

MiR-497-5p, miR-195-5p and miR-455-3p function as tumor suppressors by targeting hTERT in melanoma A375 cells

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Background: *hTERT* gene plays an important role in melanoma, although the specific mechanism involved is unclear. The aim of this study was to screen and identify the relative miRNAs with the regulation of hTERT in melanoma.

Materials and methods: Quantitative real-time polymerase chain reaction (q-PCR) and immunohistochemistry were performed to detect hTERT mRNA and protein expression in 36 formalin-fixed paraffin-embedded melanoma tissues and 36 age- and sex-matched pigmented nevi cases, respectively. Bioinformatics analysis and custom miRNA polymerase chain reaction array were determined for predicting, screening and verifying miRNAs with the regulation of the *hTERT* gene. To investigate the biological functions, miRNAs mimics or inhibitors were transfected into melanoma A375 cells. The relative expression of miR-497-5p, miR-195-5p, miR-455-3p and hTERT mRNA was determined by q-PCR. The protein expression of hTERT was detected by Western blot. 3-(4,5-Dimethylthiazolyl-2-yl)-2,5-biphenyl tetrazolium bromide and flow cytometry were employed to detect cell proliferation ability, cell apoptosis and cell cycle. Transwell and wound healing assays were used to observe cell invasion and migration abilities. A direct target gene of miRNAs was analyzed by a dual luciferase reporter activity assay.

Results: MiR-497-5p, miR-195-5p, miR-455-3p were significantly downregulated, while hTERT was upregulated in melanoma tissues. hTERT expression level was inversely correlated with miR-497-5p, miR-195-5p and miR-455-3p. Overexpression of miR-497-5p, miR-195-5p and miR-455-3p inhibited A375 cell proliferation, migration and invasion, arrested the cell cycle, induced cell apoptosis and decreased hTERT expression at both mRNA and protein levels. Suppression of miR-497-5p, miR-195-5p and miR-455-3p partially reversed the inhibitory effects. Finally, hTERT was identified as a direct target of miR-497-5p, miR-195-5p and miR-455-3p.

Conclusions: MiR-497-5p, miR-195-5p and miR-455-3p act as tumor suppressors by targeting hTERT in melanoma A375 cells. Therefore, miR-497-5p, miR-195-5p and miR-455-3p could be potential targeted therapeutic choice for melanoma.

Keywords: melanoma, miR-497-5p, miR-195-5p, miR-455-3p, hTERT

Introduction

Malignant melanoma is a highly malignant skin tumor, which originated mainly from epidermal melanocytes cells.¹ The incidence of this disease is increasing each year.² The tumorigenesis and progression of melanoma is a complex and multistep process that results from interactions between genetics and environmental factors.³ Surgical resection is an effective treatment for early stage melanoma patients, but it becomes extremely difficult to treat this disease when it progresses to the late stages.⁴ The metastasis of melanoma is the most important factor affecting its prognosis.⁵ The overall

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survival rate of metastatic melanoma patients with different stages has been improved to some extent, compared with the seventh edition of American Joint Committee on Cancer (AJCC).⁶ Although there has been significant progress in the treatment of melanoma in recent years, the current application of chemotherapy, immunotherapy and molecular biological targeting therapy, and even combined therapy, still faces enormous challenges.

The *hTERT* gene, which encodes the catalytic subunit of telomerase, is the main factor determining telomerase activity.⁷ Its expression is involved in the process of cell immortalization and cancer tumorigenesis, growth, migration, invasion and prognostic evaluation, although the underlying mechanism remains unclear.⁸ In 2013, Horn et al⁹ and Huang et al¹⁰ reported highly recurrent *hTERT* promoter mutations in melanoma. Subsequent studies have pointed out the presence of recurrent somatic mutations in the *hTERT* promoter in cancers of the central nervous system (43%), bladder (59%), thyroid (follicular cell derived, 10%) and skin (melanoma, 29%).¹¹ All of these results suggested that the *hTERT* gene plays a vital role in the occurrence and progression of melanoma. Hence, it is necessary to study the posttranscriptional regulation of the *hTERT* gene further.

MiRNAs are a class of small noncoding RNAs with ~22–24 nucleotides.¹² The complementary combination of miRNAs and the 3'UTR region of its targeted mRNA leads to mRNA degradation or protein translation inhibition.¹² miRNAs participate in the regulation of approximately a third of the human genome, including cell proliferation, differentiation, metabolism, migration and invasion.¹³ Abundant studies showed that ectopic miRNA expression was involved in the diagnosis, treatment and prognosis of melanoma.^{14–20} MiRNAs can act as oncogenes or anti-oncogenes in different types of cancer, including melanoma.^{21–23} However, the posttranscriptional regulation mechanism of *hTERT* and its related miRNAs in melanoma is not clear. The aim of this study is to screen the relative miRNAs with the posttranscriptional regulation of *hTERT* and investigate the detailed molecular regulatory mechanism between them.

Materials and methods

Human tissue samples

Thirty-six FFPE melanoma tissues and 36 age- and sex-matched pigmented nevi tissues (Table S1) were collected from Uyghur patients with melanoma and pigmented nevi

(during 2010 and 2016). This was approved by the Institutional Review Board of People's Hospital of Xinjiang Uygur Autonomous Region. The diagnosis of melanoma was made by two pathologists (Xinjiang, China) on the basis of H&E stain and IHC.

Bioinformatics analysis

We scanned the 3'UTR region of *hTERT* using the widely used online target gene prediction software program TargetScan (http://www.targetscan.org/vert_71/). The screening criteria were as follows:

1. Conserved sites and poorly conserved sites. We chose miRNAs both in conserved sites and in poorly conserved sites, because more and more functional miRNAs have been found in both conserved and poorly conserved sites.
2. Seed match. There are three types of seed match: 7mer-1a (match seed, UTR region and miRNA 1nt match position is A), 7mer-m8 (match miRNA 2-8nt) and 8mer (match miRNA 2-8, UTR region and miRNA 1nt match position is A). Generally, the extent of contribution of these three types is: 8mer>7mer-m8>7mer-1a. We filtered the relevant miRNAs according to the degree of contribution in descending order.
3. MiRBase database. We removed the miRNA which was not encoded in the miRBase database. Taking the intersection of TargetScan prediction and screening criteria as the final candidate miRNAs for miRNA PCR array.

RNA extraction

For FFPE tissues, miRNAs and mRNA were extracted by using an miRNeasy FFPE Kit (50) (Qiagen NV, Venlo, the Netherlands) in accordance with the manufacturer's protocol. For A375 cell lines, total RNA was extracted by Trizol (Thermo Fisher Scientific, Waltham, MA, USA). The concentrations were determined by 260/280 nm absorbance using a Nanodrop UV spectrophotometer (Thermo Fisher Scientific).

MiRNA PCR array

Two µL of each RNA sample was used for cDNA synthesis using the miScript II RT kit (Qiagen) according to the manufacturer's instructions. The final candidate miRNAs were quantified by means of Custom miScript miRNA PCR Array (Qiagen) performed with an ABI PRISM 7900 Sequence Detection System (Thermo Fisher Scientific) by Qiagen

(Suzhou) Translational Medicine Co., Ltd (Suzhou, China). All of the specific primers for the miRNA PCR array were synthesized by Qiagen. Results are presented as the levels of expression following normalization to human U6 small nuclear RNA using the $2^{-\Delta\Delta Ct}$ method.²⁴

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

MiR-497-5p, miR-195-5p, miR-455-3p and hTERT mRNA expressions levels were quantified by miScript SYBR Green PCR Kit (Qiagen). Approximately 3 μ g of total RNA was used in complementary DNA (cDNA) synthesis. Then, 2 μ L of cDNA was used for PCR amplification. The PCR reaction conditions were: 10 min at 95°C for one cycle, and 30 s at 95°C then 30 s at 60°C for 40 cycles. The primer sequences used are listed in [Table S2](#). Results are presented as the levels of expression following normalization to human U6 small nuclear RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) respectively, using the $2^{-\Delta\Delta Ct}$ method.²⁴

Immunohistochemistry (IHC)

Dewaxed and rehydrated sections (4 μ m) were oxidized for 5 min in 0.5% potassium permanganate solution, and then bleached for 5 min in 2% oxalic acid solution. Depigmentation was observed microscopically. Then dewaxed and rehydrated sections (4 μ m) in 10 mmol/L sodium citrate buffer (pH 6.0) were boiled in a microwave for antigen retrieval. IHC for detection of hTERT was performed using a DAB Chromogenic Kit (ZSGB-BIO, Beijing, China) and a 1:100 dilution of a polyclonal rabbit anti-human hTERT antibody (Abcam, Cambridge, MA, USA). The hTERT staining procedure produced brown cytoplasmic particles in the hTERT-positive cells. The proportion of hTERT-positive melanoma cells was scored as: <5%=0 (negative), 5%–50%=1 (+), 51%–75%=2 (++) and >75%=3 (+++). Negative controls were conducted by replacing the primary antibody with phosphate buffered saline (PBS, ZSGB-BIO).

Cell lines and cell transfection

Human melanoma A375 cells, M14 cells and HEK 293T cells were all purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Thermo Fisher Scientific), supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μ g/mL streptomycin (Thermo Fisher Scientific), at 37°C in a 5% CO₂ incubator. MiR-497-5p mimics, miR-

497-5p inhibitor, miR-195-5p mimics, miR-195-5p inhibitor, miR-455-3p mimics, miR-455-3p inhibitor, mimics negative control (mimics NC) and inhibitor negative control (inhibitor NC) were synthesized and purchased from GenePharma Co, Ltd (Shanghai, China). The sequences are listed in [Table S3](#). Human melanoma A375 cells with the concentration of 2×10^5 cells per well were seeded in 6-well plates and cultured for 24 h. Then miRNAs mimics/inhibitors/NC (50 nM) were transfected into the A375 cells using Lipofectamine 2000 (Thermo Fisher Scientific) in serum-free medium according to the manufacturer's instructions.

Cell proliferation assays

Cell proliferation assay was determined by using 3-(4,5-dimethylthiazolyl-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) in accordance with the manufacturer's instructions. MTT was purchased from Boster Biological Technology Co. Ltd (Wuhan, China). 5×10^4 cells per well with/without miRNAs mimics/inhibitors/NC were seeded into 96-well plates and cultured for 48 h. Ten μ L MTT solutions (5 mg/mL) was added into each well and incubated at 37°C in a 5% CO₂ incubator for 4 h. Then 150 μ L DMSO (Sigma-Aldrich Co., St Louis, MO, USA) was added into each well. The optical density values (OD values) at 570 nm were measured with a microplate reader (Thermo Fisher Scientific). All experiments were performed in triplicate.

Cell cycle and apoptosis assays

After 48 h of transfection, 2×10^5 cells were harvested, centrifuged for 5 min at 1,000 rpm, and washed three times by cold PBS liquid. Cell proliferation was performed by a Cell Cycle Detection Kit (KeyGen BioTECH, Nanjing, China). The A375 cells with/without miRNAs mimics/inhibitors/NC were fixed with 70% ethanol. After incubation for 30 min with RNase (50 μ g/mL), propidium iodide staining of cells protected from light at 4°C for 30 min. Cell cycle status was measured by flow cytometry (Beckman Coulter, Brea, CA, USA). For the cell apoptosis assay, after transfection for 48 h, 2×10^5 cells were harvested, centrifuged at 1,000 rpm for 5 min, and washed three times by PBS solution. Then Annexin V-APC/7-AAD Cell Apoptosis Detection Kit (KeyGen BioTECH) was used according to the manufacturer's instructions. Quantity of apoptotic cells was carried out by flow cytometry (Beckman Coulter). Annexin V-APC+/7-AAD- and Annexin V-APC+/7-AAD+ represented early apoptosis and late apoptosis/necrosis, respectively. All of the experiments were performed in triplicate.

Cell migration and cell invasion assays

Wound healing assay was performed to examine the migration ability of cells. Ten μL pipette tips were used to make scratches when transfected cells reached 90% confluence. Scratches were monitored by the microscope (Olympus Corporation, Tokyo, Japan) at different time points (0 h, 24 h). Transwell chamber with 8 μm pore size, precoated with Matrigel (BD Biosciences, San Jose, CA, USA) was used to determine cells' invasion ability. For this, 2×10^4 cells per well were added to the upper chamber of the insert with serum-free medium, and 600 μL DMEM supplemented with 10% FBS was added to the lower chamber. The cells were cultured for 24 h at 37°C in a 5% CO_2 incubator. Then noninvading cells were removed by cotton swab. The lower chamber of the insert was washed three times by PBS (Thermo Fisher Scientific), fixed with 100% ethanol for 30 min, and stained with 0.1% crystal violet (Sigma-Aldrich) for 10 min. The numbers of invaded cells were counted in five randomly visual fields by a microscope (Olympus Corporation).

Western blot

Transfected cells were treated with radio immunoprecipitation assay peptide lysis buffer supplemented with 1% protease inhibitor–phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology, Jiangsu, China). Total protein was concentrated by the BCA Protein Assay Kit (Beyotime Institute of Biotechnology), separated by sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE, Thermo Fisher Scientific), and transferred to polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked by 1×TBST solution containing 5% nonfat milk for 1 h and incubated with primary antibody–polyclonal rabbit anti-human hTERT antibody (1:1,000, Abcam), GAPDH (1:1,000, Abcam) overnight at 4°C. The membranes were washed three times by 1×TBST solution, and then incubated with secondary antibody–goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (1:5,000, ZSGB-BIO) for 2 h at room temperature. The bands were visualized using an enhanced chemiluminescence solution (Thermo Fisher Scientific). All of the experiments were performed in triplicate.

Plasmid construction and luciferase activity assay

To generate the luciferase reporter plasmid, the wild type and mutant type of hTERT 3'UTR were amplified and constructed into pYr-mirTarget vector (Biofavor Biotech Co Ltd, Wuhan, China). Then the plasmids (1 μg) and miR-497-5p or miR-195-5p or miR-455-3p mimics, mimics NC (50 nM) were cotransfected into HEK 293T cells (5×10^4)

using Lipofectamine 2000 (Thermo Fisher Scientific). At 48 h posttransfection, luciferase activity was determined using a dual-luciferase assay system (Promega Corporation, Fitchburg, WI, USA) according to the manufacturer's instructions. All experiments were performed in triplicate.

Statistical analysis

Statistical calculations were performed with IBM SPSS software, version 23.0 (IBM Corporation, Armonk, NY, USA). All data were shown as mean \pm SD. Any differential expressions between groups were determined by independent samples *t*-test. The mRNA expression correlation between miR-497-5p, miR-195-5p, miR-455-3p and hTERT were measured by Spearman's correlation analysis. All of the tests were performed as two-tailed and the level of significance was set as $p < 0.05$.

Ethics statement

This study was approved by the Institutional Review Board of People's Hospital of Xinjiang Uygur Autonomous Region. Formalin-fixed paraffin-embedded (FFPE) tissues were collected from patients after obtaining written informed consent.

Results

MiRNA prediction and screening

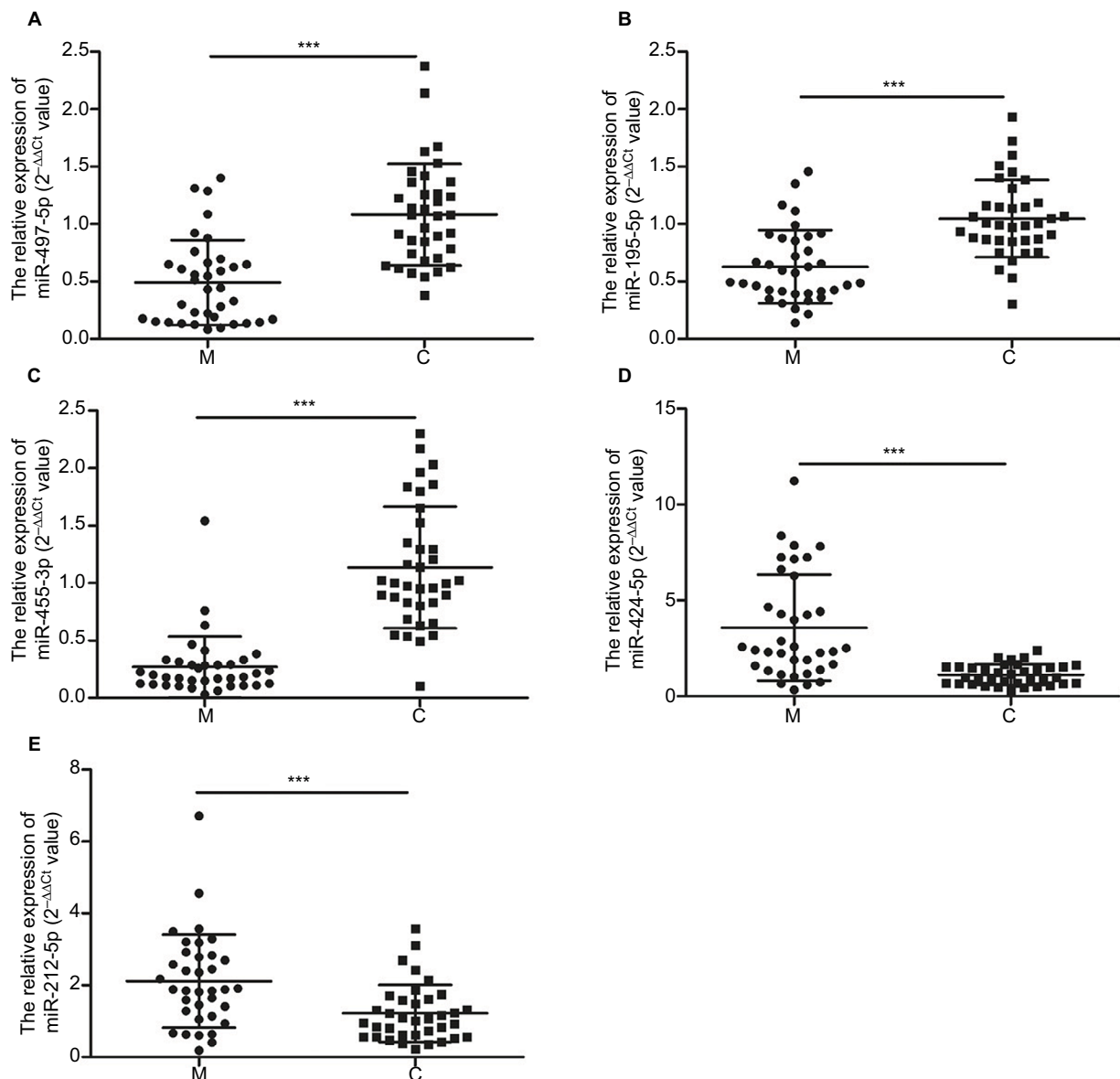
Using TargetScan software, we found 1,347 predicted target miRNAs related to the hTERT gene (http://www.targetscan.org/cgi-bin/targetscan/vert_71/view_gene.cgi?rs=ENST0000296820.5&taxid=9606&members=&showcnc=0&shownc=0&showncf1=&showncf2=&subset=1). After the intersection of screening criteria and TargetScan prediction, 14 candidate miRNAs were screened out, and the details are shown in Table 1.

Confirmation of 14 miRNAs in melanoma tissues with custom miRNA PCR array

The 14 candidate miRNAs were further validated by a custom miScript miRNA PCR array through SYBR Green q-PCR. Ct values of the positive PCR controls were 19 ± 2 across all tissue samples, indicating the uniformity of reaction conditions. As shown in Figure 1, the expression level of miR-497-5p, miR-195-5p and miR-455-3p were statistically significantly downregulated, whereas miR-424-5p and miR-212-5p were statistically significantly upregulated in the melanoma individuals compared with pigmented nevi controls. There was no significant difference in the levels of miR-15a-5p, miR-15b-5p, miR-16-5p, miR-103a-3p, miR-107, miR-143-3p, miR-338-3p, miR-665 and miR-1306-5p (Table S4). The melt curve images are shown in Figure S1.

Table 1 Information about the 14 candidate miRNAs

No.	Encoding in miRBase database	Name	Position	Site type
1	MIMAT0001341	hsa-miR-424-5p	192-198/2565-2571/2568-2574	7mer-m8
2	MIMAT0002820	hsa-miR-497-5p	192-198/2565-2571/2568-2574	7mer-m8
3	MIMAT0000461	hsa-miR-195-5p	192-198/2565-2571/2568-2574	7mer-m8
4	MIMAT0000069	hsa-miR-16-5p	192-198/2565-2571/2568-2574	7mer-m8
5	MIMAT0000068	hsa-miR-15a-5p	192-198/2565-2571/2568-2574	7mer-m8
6	MIMAT0000417	hsa-miR-15b-5p	192-198/2565-2571/2568-2574	7mer-m8
7	MIMAT0000763	hsa-miR-338-3p	195-201/1639-1645	7mer-1A
8	MIMAT0000104	hsa-miR-107	411-417/441-447	7mer-1A
9	MIMAT0000101	hsa-miR-103a-3p	411-417/441-447	7mer-1A
10	MIMAT0000435	hsa-miR-143-3p	498-504/2083-2089/2096-2102/2114-2120/2186-2192	7mer-m8
11	MIMAT0022695	hsa-miR-212-5p	566-572/990-996	7mer-m8
12	MIMAT0022726	hsa-miR-1306-5p	214-220/1299-1305/1865-1871	7mer-m8
13	MIMAT0004952	hsa-miR-665	668-674/783-789	7mer-m8
14	MIMAT0004784	hsa-miR-455-3p	786-793	8mer

**Figure 1** The relative expression levels of miR-497-5p (A), miR-195-5p (B), miR-455-3p (C), miR-424-5p (D) and miR-212-5p (E) in melanoma and pigmented nevi group tissues, respectively.**Note:** *** $p < 0.001$, compared to control groups.**Abbreviations:** M, melanoma group; C, control (pigmented nevi group).

hTERT has an inverse correlation with miR-497-5p, miR-195-5p and miR-455-3p

We determined hTERT mRNA and protein levels in melanoma and pigmented nevi tissues by q-PCR and IHC. The results showed that the hTERT protein level in the melanoma group (31/36) was significantly higher than in the control (pigmented nevi group) (4/36), ($p < 0.001$, Figure 2A). The relative expression of hTERT mRNA in melanoma and pigmented nevi tissues was 1.962 ± 0.509 and 1.170 ± 0.430 , respectively ($p < 0.001$, Figure 2B). The expression of hTERT mRNA was inversely correlated with miR-497-5p (Figure 2C), miR-195-5p (Figure 2D) and miR-455-3p (Figure 2E).

Transfection efficiency

The hTERT protein levels in A375 and M14 cells were determined by Western blot. The expression of hTERT protein in A375 cells (0.520 ± 0.064) was higher than in M14 cells (0.259 ± 0.039) (Figure 3A). Due to this result, A375 cells were chosen for further studies. To investigate the role of miR-497-5p, miR-195-5p and miR-455-3p on the biological functions of cells, miR-497-5p mimics, miR-497-5p inhibitor, miR-195-5p mimics, miR-195-5p inhibitor, miR-455-3p

mimics, miR-455-3p inhibitor, mimics NC and inhibitor NC were transfected into A375 cells. The q-PCR results showed the high transfection efficiency of miRNAs mimics and inhibitors (Figure 3B–D).

Overexpression of miR-497-5p, miR-195-5p and miR-455-3p suppressed cell proliferation

Forty-eight hours after transfection, we used MTT to evaluate the effects of miR-497-5p, miR-195-5p and miR-455-3p on A375 cell proliferation. The results showed that overexpression of miR-497-5p, miR-195-5p and miR-455-3p significantly suppressed the growth of cells (Figure 4). Additionally, the inhibitory effects were partially reversed after miR-497-5p/ miR-195-5p/ miR-455-3p inhibitor transfection (Figure 4).

Overexpression of miR-497-5p, miR-195-5p and miR-455-3p arrested cell cycle and promoted cell apoptosis

Flow cytometry was performed to detect the cell cycle distribution and cell apoptosis. As shown in Figure 5A–C, the

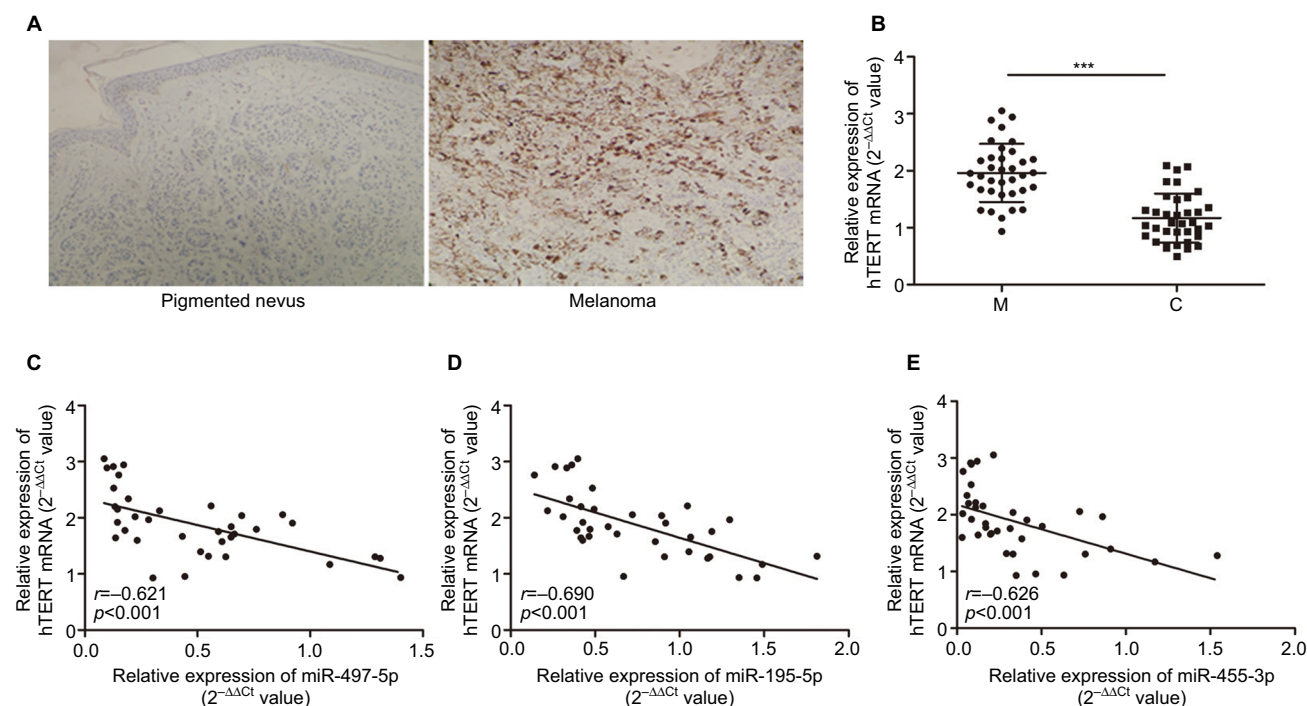


Figure 2 The relative expression of hTERT and miR-497-5p, miR-195-5p and miR-455-3p.

Notes: (A) hTERT protein expression in melanoma and pigmented nevus tissues (magnification, $\times 100$). (B) hTERT mRNA expression in melanoma and pigmented nevus tissues. hTERT mRNA expression level was inversely correlated with miR-497-5p (C), miR-195-5p (D) and miR-455-3p (E).

Note: *** $p < 0.001$, compared to control groups.

Abbreviations: hTERT, human telomerase reverse transcriptase; M, melanoma group; C, control (pigmented nevi group).

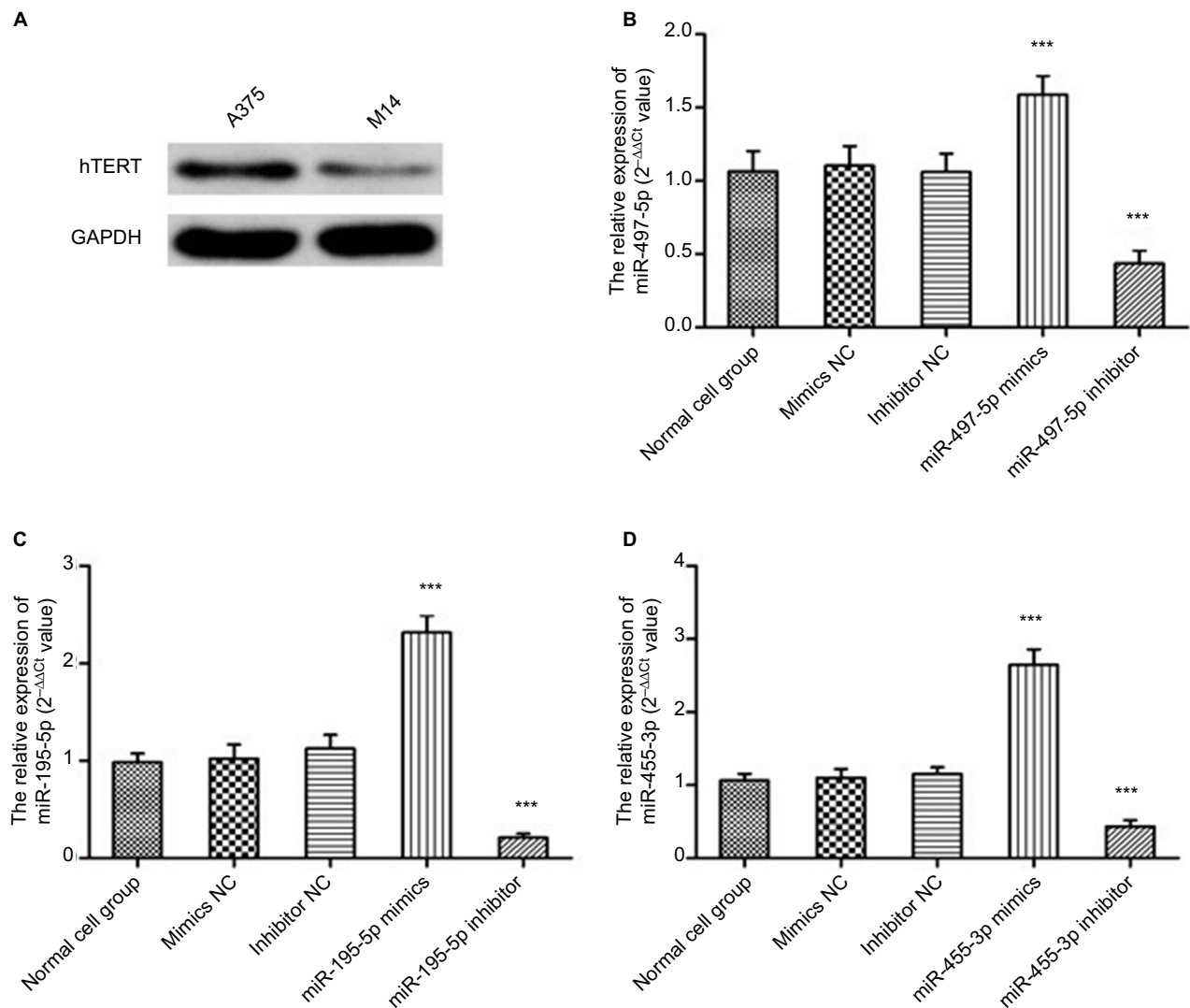


Figure 3 hTERT protein expressions levels in melanoma cell lines and transfection efficiency.

Notes: (A) hTERT protein expressions in A375 cells and M14 cells were detected by Western blot. The expression levels of miR-497-5p (B), miR-195-5p (C), and miR-455-3p (D) after transfection were determined by q-PCR. All data are presented as mean±SD, and all of these experiments were performed in triplicate. ***p<0.001, compared to control groups.

Abbreviations: hTERT, human telomerase reverse transcriptase; NC, negative control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; q-PCR, quantitative real-time polymerase chain reaction.

percentage of G0/G1 phase cells in overexpression of miR-497-5p/ miR-195-5p/miR-455-3p groups was significantly higher than in the NC groups, while S phase and G2/M phase cells were decreased. In addition, the arrest effects were partially reversed by transfecting miR-497-5p/miR-195-5p/miR-455-3p inhibitors. The cell cycle histograms are shown in [Figure S2](#). For cell apoptosis, the apoptosis rates of miR-497-5p/miR-195-5p/miR-455-3p mimic groups were significantly increased compared with NC groups (Figure 5D–F). Moreover, miR-497-5p/miR-195-5p/miR-455-3p inhibitors significantly decreased the apoptosis rate of cells (Figure 5D–F).

Overexpression of miR-497-5p, miR-195-5p and miR-455-3p inhibited cell migration and invasion abilities

Wound healing assay results showed that the mobility of A375 cells was significantly suppressed after overexpression of miR-497-5p, miR-195-5p and miR-455-3p (Figure 6A–C). However, suppression of miR-497-5p, miR-195-5p and miR-455-3p did not increase the cells' mobility significantly. The results of Transwell chambers displayed that the numbers of invasion cells were significantly reduced in the mimic groups compared to NC groups (Figure 6D–F). Furthermore, for the invasion ability, suppression of miR-497-5p, miR-195-5p

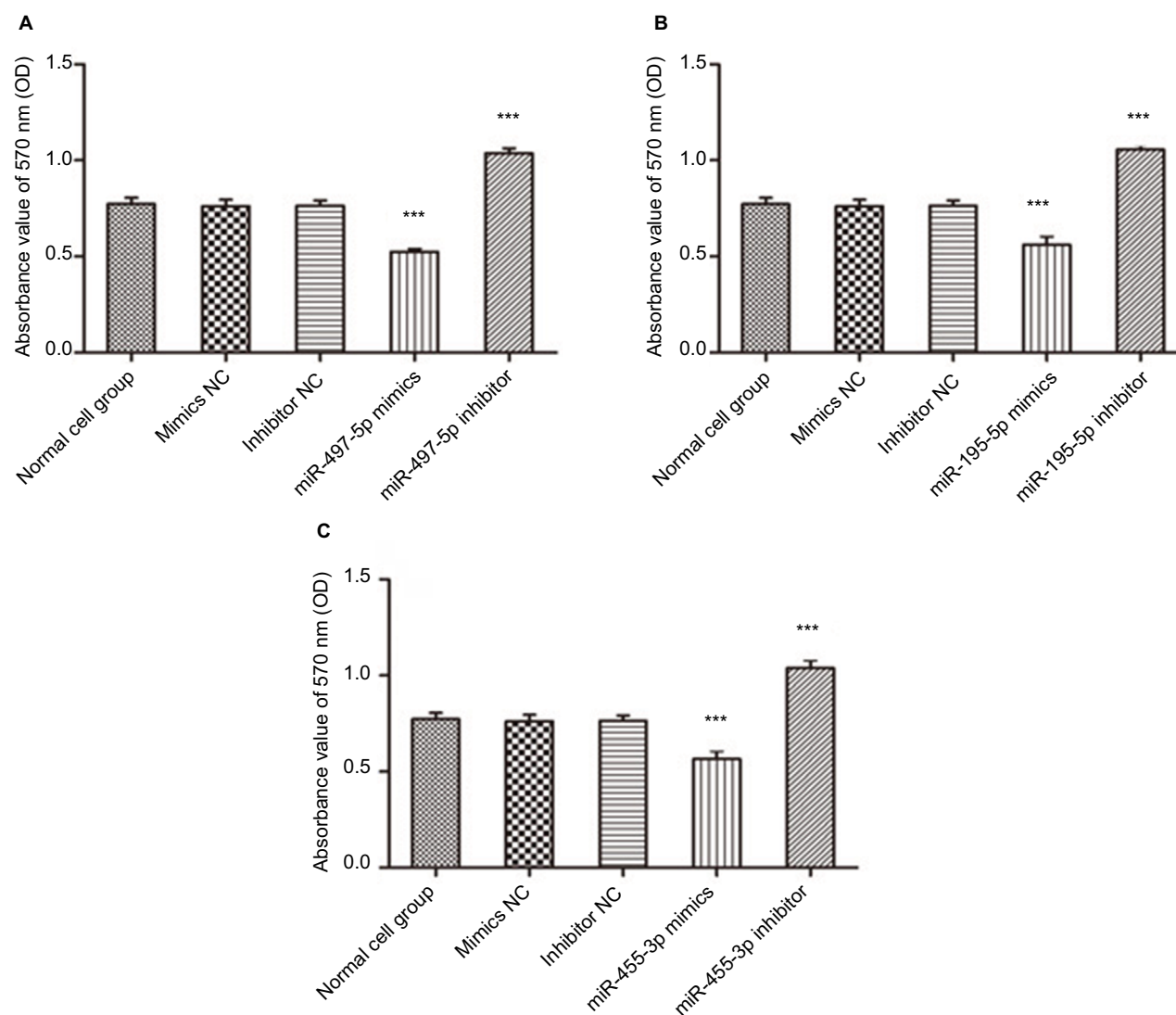


Figure 4 The effects of overexpression of miR-497-5p (A), miR-195-5p (B) and miR-455-3p (C) on cells proliferation.

Notes: All data are presented as mean±SD, and all of these experiments were performed in triplicate. *** $p < 0.001$, compared to control groups.

Abbreviations: NC, negative control; OD, optical density.

and miR-455-3p partially reversed the inhibitory effects of mimic groups (Figure 6D–F).

Identification of hTERT as the target gene of miR-497-5p, miR-195-5p and miR-455-3p

Western blot and q-PCR results showed that overexpression of miR-497-5p, miR-195-5p and miR-455-3p significantly decreased hTERT expression at both protein and mRNA levels. Similarly, with the transfection of miR-497-5p/miR-195-5p/miR-455-3p inhibitors, the inhibitory effects were partially reversed (Figure 7A–C). Additionally, a dual-luciferase reporter assay was performed, and the results showed

that the *hTERT* gene was a direct target gene of miR-497-5p, miR-195-5p and miR-455-3p (Figure 7D–G).

Discussion

MiR-497-5p and miR-195-5p belong to the miR-15/16/195/424/497 family. There have been some reports showing the vital roles that miR-497-5p, miR-195-5p and miR-455-3p play in the tumorigenesis and development of angiosarcoma,²⁵ colorectal cancer,^{26–28} colon cancer,^{29,30} thyroid cancer,³¹ osteosarcoma,^{32,33} prostate cancer,³⁴ hepatocellular carcinoma,³⁵ breast cancer,^{36,37} gastric cancer³⁸ and melanoma.^{39–42} However, the expressions of miRNAs show high tissue and cell type specificity.⁴³ Although miR-497-5p,

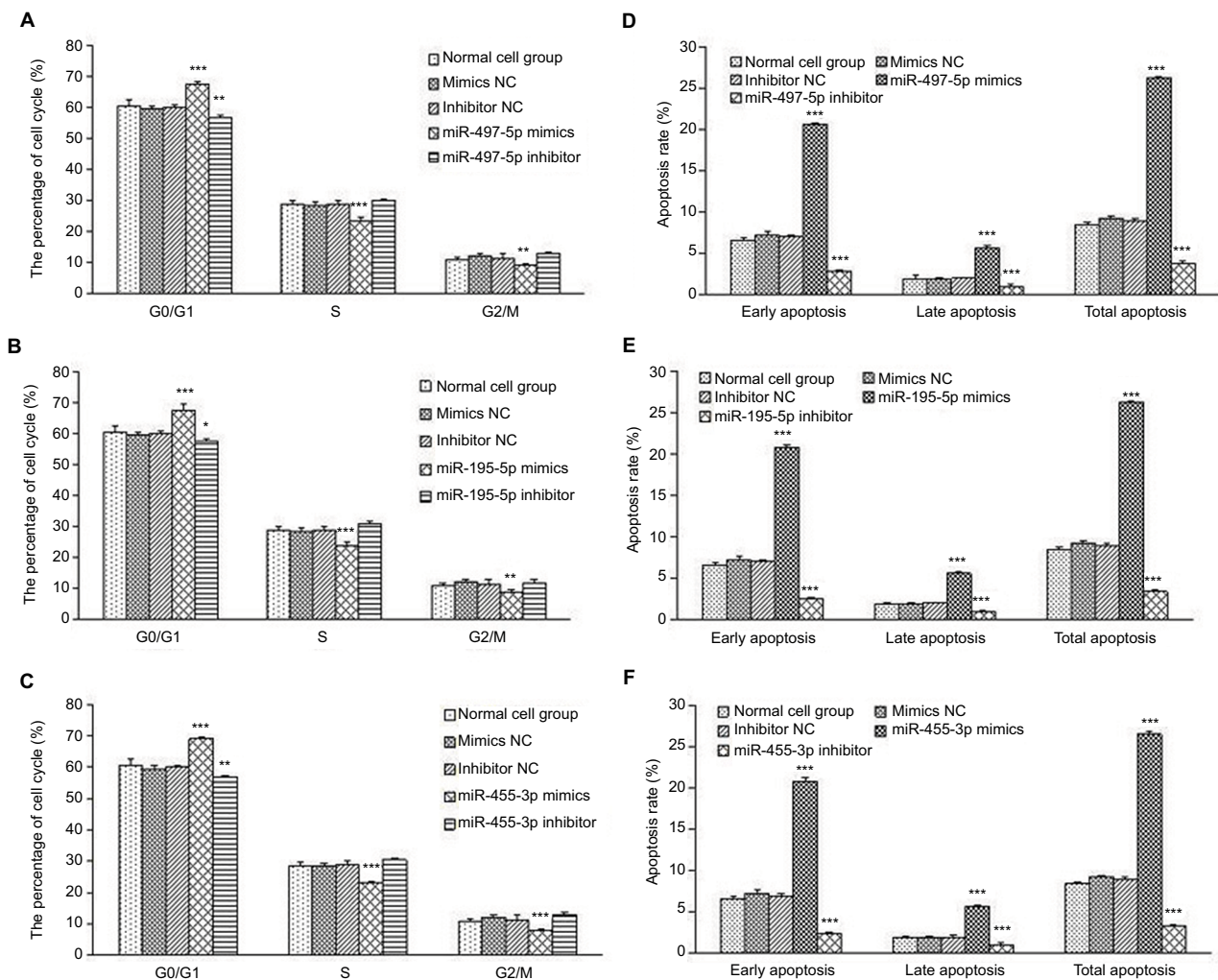


Figure 5 Overexpression of miR-497-5p, miR-195-5p and miR-455-3p arrested cell cycle and promoted cell apoptosis.

Notes: (A–C) Cell cycle analysis was performed after transfected with miR-497-5p/miR-195-5p/miR-455-3p mimics and inhibitors. (D–F) The percentage of early/late/total apoptosis cells of each group after transfection. All data were presented as mean±SD, and all of these experiments were performed in triplicate. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, compared to control groups.

Abbreviation: NC, negative control.

miR-195-5p and miR-455-3p act as anti-oncogenes in most human cancers, they could act as oncogenes in other diseases, such as glioma,⁴⁴ chronic lymphocytic leukemia⁴⁵ and triple negative breast cancer.³⁷

Although Ohira et al⁴⁶ have reported that miR-19b could regulate hTERT mRNA expression by targeting PITX1 in melanoma cells at a pretranscriptional regulation level, the posttranscriptional regulation mechanism of hTERT and its related miRNAs in melanoma is not clear. Recently, a few studies reported the postregulation role of miRNAs in hTERT.^{47–49} Mitomo et al⁴⁷ showed that miR-138 directly repressed hTERT expression in anaplastic thyroid carcinomas. Chen et al⁴⁸ identified that miR-1207-5p and miR-1266 directly suppressed hTERT expression in gastric cancer. Here, we first verified 14 candidate miRNAs by custom miRNA

PCR array after bioinformatics prediction and screening. Our results showed that miR-497-5p, miR-195-5p and miR-455-3p were all significantly downregulated in melanoma tissues samples compared with pigmented nevus samples. Then hTERT mRNA and protein levels were detected by using q-PCR and IHC.

hTERT, the rate-limiting factor of telomerase activity, encodes the catalytic reverse transcriptase subunit of telomerase.⁵⁰ hTERT mRNA was significantly expressed higher in malignant tumors, but not expressed or low expressed in normal tissues and benign tumors.⁵¹ Levels of hTERT mRNA are closely related to telomerase activity and hTERT protein expression.^{50,52} Our results displayed significantly higher expression of hTERT mRNA and protein in melanoma compared with pigmented nevi tissues. Pearson's and

Spearman's correlation analysis reflected a converse relationship between hTERT and miR-497-5p, miR-195-5p and miR-455-3p. These findings suggested that hTERT played an important role in the pathogenesis of melanoma. Also, hTERT might be involved in the pathogenesis of melanoma

via posttranscriptional regulatory mechanism interacting with miRNAs.

It is well known that abnormal proliferation of cells is crucial to the tumorigenesis of malignancies.⁵³ Cell cycle arrest is the main cause of inhibition of cell proliferation.³⁰

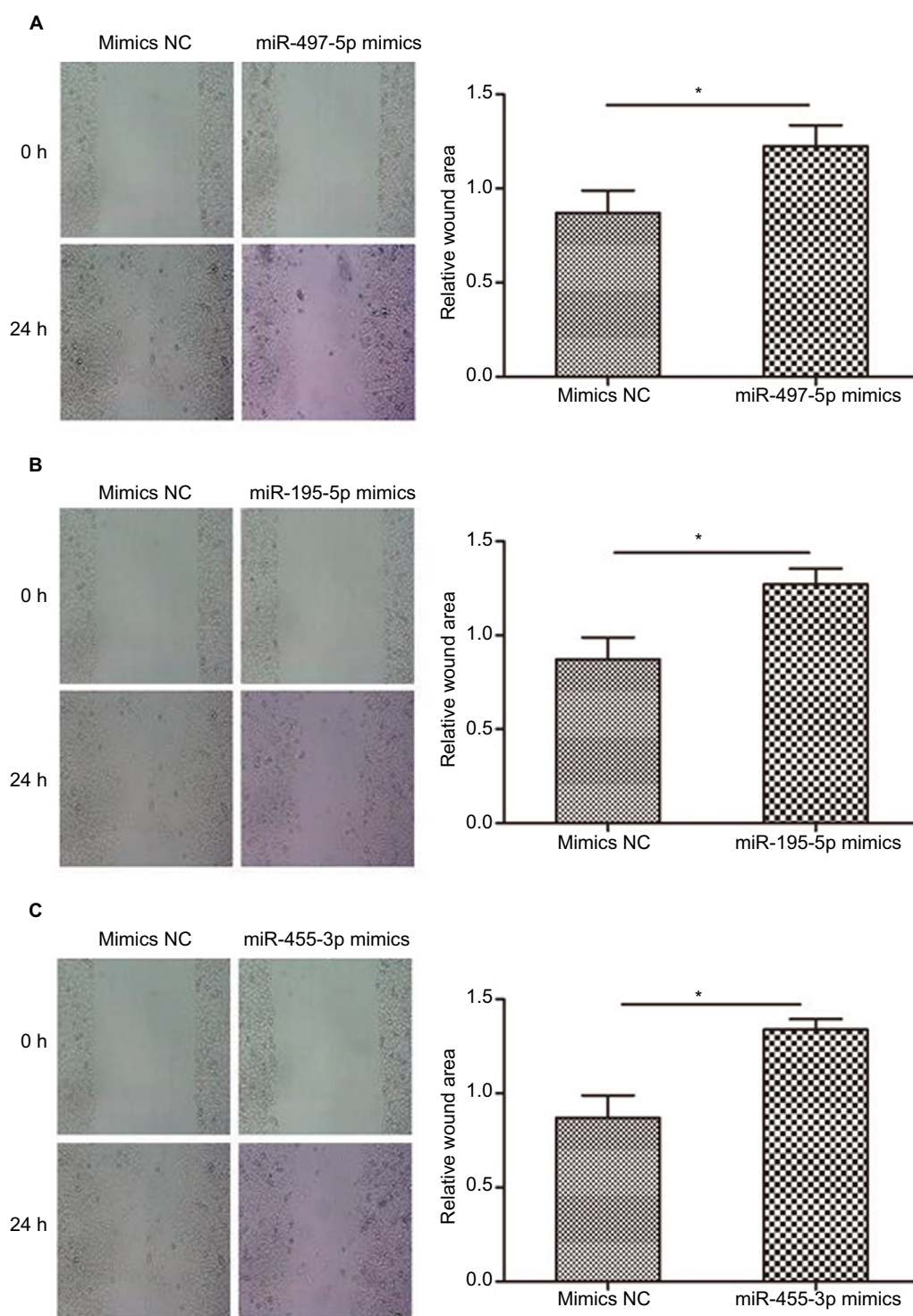


Figure 6 (Continued)

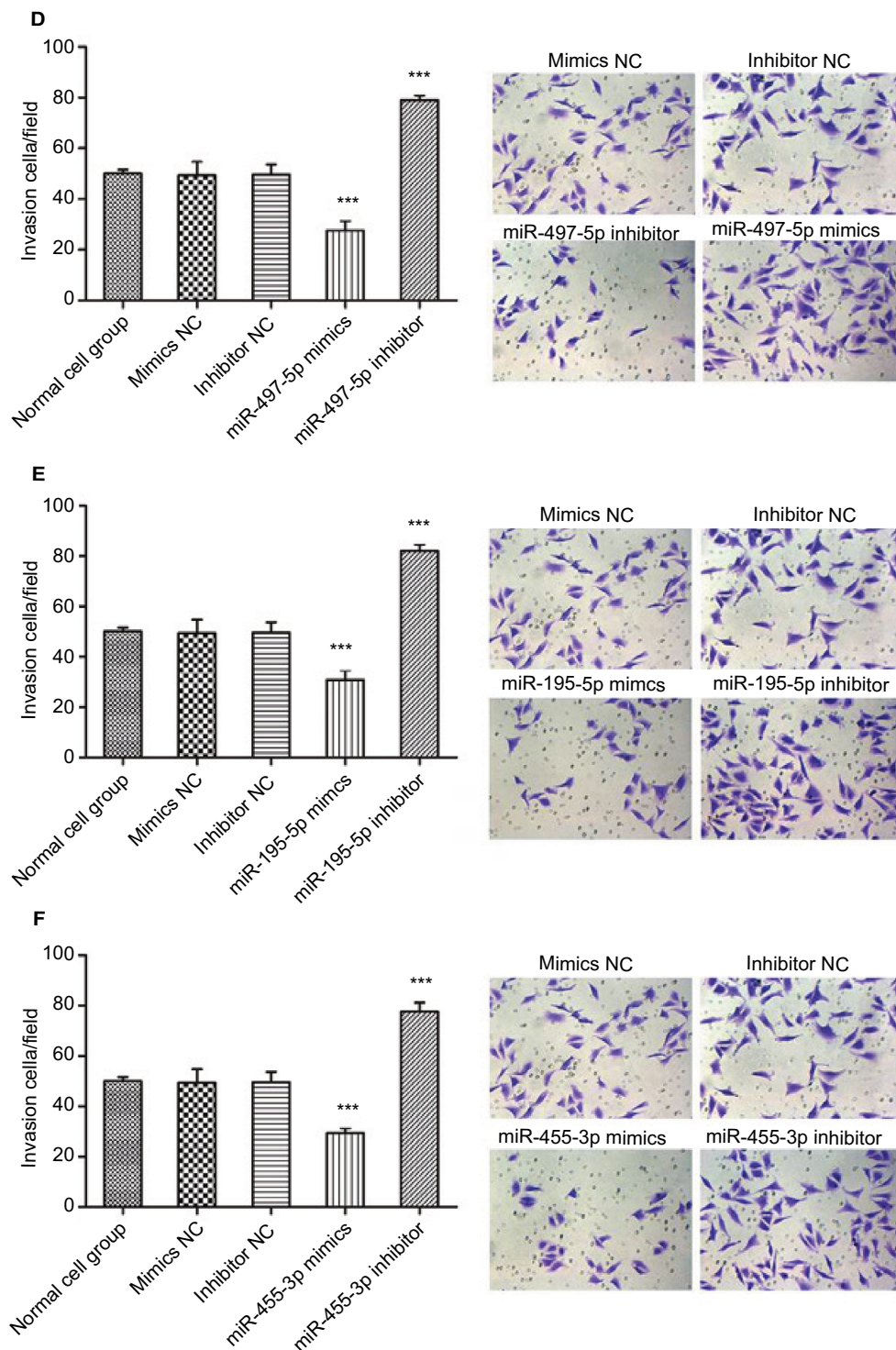


Figure 6 Overexpression of miR-497-5p, miR-195-5p and miR-455-3p inhibited cell migration and invasion abilities.

Notes: (A–C) Micrographs of 0 and 24 h (left, 100 \times) and quantification data of relative wound area (right, 24 h) were analyzed by Image J software. (D–F) Quantification data of invasion cells (left) and micrographs of crystal violet-stained cells (right, 200 \times) are shown. All data are presented as mean \pm SD, and all of these experiments were performed in triplicate. *** p <0.001; * p <0.05, compared to control groups.

Abbreviation: NC, negative control.

We detected cell proliferation ability by cell biological function experiments. MTT assay and flow cytometry assay showed that overexpression of miR-497-5p, miR-195-5p

and miR-455-3p significantly suppressed cell proliferation ability as a result of G0/G1 phase cell arrest. In addition, the inhibition of cell proliferation is closely related to the

enhancement of cell apoptosis.⁵⁴ Our cell apoptosis assay displayed the promoted apoptosis rate after treated with miR-497-5p, miR-195-5p and miR-455-3p mimics.

Invasion and metastasis are considered to be the most significant biological characteristics of malignant tumors³⁴ and are the greatest challenges in cancer treatment. In the present

study, wound healing assay and Transwell results showed that overexpression of miR-497-5p, miR-195-5p and miR-455-3p significantly suppressed cell migration and invasion abilities. Further, the promoted effects of apoptosis and the inhibitory effects of cell proliferation and cell invasion were partially reversed by transfection with inhibitors. All of these data

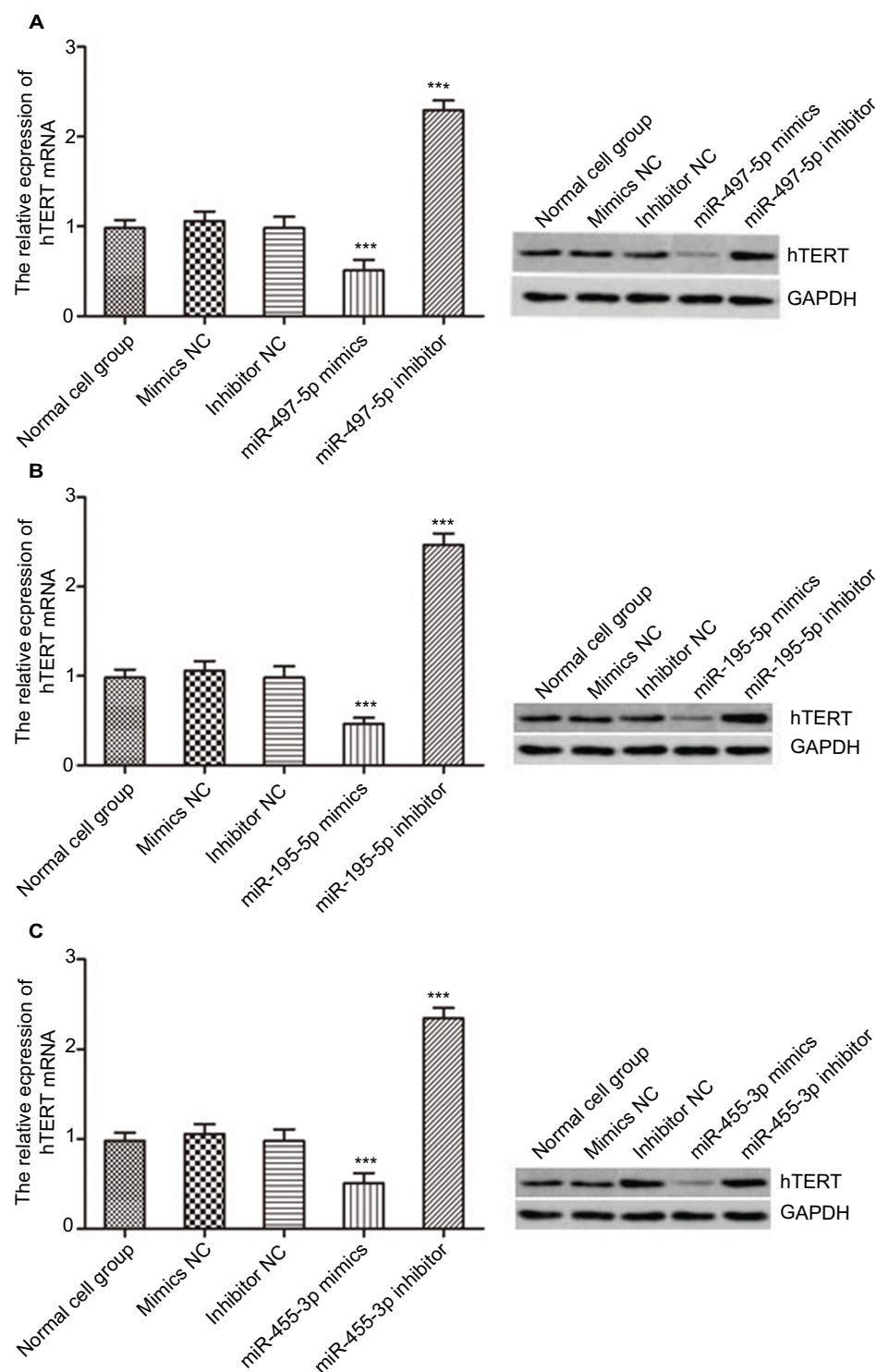


Figure 7 (Continued)

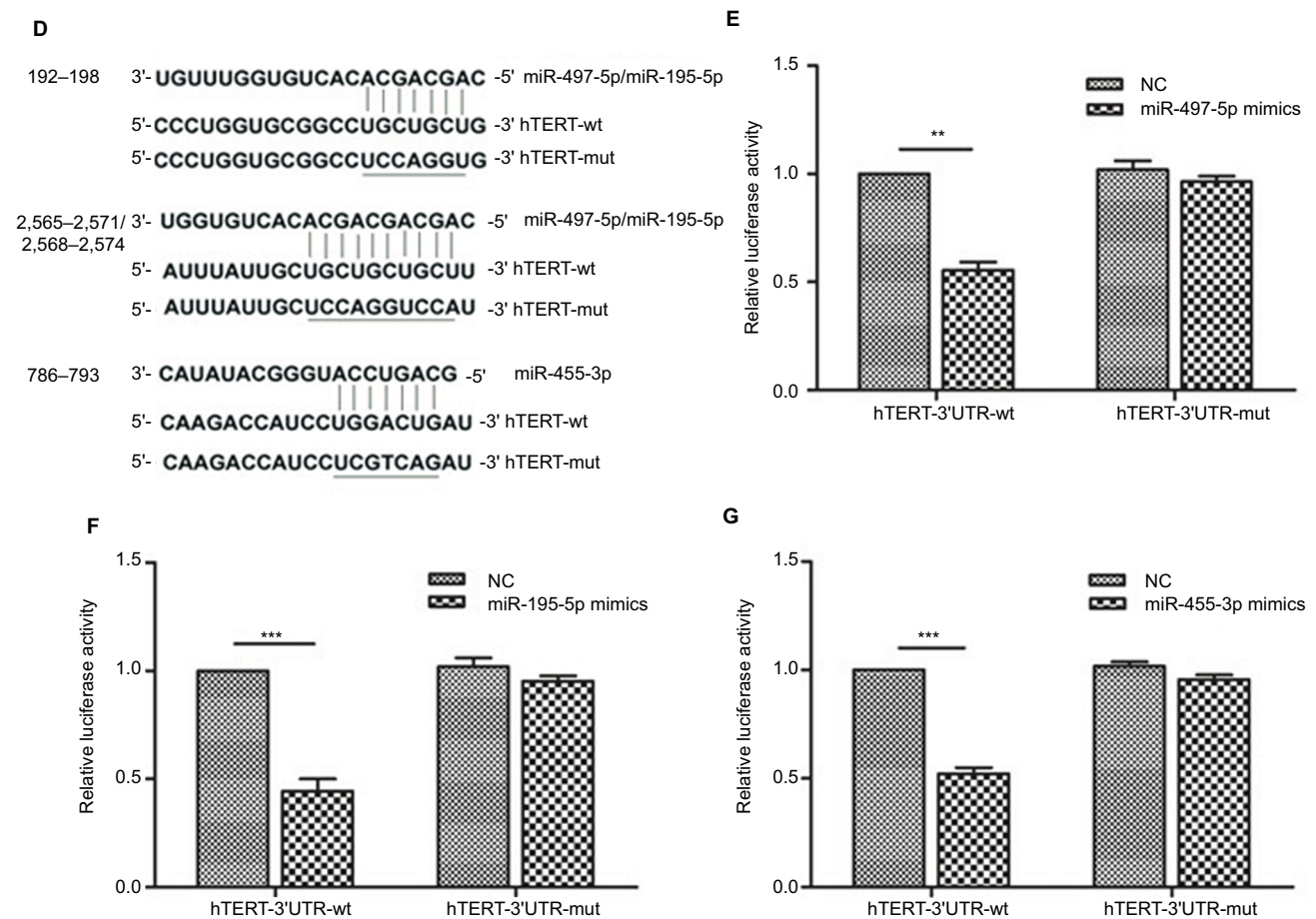


Figure 7 Overexpression of miR-497-5p (A), miR-195-5p (B) and miR-455-3p (C) decreased hTERT expression at both mRNA (left) and protein (right) levels. (D) Sequences of miR-497-5p, miR-195-5p, miR-455-3p, 3'UTR of hTERT are shown. The HEK 293T cells were cotransfected with miR-497-5p (E), miR-195-5p (F), miR-455-3p (G) mimics and luciferase reporter vectors containing a fragment of hTERT 3'UTR harboring either the binding sites (hTERT-3'UTR-wt) or a mutant sites (hTERT-3'UTR-mut).

Notes: All data are presented as mean±SD, and all of these experiments were performed in triplicate. ** $p < 0.01$; *** $p < 0.001$, compared to control groups.

Abbreviations: hTERT, human telomerase reverse transcriptase; NC, negative control; wt, wild type; mut, mutant type; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

of cell biological function experiments indicated that miR-497-5p, miR-195-5p and miR-455-3p functioned as tumor suppressors in the occurrence and development of melanoma.

Accumulating evidence has indicated that one miRNA may target multiple genes, and one gene also might be targeted by multiple miRNAs.⁵⁵ Here we showed that overexpression of miR-497-5p, miR-195-5p, and miR-455-3p significantly decreased hTERT expression at both protein and mRNA levels. Furthermore, we identified that hTERT acted as a direct target gene of miR-497-5p, miR-195-5p and miR-455-3p by a dual-luciferase reporter assay.

Conclusions

We provided evidence that miR-497-5p, miR-195-5p and miR-455-3p functioned as tumor suppressors in melanoma A375 cells by targeting *hTERT*. Overexpression of

miR-497-5p, miR-195-5p, and miR-455-3p might be a potential effective strategy for the treatment of melanoma.

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Disclosure

The authors report no conflicts of interest in this work.

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