3 could not be regulated. Recovering SERPINA3 resulted in promoting cell proliferation and invasion. This study revealed that miR-613 directly suppresses SERPINA3, which might be one of the mechanisms in inhibiting melanoma cell proliferation and invasion. This research offers a promising therapeutic strategy for treatment melanoma.

Keywords: Melanoma, miR-613, SERPINA3, proliferation, invasion

Introduction

Melanoma is an invasive cancer that originates from the abnormal growth of pigment-producing skin cells. Although early stage surgical operation had made a high recovery rates, melanoma is still the most invasive skin cancer with a very high patient mortality (> 80%) after becoming metastatic [1, 2]. Recently, the MAP Kinase inhibitors was used and which made a breakthrough progress in treatment metastatic melanoma. Moreover, the checkpoint antibodies have markedly changed the therapy landscape of melanoma. However, both of them have their specific limitations [3]. Nowadays, metastatic melanoma is a fatal disease as before, only with a median survival time of 6 to 10 months [4]. Therefore, it is very urgent to deeply understanding the molecular mechanisms in melanoma progression and metastasis so as to work out new therapeutic strategies.

MicroRNAs (miRNAs) are a kind of 21-23 nucleotide long, small non-protein coding single-stranded RNA molecules, which are the important post-transcriptional regulators of gene expression in animals and plants [5]. In animals, the regulation function of miRNAs is achieved by binding the complimentary sequences within the 3’-untranslated regions (3’-UTRs) of their cognate messenger RNA (mRNA) targets [6, 7]. MiRNAs participate in a variety of tumorigenic activities, such as proliferation, invasion and metastasis [8]. MiR-613 has been found to be a critical role in tumorigenesis and cancer progression, such as inhibited ovarian cancer proliferation and invasion [9], induced lung cancer cell cycle arrest [10]. However, the effect of miR-613 in the melanoma remains elusive.

In this study, we investigated the effect of miR-613 on melanoma cell proliferation and the potential mechanism. Results showed that the expression of miR-613 decreased in melanoma
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cell lines, and restoration of miR-613 in A-375 cells suppressed proliferation and invasion. In addition, we also found that Serpin Peptidase Inhibitor, clade A member 3 (SERPINA3) is a target gene of miR-613. The influences of miR-613 on tumor proliferation and invasion are relying on suppressing the expression of SERPINA3. All these results show the mechanism of and a new role for miR-613 as a tumor inhibitor in melanoma.

Material and methods

Human tissue samples and cell lines

Human melanoma tissue and the corresponding normal tissue samples (29 samples, age from 29 to 54, female/male is 14/15, without other diseases) were acquired from patients of our hospital and tissues were frozen at once and stored at -80°C for RNA extraction. All the study was approved by the patients and was carried out on the basis of the instructions of our institute under the surveillance of the Clinical Research Ethics Board. The normal human epidermal melanocytes (NHEM) and human melanoma cell lines A-375, A-875, MV3 were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All the cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in RPMI-1640 medium supplemented with penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively) and 10% fetal bovine serum.

Lentivirus production and infection

The LV-miR-613 mimics and mimics control were purchased from Gene-Chem, Shanghai, China. A-375 cells were seed into 24-well plates at a density of 30% and incubated for a night before transfection. Cells were transfected with miR-613 mimics or mimics control by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s protocols.

Plasmid construction and dual luciferase activity assay

For SERPINA3 overexpression the eukaryotic expression vector pcDNA3.1(+) was cloned with full-length SERPINA3 cDNA which lacking of the 3’-UTR (Invitrogen, Carlsbad, California, USA). The SERPINA3 3’UTR target site for miR-613 was amplified by PCR and cloned into the Xba site of pGL3 control (Promega, Madison, USA). This vector was called WT SERPINA3 3’UTR. The Quick-change mutagenesis kit (Stratagene, Heidelberg, Germany) was used to carry out the site-directed mutagenesis of the miR-613 target-site in the SERPINA3 3’UTR and known as Mut SERPINA3 3’UTR. For the luciferase activity assay, Wt or Mut SERPINA3 3’UTR vector and the control vector pRL-CMV ((cytomegalovirus) coding for Renilla luciferase, Promega) were cotransfected. Dual-Luciferase Reporter Assay System (Promega, Madison, USA) was used to detect the luciferase activity 36 h after transfection.

Colony formation assay

For the colony formation test, A-375 cells treated with miR-613 control, miR-613 mimics, or miR-613+ pcDNA3.1-SERPINA3 were seeded in 6-well plates. After cultured 14 days, colonies stained with methylene blue and then photographed.

Cell invasion assay

A-375 cells were transfected with the miR-613 mimics, miR-613 control and miR-613+ SERPINA3 for 24 h. Then, the cells were plated onto 24-well upper chamber with a membrane that was pre-treated with Matrigel (100 μg per well, BD Biosciences, San Jose, CA, USA). In the lower part of the chamber, fresh medium contained 10% FBS was added. After the cells were cultured for 24 h at 37°C, we carefully removed the cells in the upper chamber. Invaded cells were fixed with 4% formaldehyde, stained with 0.5% crystal violet, and counted under a microscope.

RNA extraction and quantitative reverse transcription real-time PCR (qRT-PCR)

Total RNA was extracted with TRizol reagent (Invitrogen, Carlsbad, California, USA) following the operating instructions. The miR-613 mRNA expression level was detected by the Applied Biosystems (ABI) 7500 Sequence Detection System using the TaqMan MicroRNA assay kits (Applied Biosystems, California, USA). U6snRNA was used as the normalized control. The gene expression of SERPINA3 analyzed by SYBR Green and normalized with β-actin. The specificity of primer sequences were detected by its dissociation curve, 2^{-ΔΔCt} (cycle threshold) was...
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Western blot and immunohistochemical analysis

Protein was obtained by using RIPA buffer which contains a protease inhibitor cocktail (Sigma, St. Louis, MO, USA) according to the handling instructions. 50 μg of protein samples were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA, USA) using the Bio-Rad transfer system (Bio-Rad, California, USA). Western blot was executed by using anti-β-actin and anti-SERPINA3 (CST, Denver, CO, USA). The protein levels were detected with an ECL Plus Western Blotting Substrate (Thermo Scientific, CA, USA) following the manufacturer's instructions and analyzed by Image Lab. The immunohistochemistry assay was executed as before [11].

In vivo tumor growth assay

In the present study all the animal procedures and experiments were executed in conformity to the National Institutes of Health Guide for Care and Use of Laboratory Animals. The 6-week-old nude mice [BALB/c A-nu (nu/nu)] were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and all the mice housed in the Animal Resource Facility at Laboratory Animal Centre. 5 × 10^6 melanoma cells stably expressing either miR-613 or miR-control were injected subcutaneously in the both flanks of nude mice. The palpable tumor formation was observed every two days. Thirty days after the implantation, removed the xenografts from mice and weighed the xenografts. Tumor volume was calculated using the following formula: 4π/3 × (width/2)^2 × (length/2).

Statistical analysis

All the experiment results are presented as the mean ± standard deviation (SD). All the experiments were repeated 3 times unless otherwise specified. Two-tailed Student’s t-test Datos and one-way ANOVA were used to analyze the significant differences between groups. SPSS 18.0 and Graph Pad Prism 6.0 software were used for data analysis. Differences were considered statistically significant at $P<0.05$.

Results

Expression of miR-613 is down-regulated in melanoma tissues and melanoma cell lines

The level of miR-613 in tissue samples was valued by qRT-PCR, results showed that in melanoma tissues the expression level of miR-613 was significantly decreased when compared with the normal tissues ($P<0.05$, Figure 1A). The level of miR-613 also detected in the normal human epidermal melanocytes (NHEM) as well as in melanoma cells, such as A-375, A-875 and MV3. Results demonstrated that the
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miR-613 level was decreased in all of the melanoma cells \( (P<0.05) \), and the A-375 cells had the lowest miR-613 expression \( (P<0.01, \textit{Figure 1B}) \). It was likely that the higher degree of tumor malignant the lower of the miR-613 expression level, which would be more suitable for studying the functions of miR-613. Therefore, we chosen the A-375 as the following experiments.

\textit{SERPINA3 was suppressed by miR-613}

The potential mechanism of miR-613 to regulate SERPINA3 was analyzed by using miRanda

\textbf{Figure 2.} SERPINA3 is a potential target gene of miR-613. A: Sequence alignment of miR-613 and the SERPINA3 3′-UTR, which contains one predicted miR-613-biding site. B: Luciferase assay in A-375 cell lines co-transfected with miR-613 a luciferase reporter containing the SERPINA3 3′-UTR (WT) or a mutant (Mut). C: MiR-613 transfection decreased the SERPINA3 mRNA levels \( (P<0.05 \text{ vs. the miR-NC group}) \). D, E: MiR-613 transfection affects the SERPINA3 protein levels \( (P<0.05 \text{ vs. the miR-NC group}) \).
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![Image](image-url)

**Figure 3.** MiR-613 suppresses the melanoma cells proliferation and invasion through targeting SERPINA3. A, B: Western blot showed that transfection of SERPINA3 recovered the miR-613 induced down-regulation of SERPINA3 (**P<0.05 vs. the miR-613 mimics group**). C, D: MiR-613-overexpressing melanoma cells had decreased colony forming abilities when compared with mock cells. Transfection of SERPINA3 restored the colony forming abilities of miR-613-overexpressing cells (**P<0.05 vs. the miR-613 mimics group**). E, F: Invasion assay showed that miR-613 made the invasion cell numbers reduced while SERPINA3 could increase it (**P<0.05 vs. the miR-613 mimics group**).

algorithms and targetsscan. Results showed that miR-613 recognition site in SERPINA3 3’-UTRs. In order to verify whether SERPINA3 mRNA was a direct target gene of miR-613, SERPINA3 wild-type (WT) or mutant 3’-UTR (Figure 2A) was subcloned into a luciferase
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reporter vector and co-transfected with miR-613 mimics or negative control into A-375 cells. Luciferase activity assay demonstrated that miR-613 markedly inhibited the luciferase activity of the SERPINA3 WT 3’-UTR while miR-613 had no influence on the mutant (Figure 2B). To straightly evaluate the effect of miR-613 on SERPINA3 expression, we transfected miR-613 into A-375 cells and discovered that overexpression of miR-613 diminished the mRNA and protein levels of SERPINA3 (Figure 2C-E). Together with these results indicated that SERPINA3 is a direct target for miR-613, miR-613 inhibit SERPINA3 by targeting the binding sequence of SERPINA3 mRNA in the 3’UTR.

MiR-613 suppressed melanoma cells proliferation and invasion through targeting SERPINA3 in vitro

SERPINA3 has been found to be overexpressed in many types of cancer, such as cancers of melanoma [3], colon [12], breast [13-15] and stomach [16]. Moreover, studies had been demonstrated that upregulated of SERPINA3 has a positively correlate with worsening prognosis in patients with lung [17, 18], breast and melanoma [3]. The down-regulation of miR-613 in melanoma cells, and its repressive function on SERPINA3 implies that miR-613 may play an important role in melanoma cells proliferation by down regulation of SERPINA3. We therefore researched the role of miR-613 upregulation and SERPINA3 reconstruction on melanoma cells. Western blot assay showed that restoration of SERPINA3 recovered the expression of SERPINA3 which decreased by miR-613 (Figure 3A, 3B). In the colony formation assay, overexpression of miR-613 markedly decreased the colony formation capability in melanoma cell lines when compared to the miR-613 controls (Figure 3C, 3D). The invasion assays showed that upregulation of miR-613 markedly inhibited invasive acticity in melanoma cells when compared with the mimics controls (Figure 3E, 3F). However, transfection of SERPINA3 into melanoma cell lines ameliorated the inclined in proliferation and invasion induced by stable overexpression of miR-613 (Figure 3C-F). Taken together, our results demonstrated that overexpression of miR-613 obviously decreased the level of SERPINA3 when compared with mimics control, and it was markedly improved by tranfection of SERPINA3. However, the decrease in colony formation and invasion produced by stable upregulation of miR-613 restored by transfection SERPINA3 into melanoma cell lines. To sum up, these results indicated that miR-613 remarkably inhibits invasion and proliferation at least partially, through targeting SERPINA3.

Upregulation of miR-613 inclined the growth of melanoma cells in vivo

Based on the effect of miR-613 inhibiting melanoma cell growth, we further measured the effect of miR-613 against tumor growth in vivo. The tumor volume of subcutaneous xenograft tumors in nude mice markedly decreased in
melanoma cells stable transfection of miR-613 when compared with those cells who stably transfected with LV-control (Figure 4A, 4B). Furthermore, the level of SERPINA3 was also declined by stable transfection of miR-613 (Figure 4C).

Discussion

Melanoma is the most invasive form of skin cancer and is the sixth most common cancer in the USA, it only accounts for 4% of the cases but death rates as many as 74% of all the skin cancer [19]. Some studies had demonstrated that the pathogenesis of melanoma is very complex [20, 21], so it is crucial to revealing the potential molecular mechanisms for melanoma formation and development so as to provide a new treatment strategy [22, 23]. MicroRNAs (miRs) are a kind of small endogenous non-coding RNAs that could regulate the expression level of target genes by sequence-specific interactions with the 3'-UTR of cognate mRNAs [24]. Considering that an individual miR is capable of suppressing many kinds of mRNAs, they function as elementary regulators of tumor formation and development in different kinds of cancer, containing melanoma [23].

MiR-613 was firstly reported to be taken part in lipid metabolism in macrophages [25] and HepG2 cells [26]. Recent studies showed that miR-613 had a close relationship with tumorigenesis and metastasis [9, 10, 27], and identification of tumor-related miRNAs as well as their direct target genes is of great importance for understanding the biological meaning of miRNAs in melanoma progression and metastasis [28, 29]. In this study, we observed the expression level of miR-613 by qRT-PCR, results showed that in both melanoma cell lines and tissues the levels of miR-613 decreased. Furthermore, we found that the level of miR-613 was markedly associated with the degree of malignancy; it was the lowest in metastatic melanoma cells. With the purpose of further exploring the potential therapeutic effect of upregulation miR-613 in melanoma cells, we detected the role of miR-613 in the growth of subcutaneous xenograft tumors in nude mice. In line with the in vitro researches, recovery the level of miR-613 suppressed the growth of xenograft tumors. In vitro experiments also identified that restoration of miR-613 reduced tumor proliferation and invasion. These results give evidence of the therapeutic value of recovery miR-613 expression level in the therapy of tumors.

SERPINA3 previously called as α1-antichymotrypsin, which is a 68 kDa secreted serine protease inhibitor generally generated by the liver that proteolytically inhibits the activity of various serine proteases including chymotrypsin and cathepsin G [30]. This acute phase reactant protein is taken part in a wide variety of physiological activities such as complement activation, blood coagulation, wound healing, apoptosis and embryonic development [31-33]. More and more evidence has demonstrated that SERPINA3 is overexpressed in many kinds of cancers, including melanoma [14, 15, 34]. Moreover, SERPINA3 was found to play an important role in melanoma progression [3]. In the present study, we clarified that SERPINA3 is a direct target of miR-613 by using western blot and dual luciferase reporter. Both the real-time PCR and western blot results revealed that miR-613 had the ability to negatively modulate the expression of SERPINA3, which playing the role by connecting with a site in the SERPINA3 3'-UTR. Further study found that the inhibitory effect of miR-613 on invasion and proliferation could be recovered by transfection of SERPINA3 into miR-613-overexpressing cells. Therefore, we concluded that SERPINA3 as a direct target for miR-613 and this may suggest that miR-613 is a new target for melanoma therapy.

In the present study, there are many lines of demonstrations identify the interaction between miR-613 and SERPINA3. First, the 3'-UTR of SERPINA3 have an assumed binding site for miR-613 with meaningful seed match. Second, miR-613 inhibits the activity of a luciferase reporter merged with the 3'-UTR of SERPINA3 mRNA. The last but not the least is that miR-613 suppresses the expression of SERPINA3 at both protein and mRNA levels. These discoveries make us understanding the role of SERPINA3 in modulating melanoma cells better.

Taken together, our results suggested that miR-613 is involved in melanoma cell lines and tissue specimens. In addition, we demonstrated that miR-613 functions as an important role in the malignancy of melanoma cells, at least, through inhibitory of SERPINA3 expression. This is the first study combine miR-613 with SERPINA3 in melanoma, and miR-613 could be
a potential target to treatment melanoma in the future.

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Disclosure of conflict of interest

None.

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