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ORIGINAL ARTICLE

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

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Summary

Purpose: *To explore the role of MicroRNA-22 (miR-22) in the development of malignant melanoma and the underlying mechanism.*

Methods: *Potential miRNAs binding FASN (fatty acid synthetase) were predicted by bioinformatics analysis and miR-22 was selected. Their binding relationship was confirmed by dual-luciferase reporter assay. MiR-22 and FASN levels in 40 clinical samples of melanoma were detected. Expression correlation 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, respectively. Proliferative and metastatic abilities in each group were determined by 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. Overexpression of miR-22 inhibited proliferative, migratory and invasive abilities in M21 and A375 cells. Notably, overexpression of FASN abolished the inhibitory effects of miR-22 on proliferative and metastatic abilities in melanoma.*

Conclusions: *MiR-22 is lowly expressed in the malignant melanoma samples. Overexpression of miR-22 inhibits proliferative and metastatic abilities in melanoma by targeting FASN and negatively regulating its level. MiR-22 may be a promising therapeutic target of melanoma.*

Key words: *malignant melanoma, MiR-22, FASN, proliferation, metastasis*

Introduction

Melanoma is caused by the malignant deterioration of melanocytes distributed in the stroma, most of which are formed by the canceration of normal moles and pigmented plaques [1]. Although the incidence of malignant melanoma accounts for only 1% of skin tumors, its mortality 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 improve its prognosis.

MicroRNAs (miRNAs/miRs) are a kind of short, single-stranded non-coding RNAs with a length of about 22nt. They mainly 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 evidences have indicated that dysregu-

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lated miRNAs remarkably influence tumor development [4]. For example, miR-153-3p promotes the progression of ovarian cancer by targeting MCL1 [5]. MiR-551b-3p inhibits the growth of human cholangiocarcinoma through targeted regulation of Cyclin D1 [6].

FASN (fatty acid synthetase) 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]. Silence of FASN in melanoma cells can activate 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, miR-22 is found to be the target of FASN. In this paper, we detected expression pattern of miR-22 in melanoma samples, and the involvement of miR-22/FASN axis in mediating cell phenotypes of melanoma.

Methods

Sample collection

Melanoma tissues (n=40) and skin pigmented nevi (n=40) were collected in our hospital. Tissue samples were pathologically confirmed and stored at -80°C. This study got approval by Ethics Committee of Nanfang Hospital of Southern Medical University and it was conducted after informed consent of each subject.

Cell culture and transfection

Human melanocytes HEM and malignant melanoma cell lines M21, B16 and A375 were provided by Cell Bank (Shanghai, China). HEM cells were cultured in MGM and 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 a 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 at 24 h.

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

Extracted RNAs by 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). The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. miR-22: 5′-TGGAATGTAAAGAAGTATGTAT-

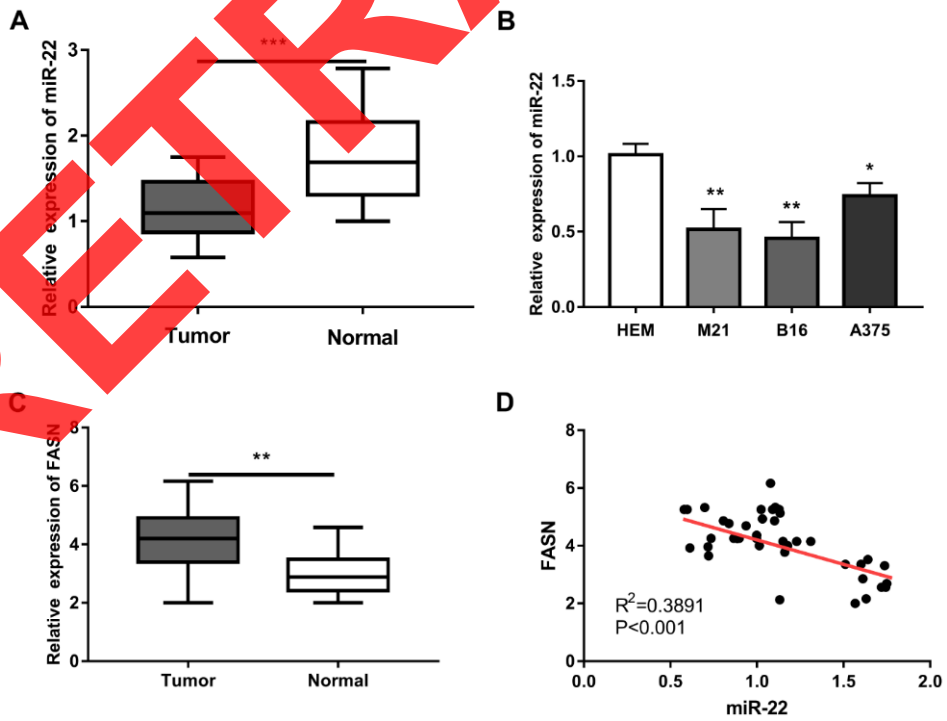

Figure 1. MiR-22 was the target of FASN and lowly expressed in melanoma. **(A)** MiR-22 levels in melanoma tissues (n=40) and skin pigmented nevi (n=40). **(B)** MiR-22 levels in melanoma cell lines. **(C)** FASN levels in melanoma tissues (n=40) and skin pigmented nevi (n=40). **(D)** A negative correlation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

3′; FASN F: 5′-AGATCCTGGAACGAGAACACGAT-3′, R: 5′-GAGACGTGTCACTCCTGGACTTG-3′; U6, F: 5′-CTCGCTTCGGCAGCACATA-3′, R: 5′- CGCTTCACGAATTTGCGTG -3′; GADPH, F: 5′-AGCCACATCGCTCAGACAC-3′, R: 5′-GCCCAATACGACCAAATCC-3′.

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 the Dual-Glo Luciferase Assay System (Promega, Madison, WI, USA).

Cell counting kit-8 (CCK-8) assay

Cells were inoculated in a 96-well plate with 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). They were 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

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. 600 μL of medium 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.

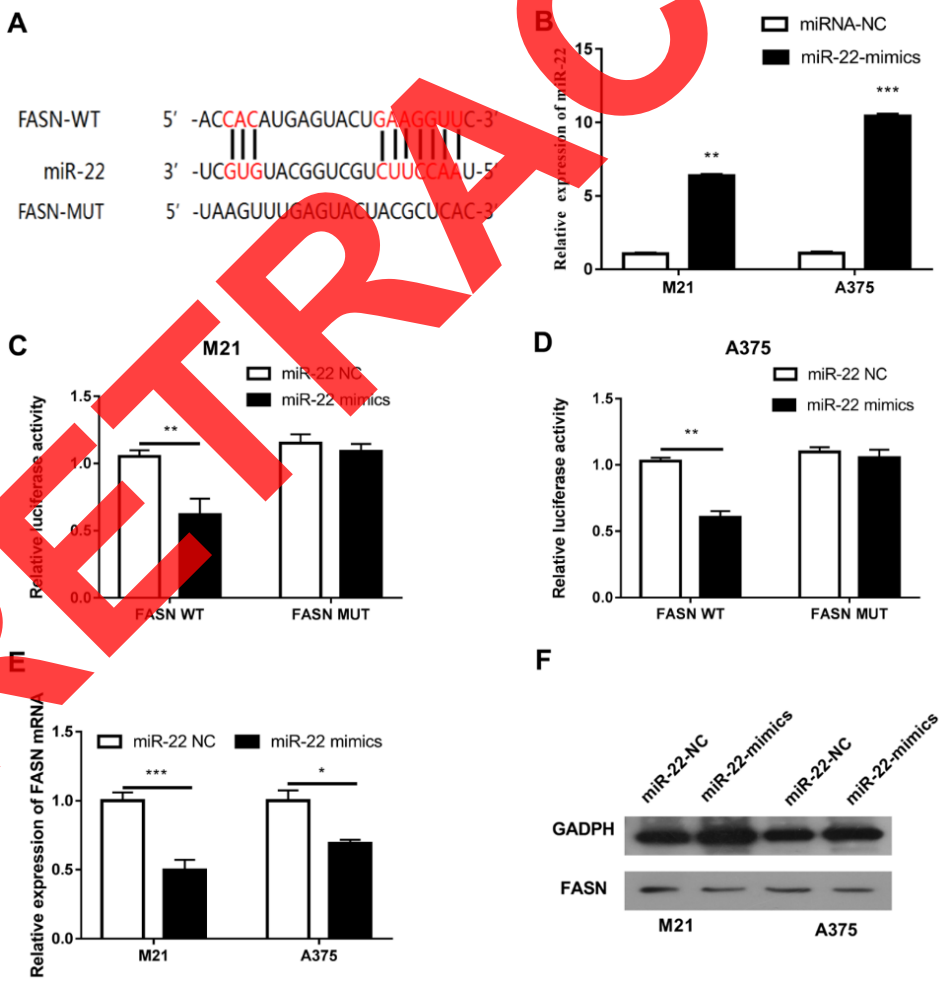

Figure 2. MiR-22 negatively regulated FASN level in melanoma. **(A)** Binding sequences in the 3'UTR of miR-22 and FASN. **(B)** Transfection efficacy of miR-22 mimics in M21 and A375 cells. **(C, D)** Luciferase activity in M21 (C) and A375 cells **(D)** co-transfected with miR-22 NC/miR-22 mimics and 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.

Western blot

Cells were lysed for isolating proteins and electrophoresed. Protein samples were loaded on polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses of grey values were finally conducted.

Statistics

SPSS) 21.0 (IBM, Armonk, NY, USA was used for data analyses and figures were depicted using GraphPad Prism 7.0 (La Jolla, CA, USA). Data were expressed as mean ± standard deviation. Differences between groups were analyzed by 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

MiR-22 was the target of FASN and lowly expressed in melanoma

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 (Figure 1A, 1B). As an oncogene, FASN was upregulated in melanoma tissues (Figure 1C). Pearson correlation test suggested that FASN level was negatively linked to miR-22 level in melanoma tissues (Figure 1D).

MiR-22 negatively regulated FASN level in melanoma

The above results indicated a regulatory relationship between FASN and miR-22 in melanoma. Subsequently, their binding ability was tested. Binding sequences in the 3'UTR of miR-22 and FASN were depicted in Figure 2A. Wild-type and mutant-type FASN vectors were constructed, and transfection efficacy of miR-22 mimics in M21 and A375 cells was verified (Figure 2B). Overexpression of miR-22 decreased luciferase activity in wild-type FASN vector, demonstrating the binding relationship between miR-22 and FASN (Figure 2C, 2D). Interestingly, both mRNA and protein levels of FASN were downregulated in M21 and A375 cells overexpressing miR-22, displaying a negative regulation (Figure 2E, 2F). It is concluded that miR-22 can regulate the transcription and translation of FASN by binding to its 3'UTR region.

MiR-22 inhibited proliferative ability in melanoma

To uncover the role of miR-22 in regulating malignant melanoma, M21 and A375 cells were

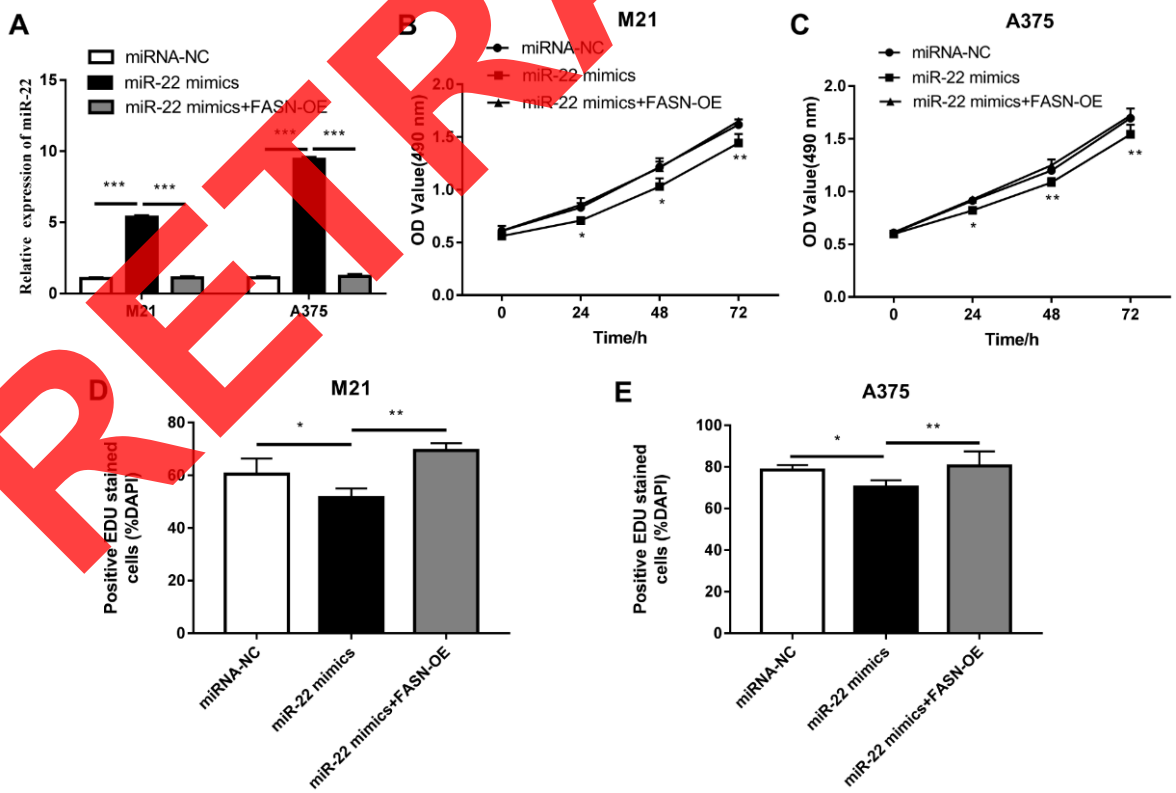

Figure 3. MiR-22 inhibited proliferative ability in melanoma. M21 and A375 cells were transfected with miRNA NC, miR-22 mimics or miR-22 mimics+FASN-OE, respectively. **(A)** Relative level of miR-22 in M21 and A375 cells. **(B, C)** Viability in M21 **(B)** and A375 cells **(C)**. **(D, E)** Positive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

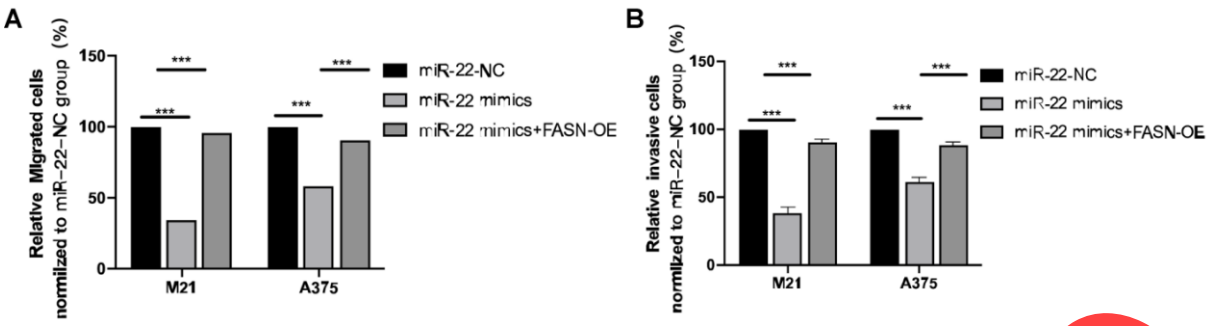

Figure 4. MiR-22 inhibited migratory and invasive abilities in melanoma. M21 and A375 cells were transfected with miRNA NC, miR-22 mimics or miR-22 mimics+FASN-OE, respectively. **(A)** Migratory cell number in M21 and A375 cells; **(B)** Invasive cell number in M21 and A375 cells. ***p<0.001.

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 (Figure 3B, 3C) and positive EdU-stained cells (Figure 3D, 3E) 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 examined 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 could be reversed by FASN overexpression.

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]. Inhibition of malignant growth and metastasis of melanoma contributes to prolong the survival. 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 is reported that miR-155 inhibits the proliferative, migratory and invasive potentials of malignant melanoma by negatively regulating CBL expression [13]. In addition, miRNA-139-5p regulates 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 inhibiting translation or directly degrading target mRNAs [15]. Previous studies have shown the involvement of miR-22 in different types of human cancers [16-18]. The potential role of miR-22 in the progression of melanoma remains largely unclear. In this paper, we 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 by 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 could be abolished by overexpressed FASN. To sum up, miR-22 alleviated the deterioration of melanoma by negatively regulating FASN level.

Conclusions

MiR-22 is lowly expressed in the malignant melanoma samples. Overexpression of miR-22 inhibits proliferative and metastatic abilities in melanoma by targeting FASN. MiR-22 may be a promising therapeutic target of melanoma.

Conflict of interests

The authors declare no conflict of interests.

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