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Original Research Article

MiR-22 alleviates the proliferation and metastasis of melanoma by targeting FASN

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Abstract

Purpose: *To determine the role of microRNA-22 (miR-22) in the development of malignant melanoma, and the underlying mechanism.*
Methods: *Potential miRNAs binding fatty acid synthase (FASN) were predicted by bioinformatics analysis, out of which miR-22 was selected. Their binding relationship was confirmed using dual-luciferase reporter assay. MicroRNA-22 and FASN levels in 40 clinical samples of melanoma were determined, and the correlation of the expression between miR-22 and FASN was assessed by Pearson correlation test. To uncover the role of miR-22 in regulating cell phenotypes of malignant melanoma, M21 and A375 cells were transfected with miRNA-NC, miR-22 mimics or miR-22 mimics + FASN-OE (FASN-over expression), respectively. Proliferative and metastatic abilities in each group were determined using cell counting kit-8 (CCK-8), 5-Ethynyl-2'- deoxyuridine (EdU) and Transwell assay, respectively.*
Results: *MiR-22 was the target gene binding the oncogene, FASN. Downregulated miR-22 and upregulated FASN were observed in melanoma tissues, showing a negative correlation between them. An overexpression of miR-22 significantly inhibited proliferative, migratory and invasive capacities in M21 and A375 cells ($p < 0.05$). Notably, overexpression of FASN abolished the inhibitory effects of miR-22 on proliferative and metastatic abilities in melanoma.*
Conclusion: *The level of expression of miR-22 in the malignant melanoma samples is low. Overexpression of miR-22 inhibits the proliferative and metastatic abilities of melanoma by targeting FASN and negatively regulating its level. Thus, miR-22 may be a promising therapeutic target of melanoma.*

Keywords: *Malignant melanoma, MicroRNA-22, Fatty acid synthase, Proliferation, Metastasis*

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INTRODUCTION

Melanoma is caused by malignant deterioration of melanocytes distributed in the stroma, most of which are formed by the carcinogenic transformation of normal moles and pigmented plaques [1]. Although the incidence of malignant melanoma accounts for only 1 % of skin tumors, its mortality rate is extremely high [2]. Melanoma rapidly progresses, leading to local or distant metastasis in a short period. Therefore, clarifying potential mechanisms of proliferation and

metastasis in melanoma contributes to improving its prognosis.

MicroRNAs (miRNAs) are short, single-stranded non-coding RNAs with a length of about 22 nucleotides. They regulate target gene expressions at the post-transcriptional level. In recent years, miRNAs have been considered to be closely related to tumorigenesis and tumor progression [3]. Increasing evidence have indicated that dysregulated miRNAs remarkably influence tumor development [4]. For example, miR-153-3p promotes the progression of ovarian cancer by targeting MCL1 [5]. MicroRNA 551b-3p inhibits the growth of human cholangiocarcinoma through targeted regulation of Cyclin D1 [6].

Fatty acid synthase (FASN) is an important functional enzyme involved in tumor development [7]. It is not only involved in energy metabolism, but also in intracellular signaling transmission [8]. Silencing of FASN in melanoma cells activates the intrinsic pathway of apoptosis [9]. N-phenylmaleimides affects adipogenesis by downregulating FASN, thus preventing against tumor [10]. It is believed that silencing FASN may be an effective gene therapy for melanoma. Through bioinformatics analysis, FASN was found to be a target of miR-22. In this study, the expression pattern of miR-22 in melanoma samples was determined, and the involvement of miR-22/FASN axis in mediating cell phenotypes of melanoma was also studied.

METHODS

Sample collection

Melanoma tissues (n = 40) and skin pigmented nevi (n = 40) were collected in Nanfang Hospital of Southern Medical University. Tissue samples were pathologically confirmed and stored at -80 ^{o}C. This study was approved by the Ethics Committee of Nanfang Hospital of Southern Medical University (approval no. 18NF#01), and was conducted after obtaining informed consent from each subject. The study followed the guidelines of Declaration of Helsinki [10].

Cell culture and transfection

Human melanocytes (HEM) and malignant melanoma cell lines; M21, B16 and A375 were provided by Cell Bank (Shanghai, China). The HEM cells were cultured in MGM, while the others were in Roswell Park Memorial Institute 1640 (RPMI 1640). 10 % fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 µg/mL streptomycin were supplemented in culture medium.

Cells with good activity were inoculated in 6-well plates and cultured to 30-40 % confluence. Transfection plasmids were provided by GenePharma (Shanghai, China). Cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Fresh medium was replaced after 24 h.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Extracted RNAs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Japan). Real-time PCR was performed using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan). Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. The primer sequences used in this study are listed in Table 1.

Table 1: Primer sequences used in PCR studies

Gene	Forward (5'-3')	Reverse (5'-3')
FASN	AGATCCTGGAACGAGAACACGAT	GAGACGTGTCACTCCTGGACTTG
U6	CTCGCTTCGGCAGCACATA	CGCTTCACGAATTTGCGTG
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC

Dual-luciferase reporter assay

Wild-type and mutant-type FASN vectors (RIBOBIO, Guangzhou, China) were constructed based on binding sequences in the 3'UTR of miR-22 and FASN. Cells were co-transfected with miR-22 NC/miR-22 mimics and FASN WT/FASN MUT, respectively. After 48 h transfection, luciferase activity was measured using Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA).

Cell counting kit-8 (CCK-8) assay

The cells were inoculated in a 96-well plate at a density of 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the CCK-8 kit

(RIBOBIO, Guangzhou, China) for plotting the viability curves.

5-Ethynyl-2'- deoxyuridine (EdU) assay

Cells were pre-inoculated in a 24-well plate (2 × 10^4 cells/well), and incubated in 4 % methanol for 30 min, followed by 10-min permeabilization in 0.5 % TritonX-100 (Beyotime, Shanghai, China), and 30-min reaction in 400 μL of 1 × ApollorR. Afterwards, cells were dyed in 1 × Hoechst 33342 for another 30 min. Positive EdU-stained (Beyotime, Shanghai, China) cells were calculated.

Transwell assay

The 3 × 10^4 cells suspended in 100 μL of serum-free medium were seeded in the upper layer of a Transwell chamber that was inserted in a 24-well plate. Culture medium (600 μL) was applied per well. On the other day, bottom cells were fixed in methanol for 15 min, stained in crystal violet in 20 min and captured using a microscope. Migratory cells were counted in 5 random fields per sample. Determination of invasive cell number was similarly conducted except for pre-coating diluted 100 μL of Matrigel on the top of each chamber.

Western blot

Total proteins of cells were prepared using lysis buffer (Beyotime, Shanghai, China), followed by centrifugation at 14,000*g* for 15 min at 4 °C. Total protein concentration was calculated using bicinchoninic acid (BCA). Protein Assay Kit (Beyotime, Shanghai, China). Protein samples were loaded on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5 % skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Primary antibodies, including FASN (dilution: 1:500, CatNOs: ab99359) and GAPDH (dilution: 1:500, CatNOs: ab37168) and secondary goat anti-rabbit (HRP) IgG antibody (dilution: 1/2000; CatNOs: ab6721) were all purchased from Abcam (Cambridge, MA, USA). The gray value was analyzed using Image J software (Version 1.38; National Institutes of Health, Bethesda, MA, USA).

Statistical analysis

Statistical analysis software (SPSS version 26.0) was used for data analyses and Figures were depicted using GraphPad Prism 7.0 (La Jolla, CA, USA). Data are expressed as mean ± standard deviation (SD). Differences between groups were analyzed using the two-tailed t-test. Pearson correlation test was applied for evaluating the expression relationship between miR-22 and FASN in melanoma tissues. $P < 0.05$ was considered as statistically significant.

RESULTS

FASN was the target of MiR-22

Potential miRNAs binding FASN were predicted on the StarBase 2.0 (http://starbase.sysu.edu.cn/agoClipRNA.php?source=mRNA), and miR-22 was selected. In both melanoma tissues and cell lines, miR-22 was lowly expressed (Figures 1). As an oncogene, FASN was upregulated in melanoma tissues (Figure 1 C). Pearson correlation test showed that FASN level was negatively linked to miR-22 level in melanoma tissues (Figure 1 D).

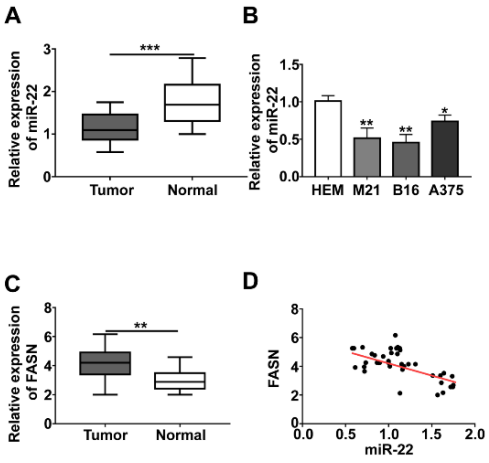

Figure 1: MiR-22 was the target of FASN and was lowly expressed in melanoma. (A) MiR-22 levels in melanoma tissues (n=40) and skin pigmented nevi (n=40). (B) MiR-22 levels in melanoma cell lines (vs. HEM group). (C) FASN levels in melanoma tissues (n=40) and skin pigmented nevi (n=40). (D) A negative correlation between expression levels of FASN and miR-22 in melanoma tissues. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

MiR-22 negatively regulated FASN level in melanoma

Binding sequences in the 3'UTR of miR-22 and FASN were depicted in Figure 2 A. Wild-type and mutant-type FASN vectors were constructed, and transfection efficacy of miR-22 mimics in M21 and A375 cells was verified (Figure 2 B). Overexpression of miR-22 decreased luciferase activity in wild-type FASN vector, demonstrating the binding relationship between miR-22 and FASN (Figure 2 C and D). Interestingly, both

mRNA and protein levels of FASN were downregulated in M21 and A375 cells overexpressing miR-22, showing a negative regulation (Figure 2 E and F). It is concluded that miR-22 can regulate the transcription and translation of FASN by binding to its 3'UTR region.

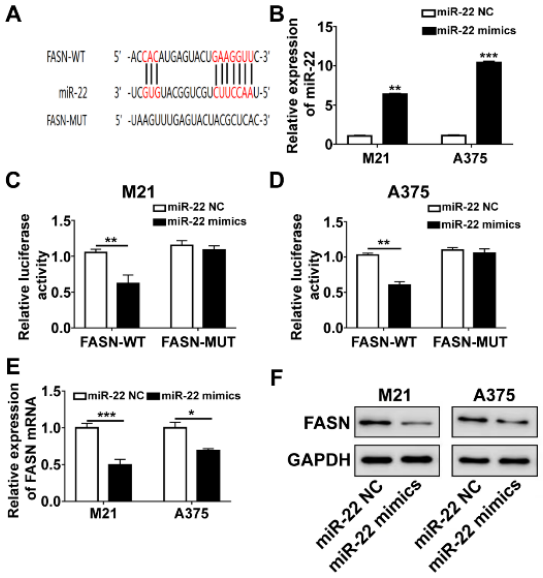

Figure 2: MiR-22 negatively regulated FASN level in melanoma. A: Binding sequences in the 3'UTR of miR-22 and FASN. B: Transfection efficacy of miR-22 mimics in M21 and A375 cells. C & D: Luciferase activity in M21 (C) and A375 cells (D) co-transfected with miR-22 NC/miR-22 mimics and FASN WT/FASN MUT. E&F: The mRNA (E) and protein levels (F) of FASN in M21 and A375 cells transfected with miR-22 NC or miR-22 mimics. *p < 0.05, **p < 0.01, ***p < 0.001, vs. miR-22 NC group

MiR-22 inhibited proliferative ability in melanoma

To uncover the role of miR-22 in regulating malignant melanoma, M21 and A375 cells were transfected with miRNA-NC, miR-22 mimics or miR-22 mimics+FASN-OE, respectively. Upregulated miR-22 level in melanoma cells overexpressing miR-22 was reduced by co-overexpression of FASN (Figure 3A). *In vitro* proliferative ability in melanoma was assessed by both CCK-8 and EdU assay. Decreased viability (Figures 3B, 3C) and positive EdU-stained cells (Figures 3 D and E) in M21 and A375 cells overexpressing miR-22 were partially reversed by co-overexpression of FASN.

MiR-22 inhibited migratory and invasive abilities in melanoma

Potential influences of miR-22 and FASN on melanoma metastasis were determined by Transwell assay. Overexpression of miR-22 markedly decreased migratory cell numbers of melanoma, and the declined trend was abolished by co-overexpression of miR-22 and FASN (Figure 4A). Similarly, decreased invasive cell number following miR-22 overexpression in M21 and A375 cells was reversed by overexpressed FASN (Figure 4B). Collectively, the inhibitory effects of miR-22 on proliferative and metastatic abilities in melanoma were reversed by FASN overexpression.

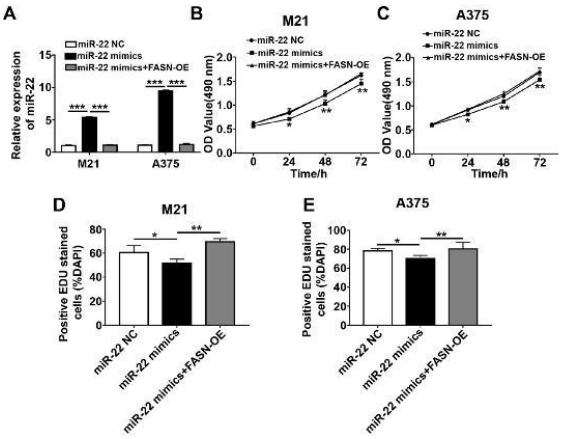

Figure 3: MiR-22 inhibited proliferative ability in melanoma. M21 and A375 cells were transfected with miRNA NC, miR-22 mimics or miR-22 mimics+FASN-OE, respectively. A: Relative level of miR-22 in M21 and A375 cells. B&C: Viability in M21 (B) and A375 cells (C). D&E: Positive EdU-stained M21 (**D**) and A375 cells (**E**). *p < 0.05, **p < 0.01, ***p < 0.001, vs. miRNA NC group

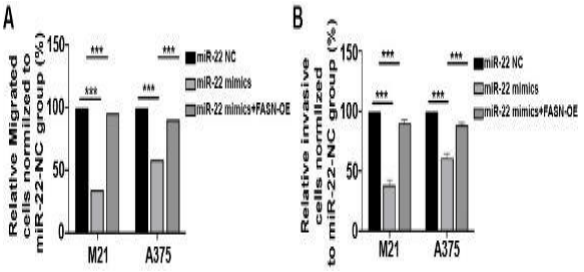

Figure 4: MiR-22 inhibited migratory and invasive abilities in melanoma. M21 and A375 cells were transfected with miRNA NC, miR-22 mimics or miR-22 mimics+FASN-OE. A: Migratory cell number in M21 and A375 cells; B: Invasive cell number in M21 and A375 cells. ***p < 0.001, vs. miR-22 NC group

DISCUSSION

Distant metastasis of melanoma through hematogenous transmission occurs at an early stage. Besides, melanoma is not sensitive to radiotherapy and chemotherapy, resulting in the poor prognosis [11]. The interaction between miRNAs and melanoma development has been

continuously reported. Multiple miRNAs are abnormally expressed in melanoma profiling, serving as oncogenes or tumor suppressors [12]. It has been reported that miR-155 inhibits the proliferative, migratory and invasive potential of malignant melanoma by negatively regulating CBL expression [13]. In addition, miRNA-139-5p regulate the growth and metastasis of malignant melanoma cells by binding to IGF1R *via* the activated PI3K / AKT pathway [14]. The above studies indicated that there are multiple regulatory mechanisms of miRNAs affecting the occurrence and development of malignant melanoma, which are required to be further explored.

MiRNAs are vital regulators in tumorigenesis through the inhibition of translation, or directly degrading target mRNAs [15-17]. Previous studies have shown the involvement of miR-22 in different types of human cancers [18-20]. The potential role of miR-22 in the progression of melanoma remains largely unclear. Shi *et al* [21] demonstrated that miR-22 served as a tumor suppressor in melanoma progression through the targeting of FMNL2.

In this study, it was found that miR-22 was downregulated in melanoma tissues and cell lines, indicating its involvement in the development of melanoma. Furthermore, experimental results demonstrated the inhibitory effects of miR-22 on proliferative, migratory and invasive abilities in melanoma cell lines M21 and A375.

The binding relationship between miR-22 and FASN was confirmed with both bioinformatics analysis and dual-luciferase reporter assay. FASN level was negatively regulated by miR-22 in melanoma cells. Notably, the inhibitory effects of miR-22 on malignant phenotypes of melanoma was abolished by overexpression of FASN. To sum up, miR-22 alleviated the deterioration of melanoma by negatively regulating FASN level. This study also has some limitations. For example, further animal experiments were not conducted to verify the findings. In the future, plans will be made to verify the effects of miR-22 on the growth of melanoma through nude mice experiments, and to further study its molecular mechanism.

CONCLUSION

The expression of MiR-22 in malignant melanoma samples is low. Overexpression of miR-22 inhibits proliferative and metastatic abilities in melanoma by targeting FASN. Thus, MiR-22 may be a promising therapeutic target of melanoma.

DECLARATIONS

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This manuscript was retracted from Journal of BUON (https://jbuon.com/archive/26-6-2614.pdf) as per our request (Retraction notice: https://jbuon.com/archive/26-6-2614-retraction-notice.pdf) due to the reason that we were not informed of the decision regarding the delisting of this journal from Web of Science. The editor-in-chief agreed with the retraction, apologized to us and informed us that we should feel free to submit this manuscript elsewhere.

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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 done 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. Jun Qiu and Yanhua Yi contributed equally to this work.

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