MIR-520f Regulated Itch Expression and Promoted Cell Proliferation in Human Melanoma Cells

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Abstract
Accumulating evidence suggests that abnormal expression and dysfunction of microRNA is involved in development of cancers. However, the function of miR-520f especially in human melanoma remains elusive. In the current study, the underlying function of miR-520f in human melanoma was investigated. Our study demonstrated that the miR-520f level in human melanoma cell lines and clinical tissues was increased. Overexpression of miR-520f promoted cell proliferation by using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay, colony formation, anchorage-independent growth assay, and 5-bromo-2-deoxyuridine assays. Furthermore, we revealed that miR-520f could interact with circular RNA Itchy E3 ubiquitin protein ligase (ITCH) 3'-untranslated region and suppress ITCH expression in human melanoma cells. The inhibitory effect of miR-520f-in could be partially restored by knockdown of ITCH in human melanoma cells. In summary, this study provides novel insights into miR-520f as a crucial role in the regulation of human melanoma cell growth via regulating ITCH, which might be a potential biomarker and therapeutic target of human melanoma.

Keywords
miR-520f, human melanoma, ITCH, cell proliferation

Introduction
Human melanoma, an aggressive skin cancer type, is the fifth and seventh most common cancer in males and females, respectively.¹ Although achieving significant progress in therapeutic strategies, such as surgical resection, radiotherapy, chemotherapy, and immunotherapy, the survival rates for this disease have not been effectively improved.²-⁵ Accordingly, it is urgently to explore the genetic and epigenetic alterations underlying human melanoma development may be promising for identifying novel therapeutic targets for human melanoma.

MicroRNAs (miRNAs), an essential class of small noncoding RNAs, act as posttranscriptional regulators in various biological processes such as cell proliferation, apoptosis, metastasis, and differentiation.⁶-⁸ Accumulating evidences have indicated that miRNAs play diverse roles in cancer progression by interacting with 3'-untranslated regions (UTRs) of target messenger RNAs (mRNAs).⁹,¹⁰ Accumulating evidence has shown that miRNAs have received great attention in human melanoma. MicroRNA-610 was reported to suppress tumor growth of human melanoma by targeting low-density lipoprotein receptor–related proteins 6.¹¹ MicroRNA-195 promoted cell proliferation of human melanoma by targeting prohibitin 1.¹² MicroRNA-326 suppresses human melanoma progression by targeting Kirsten rat sarcoma and inactivating the protein kinase B and extracellular signal–regulated protein kinase signaling pathways.¹³ MicroRNA-767 contributes to cell proliferation of human melanoma by repressing cylindromatosis expression.¹⁴ MicroRNA-137 suppresses glutamine catabolism and cell growth of malignant melanoma by targeting glutaminase.¹⁵ MicroRNA-769 promoted cell proliferation of human melanoma by suppressing glycogen synthase kinase-3β expression.¹⁶ However, the contribution of miR-520f in the tumorigenesis and progression of human melanoma has
not been fully clarified. In the current study, the expression of miR-520f and Itch E3 ubiquitin protein ligase (ITCH) in human melanoma was examined. The purpose is to explore the role of miR-520f in human melanoma cell proliferation regulation.

**Materials and Method**

**Cell Culture**

Human melanoma cells lines, including UACC257, WM-115, A7, MeWo, A375, NHEM, and WM-115, were purchased from Chinese Academy of Sciences (Shanghai, China) and were cultured in roswell park memorial institute (RPMI)-1640 supplemented with 10% fetal bovine serum (HyClone, Logan, Utah). Primary Human Epidermal Melanocytes (PEM; PromoCell, Germany) from adult skin were maintained in serum- and phorbol 12-myristate 13-acetate (PMA)-free melanocyte growth medium M2 (PromoCell). All cells were cultured at 37°C in an atmosphere of 5% CO2 and 95% air.

**Clinical Specimens**

Ten human melanoma tissues and tumor adjacent normal tissues (TAT) were obtained from Shandong Provincial Hospital Affiliated to Shandong University (Shandong, People’s Republic of China), and all samples were immediately frozen in liquid nitrogen. Prior written informed consent was obtained from each patient, this study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University (Shandong, People’s Republic of China).

**Plasmids, Small Interfering RNA, and Transfection**

MicroRNA-520f mimics, miR-520f-in, and the relative negative controls (miR-cons) were purchased from RiboBio Company (miR20002830-1-5; Guangzhou, China) and transfected into human melanoma cell lines A375 and WM-115 using Lipofectamine 2000 (PromoCell). MicroRNA-520f mimics, miR-520f-in, and the relative negative controls (miR-cons) were purchased from RiboBio Company (miR20002830-1-5; Guangzhou, China) and transfected into human melanoma cell lines A375 and WM-115 using Lipofectamine 2000 (PromoCell). According to the manufacturer’s instructions.

For ITCH depletion, small interfering RNAs (siRNAs) specifically for ITCH and the control siRNAs were purchased from RiboBio Company (Guangzhou, China) and were transfected into A375 and WM-115 cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol.

**RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction**

Total miRNA from cultured cells was extracted using the mirVana miRNA Isolation Kit (Ambion, Austin, Texas), according to the manufacturer’s instructions. Polymerase chain reaction (PCR) amplifications for miR-520f were performed with the Kit of TaqMan Human MiRNA Assay (GeneCopoeia, Guangzhou, China). Total cellular RNA was extracted from harvested cells using TRIzol reagent (Life Technologies, Grand Island, New York) according to the manual. Polymerase chain reaction amplifications for miR-520f were performed with the SYBR Premix Ex Taq Kit (Takara, Shiga, Japan). The primers of miR-520f, myelocytomatosis oncogene (MYC), cyclin D1, RNU6B, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were bought from Gene Copoeia (Guangzhou, China). U6 was used as the control gene for the relative level of miR-520f, while GAPDH served as internal control for MYC and cyclin D1. Relative expression levels were calculated using the 2−ΔΔCT method. All experiments were performed in triplicate.

**MTT Assays and Colony Formation**

Cell growth was measured by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 5000 transfected A375 and WM-115 cells/well were plated in triplicate in 96-well microplates, and MTT assays were performed according to the manufacturer’s protocol. The absorbance was measured in a Thermo Scientific Multiskan (Ambion, Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) at a wavelength of 490 nm.

For colony formation assay, the transfected cells were seeded into three 6-cm cell culture plates (1 × 10^5 cells per well) and incubated at 37°C with 5% CO2 for 14 days until visible colony formation was observed and washed with PBS and stained with 0.1% crystal violet solution for 5 minutes after fixation with 10% formaldehyde for 20 minutes. Cell colonies with >50 cells were counted and imaged.

**Anchorage-Independent Growth Assay**

Cells (1 × 10^3) were trypsinized and mixed with equal volume of 0.7% agarose and plated on top of a bottom layer consisting of 1% agar in complete medium. The plates were cultured at 37°C in a humid atmosphere of 5% CO2 for 14 days. Surviving colonies containing more than 50 cells were counted and photographed with a Qimaging Micropublisher 5.0 RTV microscope camera (Olympus, Tokyo, Japan).

**5-Bromo-2-Deoxyuridine Assay**

The 5-bromo-2-deoxyuridine (BrdU) assay was performed using BrdU Cell Proliferation Assay Kit (Cell Signaling Technology Inc, Denver, Colorado), according to the manufacturer’s instructions. Fluorescent signals were observed under a fluorescence microscope (Olympus).

**Luciferase Assays**

Cells were seeded in triplicate in 24-well plates (5 × 10⁴/well) and cultured for 24 hours. The pGL3-luciferase reporter gene plasmids pGL3-ITCH-3’-UTR were cotransfected into the cells in the presence of miR-520f or miR-520f-in or miR-520f-mut or the relative negative controls. Luciferase and Renilla activities were assayed 48 hours after transfection using a Dual Luciferase Reporter Assay Kit (Promega, Madison, Wisconsin), according to the manufacturer’s protocol.
Western Blotting

Equal quantities of protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, gels were transferred onto polyvinylidene fluoride membranes. The membrane was incubated overnight with anti-ITCH (Cell Signaling Technology), anti-cyclin D1 (Cell Signaling Technology), and anti-C-Myc (Cell Signaling Technology) after blocking with 5% nonfat milk and followed by horseradish peroxidase–conjugated secondary antibody (Santa Cruz Biotechnology, California). Protein expression levels were visualized using the chemiluminescence (Great EscAPE™ SEAP Chemiluminescence Kit 2.0), and anti-α-tubulin antibody (Santa Cruz Biotechnology) was used as a loading control.

Statistical Analysis

All statistical analyses were carried out using the SPSS 19.0 (SPSS Inc, Chicago, Illinois). Student t test was used to evaluate the significance of the differences between 2 groups of data in all the pertinent experiments. P value <.05 was thought to be statistically significant.

Result

MicroRNA-520f Expression Was Overexpression in Human Melanoma Tissues and Human Melanoma Cell Lines

To investigate the role of miR-520f in human melanoma development, we analyzed the miR-520f expression data from the Gene Expression Omnibus (GEO; accession number GSE 25653). A significant increased miR-520f expression was identified in melanoma cells (tumor cells) as compared to normal cells (N, P < .01; Figure 1A). Next, the expression of miR-520f in human melanoma cells was detected by quantitative reverse transcription PCR (qRT-PCR) analysis. The results showed that miR-520f expression was higher in all 7 human melanoma cell lines (UACC257, WM35, A7, MeWo, A375, NHEM, and WM-115) compared to PEM cells (Figure 1B). In addition, the expression of miR-520f in human melanoma biopsy tissues that were obtained from patients of our hospital was also detected by qRT-PCR analysis (Figure 1C), and the result demonstrated that the expression of miR-520f was upregulated melanoma clinical tissues (T) compared with paired tumor adjacent normal tissues (TAT). These results indicated that increased in miR-520f may be associated with human melanoma progression.

MicroRNA-520f Promoted Human Melanoma Cell Proliferation

To overexpress miR-520f, miR-520f mimic or vector was transiently transfected into A375 and WM35 cells. Relative miR-520f expression was verified using qRT-PCR after 48 hours of transfection, and the result indicated that miR-520f was successfully overexpressed in A375 and WM35 cells (Figure 2A). Result of MTT assay and colony formation assay indicated that the overexpression of miR-520f significantly promoted the proliferation of A375 and WM35 cells (Figure 2B and C). Anchorage-independent growth assay showed the overexpression of miR-520f promoted the anchorage-independent growth of A375 and WM35 cells (Figure 2D). Moreover, BrdU assay indicated that miR-520f mimic increased the number of BrdU-positive cells compared to controls (Figure 2E).

MicroRNA-520f-in Reduced Human Melanoma Cell Proliferation

To knockdown miR-520f expression, miR-520f-in or NC inhibitor was transiently transfected into A375 and WM35 cells. Relative miR-520f expression was verified using qRT-PCR (Figure 3A). The effect of miR-520f-in on cell proliferation human melanoma A375 and WM35 cells was detected. MTT assay and colony formation assay results showed that the
proliferation of A375 and WM35 cells transfected with miR-520f-in was strongly inhibited (Figure 3B and C). The anchorage-independent growth ability of A375 and WM35 cells was reduced after transfected with miR-520f-in (Figure 3D). Furthermore, the number of BrdU-positive cells was decreased after transfected with miR-520f-in compared to controls (Figure 3E). These results showed that miR-520f promoted human melanoma cell tumorigenicity in vitro.

**MicroRNA-520f Directly Targets ITCH by Binding to Its 3’-UTR**

For further identifying how miR-520f influenced cell proliferation of human melanoma, we used TargetScans to search for potential targets of miR-520f and identified ITCH as its possible target (Figure 4A). The regulatory function of miR-520f on ITCH was determined by Western blot assays, and the results

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**Figure 2.** miR-520f upregulation promoted human melanoma cell proliferation. A, Validation of miR-520f expression levels after transfection in A375 and WM35 cells by PCR analysis. B, the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assays revealed that upregulation of miR-520f promoted growth of A375 and WM35 cells. C, Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. D, Upregulation of miR-520f promoted the anchorage-independent growth of A375 and WM35 cells. Representative micrographs (left) and quantification (right) of colonies that were >0.1 mm (right). E, Representative micrographs (left) and quantification (right) of the 5-Bromo-deoxyuridine incorporation assay in A375 and WM35 cells. Each bar represents the mean of 3 independent experiments. *P < .05. PCR, polymerase chain reaction.
indicated that the overexpression of miR-520f significantly downregulated ITCH protein levels, while miR-520f-in led to an increased protein expression of ITCH (Figure 4B). Luciferase reporter assays indicated that compared with control, miR-520f could led to a significant decrease in luciferase activity of the wild-type ITCH 3'-UTR, while the repressive effect of miR-520f-in increased wild-type ITCH luciferase activity (Figure 4C). Next, we examined the expression of the key regulators of cell proliferation, P21 and MYC, by real-time PCR and Western blotting in the context of overexpression or downregulation of miR-520f (Figure 4D and E). The results demonstrated that P21 was significantly downregulated in response to miR-520f expression, while C-myc was significantly upregulated, and miR-520f-in significantly increased the expression of P21 expression, decreased the C-myc expression. The above observation indicated that miR-520f functionally modulated cellular proliferation regulators, P21 and C-myc, thus relevant to cell proliferation.

Figure 3. miR-520f-in inhibited human melanoma cell proliferation. A, Validation of miR-520f expression levels after transfection in A375 and WM35 cells by PCR analysis. B, the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assays revealed that inhibition of miR-520f suppressed growth of in A375 and WM35 cells. C, Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. D, Inhibition of miR-520f inhibited the anchorage-independent growth of in A375 and WM35 cells. Representative micrographs (left) and quantification of colonies that were >0.1 mm (right). E, Representative micrographs (left) and quantification (right) of the 5-Bromodeoxyuridine incorporation assay in A375 and WM35 cells. Each bar represents the mean of 3 independent experiments. *P < .05. PCR, polymerase chain reaction.
Itchy E3 Ubiquitin Protein Ligase Upregulation Is Required for MiR-520f-in-Induced Proliferation of Human Melanoma Cells

To further investigate the role of ITCH promotion in miR-520f-in-induced cell proliferation of human melanoma, ITCH siRNAs were employed for ITCH silencing in A375 and WM35 cells with miR-520f-in. Western blot assays indicated that ITCH siRNAs remarkably decreased the expression of ITCH in A375 and WM35 cells with miR-520f-in (Figure 5A). Colony formation assay and anchorage-independent growth assays showed that knockdown of ITCH abolished the inhibitory effects of miR-520f-in on human melanoma cells with increased cell proliferation (Figure 5B and C).

Discussion

Growing number of studies have demonstrated that aberrant expression of miRNAs implicated in the progression of various human cancers. For instance, Long et al found that miR-
219-5p was distinctly reduced in melanoma tissues and cell lines and suppressed the growth and metastasis of melanoma. MicroRNA-9 was reported to suppress the proliferation and migration of malignant melanoma cells. Finding by Fang et al indicated that miR-625 might occupy an irreplaceable role in suppressing proliferation, migration, and invasion in malignant melanoma. Additionally, it was demonstrated by Wu et al that miR-485-5p was significantly downregulated in melanoma was associated with the development and progression of melanoma. Taken together, the above study indicated that miRNAs were implicated in the progression and development of melanoma.

In the current study, the data from the GEO indicated that miR-520f expression in melanoma cells was significantly increased compared to normal cells. Consistent with the results, result of RT-PCR indicated that miR-520f expression was higher in human melanoma cell lines compared to PEM cells, and miR-520f expression was upregulated in human melanoma biopsy tissues that were obtained from patients of our hospital, and then we focused on the functional significance of miR-520f in human melanoma, we performed gain-of-function studies by transfecting miRNA mimics into A375 and WM35 cells. The MTT, colony formation, and the soft agar growth assays demonstrated that

![Image](image_url)

**Figure 5.** ITCH downregulation is required for miR-520f-induced proliferation of human melanoma cells. A, Western blot analysis verified that silencing ITCH effectively decreased the expression of ITCH in miR-520f-in-transfected A375 and WM35 cells. B, miR-520f-in-transfected A375 and WM35 cells after transfection with ITCH-siRNAs promoted cell colonies formation. C, miR-520f-in-transfected A375 and WM35 cells after transfection with ITCH-siRNAs promoted the anchorage-independent growth. Representative quantification of colonies that were >0.1 mm. Each bar represents the mean of 3 independent experiments. *p < .05. ITCH, Itchy E3 ubiquitin protein ligase. siRNA, small interfering RNA.
the ectopic expression of miR-520f significantly suppressed cell viability, colony formation, and the anchorage-independent growth. These findings suggested that miR-520f promoted the proliferation of human melanoma in vitro.

Growing evidences from studies indicated that miRNAs regulate gene expression by binding to the 3′-UTR of target mRNAs. To investigate the role of miR-520f, we searched for miR-520f target genes in human melanoma and identified that ITCH as a potential target. Itchy E3 ubiquitin protein ligase, a vital negative regulator of canonical-Wnt signaling and nuclear factor kappa B signaling, which was involved in inducing aggressiveness of cancer cells. Itchy E3 ubiquitin protein ligase has been indicated to be important regulators of cell growth and apoptosis. Our results showed miR-520f negatively regulates ITCH by directly targeting its 3′-UTR. To further explore the molecular mechanism of growth promotion induced by miR-520f, cell proliferation regulators, such as MYC and cyclin D1 were detected. Following transfection with miR-520f, the expression (mRNA and protein) of cyclin D1 and c-Myc was all markedly upregulated. By contrast, the expression (mRNA and protein) of cyclin D1 and c-Myc was all markedly downregulated by the miR-520f inhibitor.

In summary, our study revealed that the expression of miR-520f was upregulated in human melanoma. Moreover, our study suggested that miR-520f promoted cell growth by inhibiting ITCH. Taken together, our findings provided that a better understanding of the role of miR-520f in the development of human melanoma.

Authors’ Note
All authors designed the study together, performed the experiment together, analyzed the data, and wrote the paper; all authors approved the final manuscript.

Declaration of Conflicting Interests
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