miR-383-5p Inhibits Human Malignant Melanoma via Targeting CENPF

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Research

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Abstract

Background: Human malignant melanoma (MM) is one of the skin cancers with the highest mortality. In this study, we investigated the role of miR-383-5p on human MM cells.

Methods: The expression of miR-383-5p was measured by quantitative real-time PCR assay. The cell proliferation, invasion and migration were detected by CCK8, clone formation and transwell assays. Flow cytometry assay was used to detect apoptosis. The binding of miR-383-5p and 3'UTR of CENPF mRNA was indicated by dual-luciferase assays.

Results: We found that miR-383-5p inhibited the cells proliferation, migration and invasion, and promoted apoptosis of M14 and A375 cells. Biochemical analysis revealed that the expression of miR-383-5p was negatively correlated with CENPF expression in human MM, and the doul-luciferase report showed that miR-383-5p could effectively bind to the 3'UTR of CENPF. CENPF expression was up-regulated and predicted the prognosis of MM. In addition, high expression of CENPF can effectively remedy the resistance of cell proliferation and vitality caused by miR-383-5p.

Conclusion: In conclusion, miR-383-5p acts as a tumor suppressor in human MM by targeting CENPF, suggesting that CENPF may be a potential therapeutic target for human MM.

Introduction

Human malignant melanoma (MM) is a highly malignant tumor with approximately 20,0000 new cases each year in the world(1). And the incidence of MM is increasing(1). According to the stage and severity of the disease, treatment methods are different, including chemotherapy, radiotherapy, immunotherapy and biotherapy(2). However, the current treatment outcomes are not ideal(3). Studying the pathogenesis of MM is essential to improve the efficacy of MM.

MicroRNAs (miRNAs) are a class of endogenous, single-strand, short noncoding RNAs. miRNAs can bind to the 3'-untranslated region (3'UTR) base of the target mRNA to mediate translation inhibition and mRNA degradation(4). It has been reported that miRNAs can regulate the expression of more than 50% of human protein coding genes and play an important role in the biological processes of cell differentiation, metabolism, proliferation, apoptosis, and tumorigenesis(5–7).

CENPF (centromere protein F) can regulate the movement of centromere, its expression changes with the cell cycle(8). CENPF has a wealth of biological functions, including mitotic regulation, microtubule mechanics, gene regulation, myocyte differentiation, histone methylation and so on(9). It was reported that CENPF is highly expressed in a variety of tumors. In some tumors, it is also related to clinical indicators, such as stage and prognosis(10, 11).

In this study, we found that CENPF is highly expressed in MM tissues, and the high expression of CENPF predicted a poor prognosis. In addition, we found that miR-383-5p could directly target CENPF to play the

role of tumor suppressor gene in human MM.

Materials And Methods

Agents

Primers were ordered from Genewiz Company (Beijing, China). Antibodies, including anti-CENPF (Ag29688), anti-GAPDH (10494–1-AP, 1:5,000), and HRP sheep anti-rabbit/mouse (1:5,000) were ordered from PTG Company (Bellevue, WA, USA); Active-Caspase3 (#ab32042, 1:1,000), Bcl-2 (ab32124, 1:1,000), Bax (ab32503, 1:1,000), Cyclin D1 (ab134175, 1:1,000), p-AKT (ab38449, 1:1,000), and p-mTOR (ab109268, 1:1,000) were purchased from Abcam (Cambridge, United Kingdom).

Cell culture and transfection

Human MM cell lines (A375 and M14) were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Science (Shanghai, China) and were cultured in 10% fetal bovine serum (FBS), streptomycin (100 μg/mL), and penicillin (100 U/mL) DMEM medium at 37°C with 5% CO_2. The cells were divided into three groups at the logarithmic growth stage, then transfected with miR-383-5p mimics (miR-383-5p), miR-NC (NC) and miR-383-5p mimics+pcDNA 3.1-CENPF (miR-383-5p+CENPF) by using Lipofectamine 2000 (TMO, Waltham, MA, USA) according to the manufacturer's guidelines. miR-383-5p mimics sequence is 5'-AGAUCAGAAGGUGAUUGUGGCU-3'.

Reverse transcription quantitative PCR (qPCR)

Ultrapure RNA Kit (CwBio, Beijing, China) was performed to isolate the total RNA after 48 h of transfection and cDNA was synthesized using HiFiScript cDNA Synthesis Kit (CwBio, Beijing, China). The expression level of miR-383-5p was calculated by $2^{-\triangle Ct}$ method.

Reverse transcription primer of miR-383-5p: 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAGCCAC-3'.

The PCR primers for miR-383-5p are listed as follows:

Forward: 5'-GGGAGATCAGAAGGTGATTGTGGCT-3'

Reverse: 5'-CAGTGCGTGTCGTGGAGT-3'

The primer sequence of U6 as internal reference is as follows:

Forward: 5'-CTCGCTTCGGCAGCACA-3'

Reverse: 5'-AACGCTTCACGAATTTGCGT-3'

Cell growth assays

Cell Counting Kit-8 (CCK8) (Solarbio, Beijing, China) assay and colony formation assay were carried out to evaluate the proliferation and viability ability of A375 and M14 cells. About 3000 cells were seeded into each well of 96-well-plate. OD values were detected every 24 hours. 10 μl CCK8 solution was added to the well two hours before the detection. Then the growth carve was drawn according the OD values. For the colony formation assay, 500 cells were planted in each well of a 6-well plate, and were cultured for 10 days. Then colonies were fixed with 10% neutral formalin for 1 h and dyed with crystal violet (Beyotime, Haimen, China). The cells were photographed under a microscope (Olympus, Tokyo, Japan).

Cell apoptosis assay

Flow cytometer (BD FACSC anto II, BD Biosciences, San Jose, CA, USA) was used to detect the apoptosis of A375 and M14 cells. After 24 hours of transfection, cells were culture in serum-free medium for 24 hours and collected. The apoptosis rate was analyzed by annexin V/FITC (4A Biotech Company, Beijing, China) according to the instructions. Annexin V/FITC and propidium iodide were used to evaluate the percentage of apoptosis. The flow results were analyzed and processed by Flowjo software.

Cell invasion and migration assays

The invasion and migration ability of A375 and M14 cells were evaluated with transwell chamber (Millipore, Billerica, MA, USA). For invasion assay, the frozen matrigel (356234, BD, Franklin Lakes, NJ, USA) was diluted to 1:6 in serum-free medium, and then the 40 μl matrigel was applied to the upper chamber. The 150 μl serum-free medium and 1×10^4 cells were added to the upper chamber, 500 μl complete medium was added to the lower chamber. After 24 hours of incubation, the surface of each upper chamber was wiped gently with a cotton swab. The cells of lower chamber were washed with PBS and fixed with 4 % PFA for 30 min, then stained with 0.1 % crystal violet for 20 min. For migration assay, there are no matrigel coating and the rest was the same as the invasion assay.

Luciferase reporter assay

Based on bioinformatics, we predicted a complementary relationship between miR-383-5p and 3'UTR of CENPF mRNA. The mutation vector of CENPF 3'UTR was constructed by point mutation. miR-383-5p mimics and CENPF 3'UTR wild type (WT), miR-NC and CENPF 3'UTR wild type (WT), miR-383-5p mimics and mutant vector (Mut), miR-NC and mutant vector (Mut) were transfected into A375 and M14 respectively. 48 hours later, the cells were collected and the luciferase activity was detected with the doul-luciferase reporter gene assay kit (Beyotime, China) according to the instructions.

Western blot assays

The protein of cells was extracted with RIPA (protease inhibitor, CwBio, Beijing, China) buffer. 20 μg protein samples were added into each lane containing 10% SDS-PAGE gel. After transfer, the polyvinylidene fluoride (PVDF) membrane was sealed with 5 % skimmed milk for 1 hour and probed with primary and secondary antibodies. After washing the membrane, ECL developer (B500024, Proteintech, Chicago, IL, USA) was added and Quantity One Software was used to assess the results.

Statistical analyses

The data were analyzed using SPSS software. Each experiment was repeated at least three times. The results were presented as mean ± SD. Differences between two groups were tested by Student's t-test, differences among more than two groups were tested by one-way ANOVA. $P<0.05$ was considered significant difference.

Results

miR-383-5p inhibits human MM cell proliferation, migration and invasion

Firstly, we analyzed the expression level of miR-383-5p between human normal melanocytes (HEM) and MM cell lines (uacc62, M14, M21, and A375) with qPCR. As the results of Fig. 1A, the expression level of miR-383-5p was down-regulated in MM cell lines compared with HEM. Then, we chose A375 and M14 cell lines for further study. A375 and M14 cells were transfected with miR-383-5p mimic (miR-383-5p group) or miR-NC (NC group) to investigate the role of miR-383-5p in human MM. The transfection efficiency was detected with qPCR. After transfection of miR-383-5p mimic, the expression level of miR-383-5p was significantly up-regulated (Fig. 1B, $P < 0.05$). CCK8 and colony analysis showed that miR-383-5p inhibited the proliferation and growth ability of A375 and M14 cells (Fig. 1C and D). In order to further elucidate the mechanism of miR-383-5p resistance in MM cells, apoptosis was analyzed. As shown in Fig. 2A, miR-383-5p promoted the cell apoptosis ($P < 0.05$). In addition, miR-383-5p inhibited the migration and invasion ability of A375 and M14 cells (Fig. 2B, $P < 0.05$).

miR-383-5p directly targets CENPF in MM cells

Furthermore, Starbase v3.0 online database was used to analyzed the co-expression of CENPF and miR-383-5p in human MM. As shown in Fig. 3A, the expression of miR-383-5p in skin cutaneous melanoma (SKCM) tissues was negatively correlated with CENPF expression. It has been reported that miRNAs can mediate translation inhibition and mRNA degradation by pairing with the 3’-untranslated region (3’UTR)

base of the target mRNA. Therefore, miR-383-5p was predicted to bind to the 3'UTR of CENPE to reduce the expression of CENPF in SKCM through Starbase v3.0 database (Fig. 3B). The results of dual-luciferase reporter showed that the fluorescence intensity of the co-transfection group of miR-383-5p mimics and CENPF-WT was significantly lower than that of other co-transfection groups (Fig. 3B). Furthermore, the results of western blot showed that miR-383-5p inhibited the protein expression of CENBP (Fig. 3C). These results suggested that miR-383-5p could promote the translation inhibition and mRNA degradation of CENPF through base complementary.

CENPF expression is up-regulated and predicts the prognosis of MM

The expression patten of CENPF in SKCM tissues was analyzed basing on gene expression profiling interactive analysis (GEPIA). As shown in Fig. 3D, the expression of CENPF was significantly up-regulated in human SKCM tissues than that in normal tissues. Moreover, we analyzed the overall survival rate (OS) of SKCM patients with higher or lower expression level of CENPF. The results showed that the high expression level of CENPF predicted a poor prognosis (Fig. 3E).

Upregulation of CENPF reverses the effects of miR-383-5p on proliferation and migration

To further study the interaction between miR-383-5p and CENPF on MM cells, the pcDNA 3.1-CENPF and miR-383-5p mimics (miR-383-5p + CENPF) were transfected into A375 and M14 cells. As shown in Fig. 4F and G, the recovery of CENPF expression reversed the inhibitory effect of miR-383-5p on the proliferation and migration of MM cells. Taken together, CENPF could remedy the proliferation and migration resistance of MM cells induced by miR-383-5p.

Discussion

As an oncogene or tumor suppressor, miRNA provides new regulatory mechanisms(12) based on the biological function of target genes, and can be used for the classification and prognosis prediction of cancer(13). According to reports, miR-383-5p acts as a tumor suppressor in a variety of human cancers by modulating multiple target genes(14–18). Many studies have shown that the expression of miR-383-5p is down-regulated in various human cancer, and the reduction of miR-383-5p is considered a potential biomarker for metastasis and poor prognosis(14–18). In addition, miR-383-5p inhibits cell proliferation and mobility of various cancer cells, and induces apoptosis(14–18). Furthermore, the over-expression of miR-383-5p enhances the chemosensitivity of ovarian cancer cells(19) and prompts a better response outcome in sorafenib targeting therapy for hepatocellular carcinoma cells(20). However, nothing is known about the role of miR-383-5p in MM.

In this study, we found that the over-expression of miR-383-5p inhibited the proliferation, migration and invasion of M14 and A375 cells, and induced cell apoptosis. Our results are consistent with previous

studies that miR-383-5p functions as a tumor suppressor.

TargetScan 7.1 database was used to predict the target gene of miR-383-5p, and CENPF as one of the target genes attracted our attention. Recent studies have revealed that the high expression of CENPF promotes cancer cell mitosis and cell cycle(21). The dual-luciferase reporter was carried out to verify whether CENPF is the direct target of miR-383-5p in human MM cells. The results showed that miR-383-5p could effectively bind to the 3'UTR of CENPF, indicating that miR-383-5p has direct interaction with CENPF. CCK8 and transwell experiments proved that high expression of CENPF can effectively remedy the resistance of cell proliferation and vitality caused by miR-383-5p.

Conclusion

In summary, the expression of miR-383-5p was negatively correlated with the expression of CENPF in human MM, and miR-383-5p regulated the development of human MM by targeting CENPF, which provides a new strategy for the treatment of human MM.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

FC analyzed and interpreted the patient data regarding the hematological disease and the transplant. RH performed the histological examination of the kidney, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Figures

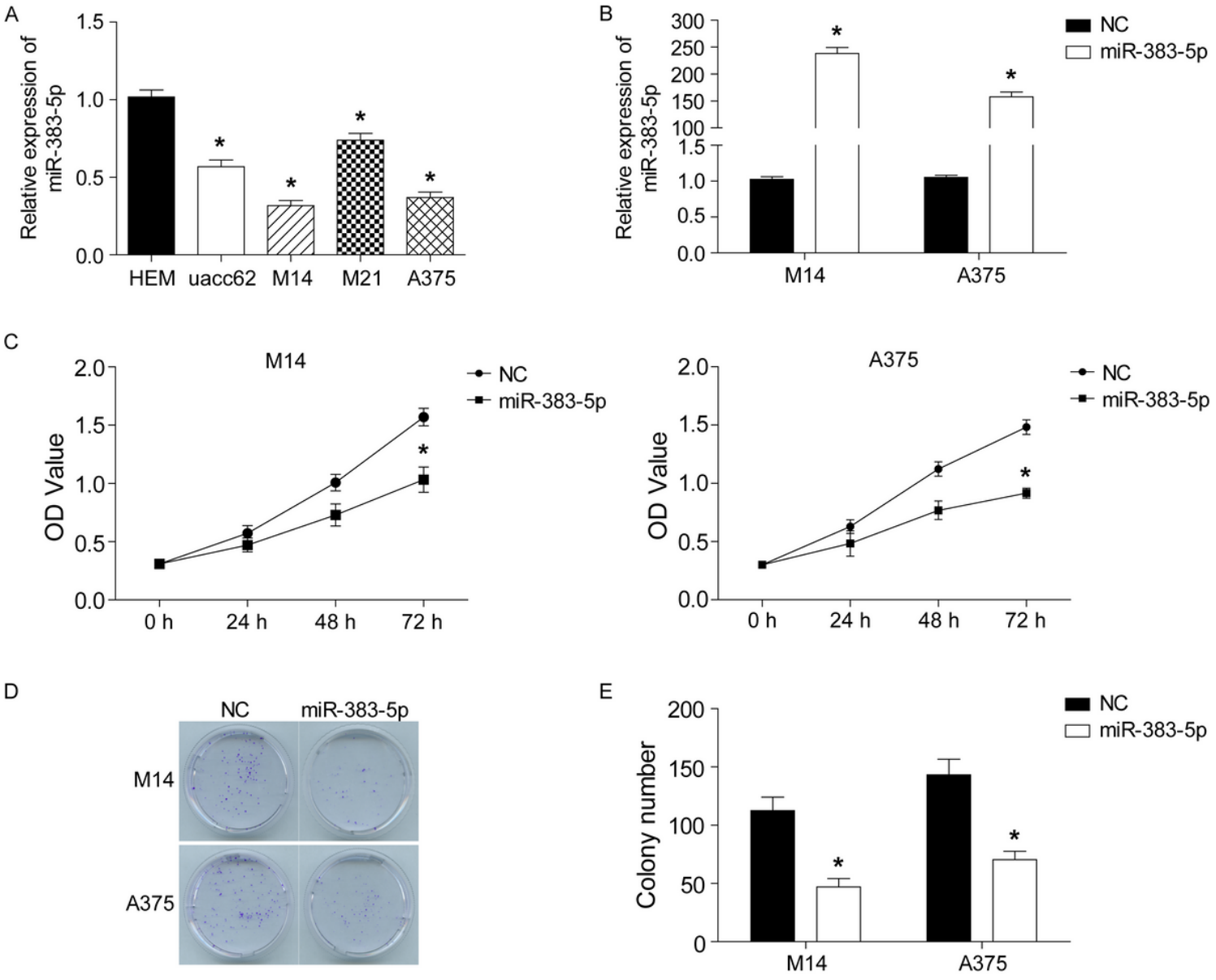

Figure 1

miR-383-5p inhibits human MM cell proliferation. Notes: (A) The expression of miR-383-5p in four human melanoma cell lines, uacc62, M14, M21, and A375, and in one human normal melanocyte cell line, HEM. (B) The expression level of miR-383-5p was evaluated with qPCR. CCK8 assay (C) and plate clonality assay (D) was performed to assess the cell proliferation of M14 and A375 cells. *P<0.05.

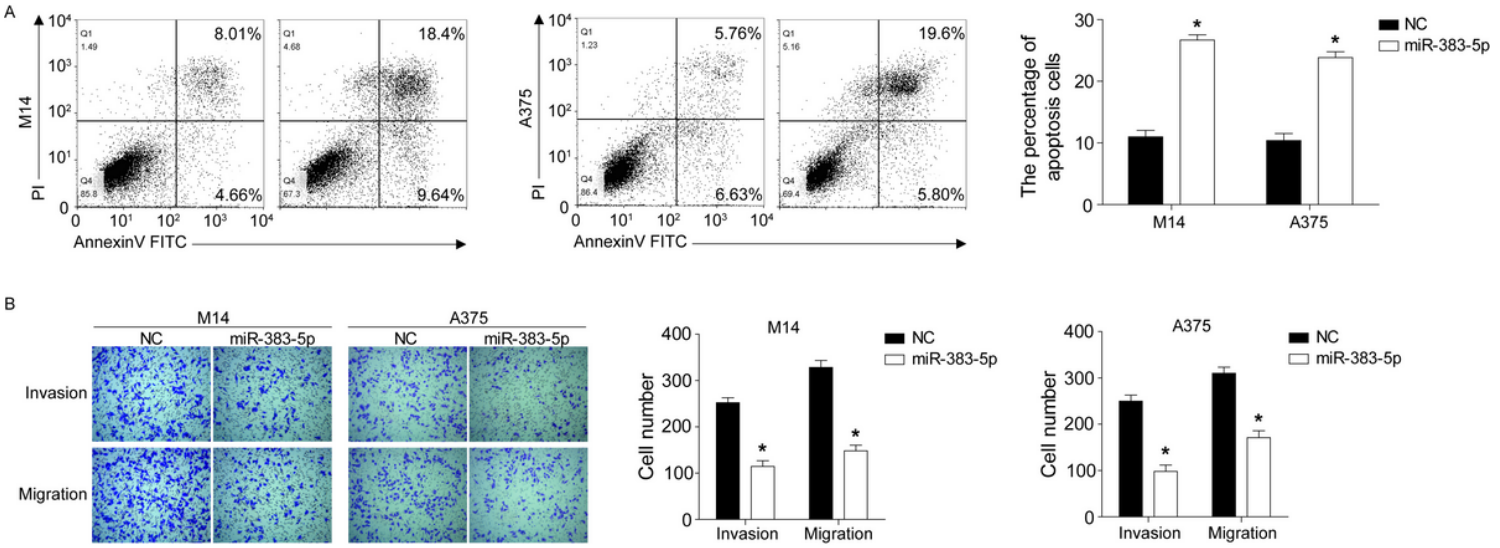

Figure 2

miR-383-5p induces apoptosis and inhibits proliferation of human MM cell. Notes: (A) Flow cytometric analysis was performed to estimate the apoptosis of A375 and M14 cells. (B) The invasion and migration of A375 and M14 was detected by transwell assay. *P<0.05.

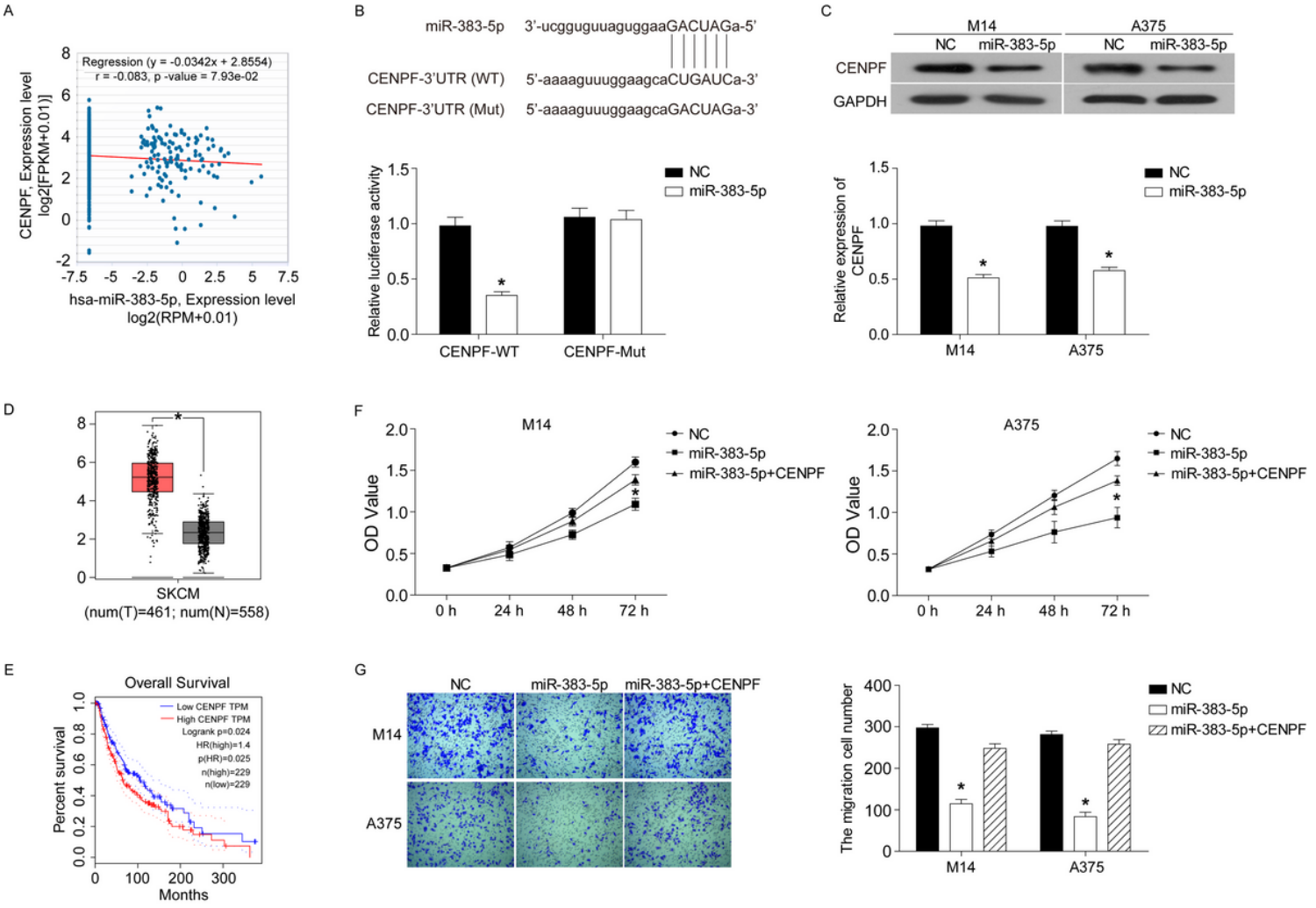

Figure 3

Upregulation of CENPF reverses the effects of miR-383-5p on proliferation and migration. Notes: (A) The co-expression of CENPF and miR-383-5p in skin cutaneous melanoma (SKCM) tissues was assessed by using the starbase v3.0 online database. (B) upper line: The binding site of miR-383-5p on 3'UTR of CENPF; lower line: Dual-luciferase reporter analysis was performed in M14 cells. (C) The expression level of CENPF was evaluated with western blotting. (D) CENPF expression was significantly increased in SKCM tumor tissues compared with normal skin tissues, which from GEPIA. (E) The survival curve showed that SKCM patients with low CENPF level had better OS than those with high CENPF level. (F) Cell proliferation ability was detected by CCK8 assay. (G) Cell migration ability was assessed by transwell assay. *P<0.05.