

MiR-498 targets UBE2T to Inhibit Proliferation and Metastasis in Malignant Melanoma

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Research

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Abstract

Background

Malignant melanoma (MM) is a common malignant tumor and also one of the fastest growing malignancies. Considering that UBE2T could be a direct target of miR-498, we explore the ability of miR-498 to regulate MM cell progression by targeting UBE2T.

Methods

We detected the expression of miR-498 by quantitative real-time PCR assay. The cell proliferation, invasion and migration in MM cells were measured by cell counting kit-8, clone formation and transwell assays. Flow cytometry assay was used to detect the percentage of apoptotic cells. We explored the correction of miR-498 and UBE2T by dual-luciferase assays.

Results

During the study, we found that the overexpression of miR-498 inhibited proliferation, invasion, migration and induced cell apoptosis of M14 and A375 cells. In addition, the expression of epithelial-mesenchymal transition (EMT)-related factors was altered by overexpression of miR-498. Furthermore, UBE2T was differentially expressed in MM and normal tissues and high expression of UBE2T was strongly correlated with poor overall survival in patients with MM. We highlighted that overexpression of UBE2T reversed the inhibitory effects of miR-498 on MM cell progression.

Conclusions

Altogether, our findings demonstrated that miR-498 significantly inhibited cell proliferation, invasion, migration and induced apoptosis, whilst confirming that miR-498 regulated MM cell progression by targeting UBE2T.

Introduction

Malignant Melanoma (MM) is a common malignant tumor of the skin, mucous membranes and pigmented membranes. It is also one of the fastest growing malignancies with an annual growth rate of 3–5%(1). Therefore, MM has become a serious threat to the health of our people(2). At present, systemic chemotherapy is still used to treat metastatic MM in clinical practice(3). Despite some effects, the 10-year survival rate of patients is still less than 10% (4). Although the research on the pathogenesis and related signal pathway of MM has made remarkable progress(5), the precise mechanism of the occurrence and development of MM is still not completely clear up to now.

MicroRNA is a short-chain uncoded RNA(6). It mainly roles in regulating the expression of target gene mRNA, which is closely related to the occurrence and development of various diseases. miR-498 is located at 19q13.41(7). Recently, growing evidence showed that miR-498 plays an important role in the

onset of diseases, including ovarian cancer, breast cancer, lung cancer, and colorectal cancer(7–9). Additionally, recent studies have reported the effect of microRNA on MM cell biological processing, such as miR-148b, miR-33a, miR-17-92 and miR-211(10–13). The role of miR-498 in MM has not been studied.

The ubiquitin-conjugating enzyme E2T (UBE2T) is located on human chromosome 1q32.1 and encodes a total of 197 amino acids. Many studies have confirmed that UBE2T can mediate the ubiquitination of proteins such as FANCD2 and FANCI, and then participate in the repair of DNA damage(14). It has been reported in recent years that UBE2T can be involved in the occurrence and development of many tumors, including breast cancer, lung cancer, prostate cancer, and bladder cancer(14–16).

In this study, we analyzed the function of miR-498 in MM cells and found that overexpression of miR-498 inhibited MM cells growth, invasion, migration, and induced cell apoptosis. Additionally, the effects of miR-30b as tumor suppressor on MM cells could be attenuated when UBE2T was reintroduced. Our findings suggested the potential utility of miR-498 for the treatment of MM.

Materials And Methods

Cell lines and cell treatment

Human malignant melanoma cell lines HEM, A375, M21, M14, and uacc62 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). miR-498 cDNA sequences were inserted into pcDNA3.1 plasmid to generate the pcDNA3-miR-498 plasmid then transfected into M14 and A375 cells using Lipofectamine 2000 (miR-498 group), and the empty plasmid of pcDNA3.1 was used as negative control (NC). Similarly, M14 and A375 cells were transfected with miR-498 and UBE-2T. Infected cells were harvested for 48 h.

Quantitative real-time PCR analysis (qRT-PCR)

Total RNA was extracted from infected cells using Trizol and cDNA was synthesized using Revert Aid first strand cDNA synthesis Kit (CW BIO, Beijing, China). The expression of miR-498 was detected using ABI Prism 7300 sequence detector (Applied Biosystems) and SYBR Green reagent. Primers were listed below: miR-498 primers, 5'- AAGCCAGGGGCGTTT - 3' (upstream) and 5'- GAACATGTCTGCGTATCTC - 3' (downstream); U6 primers, 5'- CTCGCTTCGGCAGCACA - 3' (upstream) and 5'- AACGCTTCACGAATTTGCGT - 3' (downstream). The $2^{-\Delta\Delta Ct}$ method was used to calculate the data. Each experiment included triplicate measurements.

Cell counting kit-8 assay

Briefly, 1×10^3 cells were seeded into per well of 96-well plates. After every 24 h, 10 μ l CCK8 solution (Solarbio Science & Technology, Beijing, China) was added to detect cell proliferation. After 2 h of incubation, enzyme standard instrument was used to measure OD value at 450 nm to drawn proliferation curve. This assay was performed in triplicate.

Colony formation assay

About 3×10^2 transfected cells were planted into a 6 cm dish containing 5 ml medium and maintained at 37°C in 5% CO₂. When cells formed visible clones, 4% paraformaldehyde solution and 0.1% crystal violet were used to fix and stain clones, respectively. Finally, the clones were taken a image and counted. The assay was performed three times.

Transwell assay

The invasion and migration of M14 and A375 cells was determined using matrigel-coated transwell chambers (BD Biosciences). Briefly, 2×10^4 cells with serum-free DMEM medium were transferred to the upper chamber while complete medium was added to the lower chamber. After 24 h of incubation, the invasive and migrated cells were fixed with 4 % paraformaldehyde and then stained with 0.1 % crystal violet. Finally, the cells were photographed and counted under a microscope. The migration experiment procedure was similar to the invasion assay, but the cubicles were coated with uncoated filters. Transwell assay included triplicate measurements.

Apoptosis detecting assay

Apoptosis of cells was determined using Annexin V-FITC Apoptosis Detection kit I (Thermo Fisher Scientific, Shanghai, China) according to the manufacturer's instructions. Briefly, after 48 h of incubation, the cells were collected and mixed with 10 µl Annexin V-FITC and 5µl propidium iodide (PI) at room temperature in the dark for 5 min. Apoptosis of cells were measured using flow cytometry. This assay was performed three times.

Western blot assay

After 48 h of incubation, cells were centrifuged at 4°C and the protein was extracted with RIPA lysis buffer. Subsequently, 50 µg proteins were separated using SDS-PAGE and then transferred to PVDF membrane. Then, the membrane and antibody to Bcl-2, Bax, cleaved caspase3, E-cad, N-cad, Vimentin, UBE2T, GAPDH were incubated overnight. Finally, the membrane was incubated with HRP-conjugated secondary antibodies. Each experiment included triplicate measurements.

Dual-luciferase assay

The pmirGLO-UBE2T-3'UTR wild type (wt) or mutant (mut) reporter plasmid were co-transfected into cells using Lipofectamine 2000. After 48 h of transfection, cells were lysed and luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA). Renilla luciferase activity was used for standardization. Each experiment was performed three times.

Statistical analysis

Data was obtained from three independent experiments and analyzed using SPSS 18.0. The comparison of two groups and multiple groups was analyzed using Student's t-test and ANOVA test, respectively. Differences with *P* values of less than 0.05 was considered statistically significant.

Results

miR-498 inhibits MM cell proliferation.

miR-498 expression levels in four human MM cell lines (A375, M21, M14, and uacc62) and primary HEM cells were analyzed by qRT-PCR assay. As shown in Fig. 1A, miR-498 expression was significantly down-regulated in MM cell lines compared to normal HEM cells ($P < 0.05$). We further explored the function of miR-498 in M14 and A375 cells. As shown in Fig. 1B, miR-498 mRNA level was significantly increased by miR-498 mimics in M14 and A375 cells ($P < 0.05$). Moreover, the results in Fig. 1C and 1D showed that the OD value of M14 and A375 cells infected with miR-498 was significantly decreased ($P < 0.05$). Clone formation assay also showed similar results in Fig. 1E that the clonogenic abilities of M14 and A375 cells transfected with miR-498 was significantly decreased compared with NC ($P < 0.05$).

miR-498 inhibits MM cell invasion and migration and induces cell apoptosis

The effect of miR-498 on MM cell invasion and migration was detected using transwell assay. As shown in Fig. 2A, the number of invaded and migrated M14 cells in miR-498 group was significantly decreased compared to those in NC group ($P < 0.05$). Then as shown in Fig. 2B, transwell assay also showed similar results that the number of invaded and migrated A375 cells in miR-498 group was significantly declined in comparison to those in NC group ($P < 0.05$). The apoptosis of M14 and A375 cells transfected with miR-498 were detected by flow cytometry assay. As shown in Fig. 2C, the percentage of apoptotic cells was significantly increased from 14.38% or 10.28–25.00% or 19.03% in M14 and A375 cells respectively in miR-498 group compared to NC ($P < 0.05$). Western blot was further used to detect cell apoptosis. As shown in Fig. 3A, the expression of anti-apoptotic protein Bcl2 in miR-498 group was strongly decreased compared with the NC group, while the expression of pro-apoptotic proteins Bax, cleaved caspase3 was significantly increased ($P < 0.05$).

miR-498 regulates epithelial-mesenchymal transition (EMT) of MM cells

The expression of EMT-related proteins was detected using western blot. We observed significant up-regulation of E-cad in miR-498 group in M14 and A375 cells in comparison to that in NC group ($P < 0.05$), while N-cad and Vimentin were significantly down-regulated in miR-498 group (Fig. 3B), which suggested that miR-498 regulated EMT process in MM cells.

Higher level of UBE2T is associated with poor prognosis in MM cells

TCGA is initiated by the National Cancer Institute (NCI) and the National Human Genome Institute (NHGRI), and has now generated genomic data sets for 33 cancers. Based on the TCGA database, we analyzed and screened the poor prognosis genes for MM, and found that UBE2T was a new poor prognosis index for patients with MM. As shown in Fig. 4A, UBE2T expression was markedly higher in

SKCM compared with normal samples ($P < 0.05$). The result showed in Fig. 4B that the survival rate of SKCM patients with high expression of UBE2T was significantly lower compared to those with low expression of UBE2T ($P < 0.05$). It suggested that UBE2T might play a role in promoting MM cell progression.

UBE2T is a novel target gene of miR-498 and overexpression of UBE2T reverses the inhibitory effects of miR-498 on MM cell progression

Based on TargetScan, we predicted UBE2T could be a direct target of miR-498 (Fig. 5A). In order to further determine whether UBE2T was a direct target of miR-498, we performed dual-luciferase assay. As shown in Fig. 5B, miR-498 significantly inhibited the luciferase activity of reporter genes wild-type of 3'-UTR of UBE2T ($P < 0.05$). Then western blot results in Fig. 5C confirmed that UBE2T expression was significantly decreased in M14 and A375 cells transfected with miR-498 ($P < 0.05$). Subsequently, cell counting kit-8 and transwell assays were used to identify whether miR-498 regulated proliferation and migration of MM cells by targeting UBE2T. The result showed in Fig. 5D that UBE2T reversed the effect of miR-498 on inhibiting tumor growth of M14 and A375 cells ($P < 0.05$). As shown in Fig. 5E, UBE2T reversed the effect of miR-498 on inhibiting tumor migration of M14 and A375 cells ($P < 0.05$). Together, these results indicated that miR-498 could inhibit tumor cell growth, invasion, and migration by directly targeting UBE2T in human MM.

Discussion

EMT is an important factor leading to an increase in the malignancy of tumors(17, 18). When EMT occurs, the expression of epithelial-derived markers E-cadherin and α 1-catenin is reduced, and the expression of mesenchymal-derived markers N-cadherin, fibronectin and vimentin is increased(19). Previous studies have shown that when EMT occurs in tumor cells, due to changes in the expression of cytoskeletal proteins and decreased expression of interstitial adhesion proteins such as E-cadherin, the morphology of tumor cells will change, and the ability of cell migration and invasion will increase(20). Here, we found that the overexpression of miR-498 significantly inhibited the activation of EMT including upstream protein E-cad and downstream proteins N-cad and Vimentin, and inhibited cell proliferation, invasion and migration in M14 and A375 cells.

miR-498 is a tumor suppressor miRNA, which can regulate a variety of tumor suppressor genes or proteins related to the regulation of tumor growth, invasion and metastasis, thereby regulating the occurrence and development of various tumors(7, 21, 22). As shown in previous study, miR-498 expression is reduced in colon cancer tissues, and miR-498 can inhibit the proliferation of colon cancer cells(9). It has been reported that miR-498 can inhibit the proliferation and migration of triple-negative breast cancer cells by down-regulating the BRCA1 gene(7). Consistent with the above findings, we used M14 and A375 cells transfected with miR-498 mimics and found that miR-498 inhibited the proliferation, invasion and migration of MM cells, suggesting that miR-498 was a tumor suppressor miRNA in MM.

Previous studies have found that UBE2T is highly expressed in multiple tumors tissues, and its expression level is significantly related to patient survival(23). In breast cancer, UBE2T can not only be used as a molecular marker to predict the prognosis of breast cancer patients, but also promote the occurrence and development of tumors by participating in the ubiquitination degradation of breast cancer susceptibility protein 1. In gastric cancer, interfering with UBE2T expression not only causes gastric cancer cell cycle arrest and apoptosis, thereby inhibiting the growth of tumor cells, but also inhibits the migration and invasion of tumor cells through EMT. Clinically, high expression of UBE2T often indicates a poor prognosis in patients with gastric cancer. In hepatocellular carcinoma (HCC), previous studies analyze TCGA and Oncomine database data and find that the expression level of UBE2T in HCC tissues is significantly higher than that in adjacent tissues. The high expression of UBE2T in HCC is related to intrahepatic metastasis of the tumor, and can promote the migration and invasion of HCC cells(24). In this study, we found that UBE2T mRNA expression in SKCM tissues was significantly higher than in normal tissues by searching on GEPIA. We explored the relationship of miR-498 and UBE2T and first found that overexpression of UBE2T reversed the inhibitory effects of miR-498 on MM cell progression.

Conclusion

In summary, the results of our study indicate that miR-498 can inhibit the EMT of M14 and A375 cells by targeting UBE2T, providing new ideas for the treatment of MM.

Declarations

Consent for publication

Not applicable.

Data statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of Interests Statement

The authors declare that they have no competing interests, and all authors should confirm its accuracy.

Authors' contributions

ND performed the experiments, analyzed the data and wrote the paper. WC and PL assisted with the experiments and data analysis. SH helped analyzed the data and modify the paper. All authors have edited and approved the final manuscript.

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Ethical Statement

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Figures

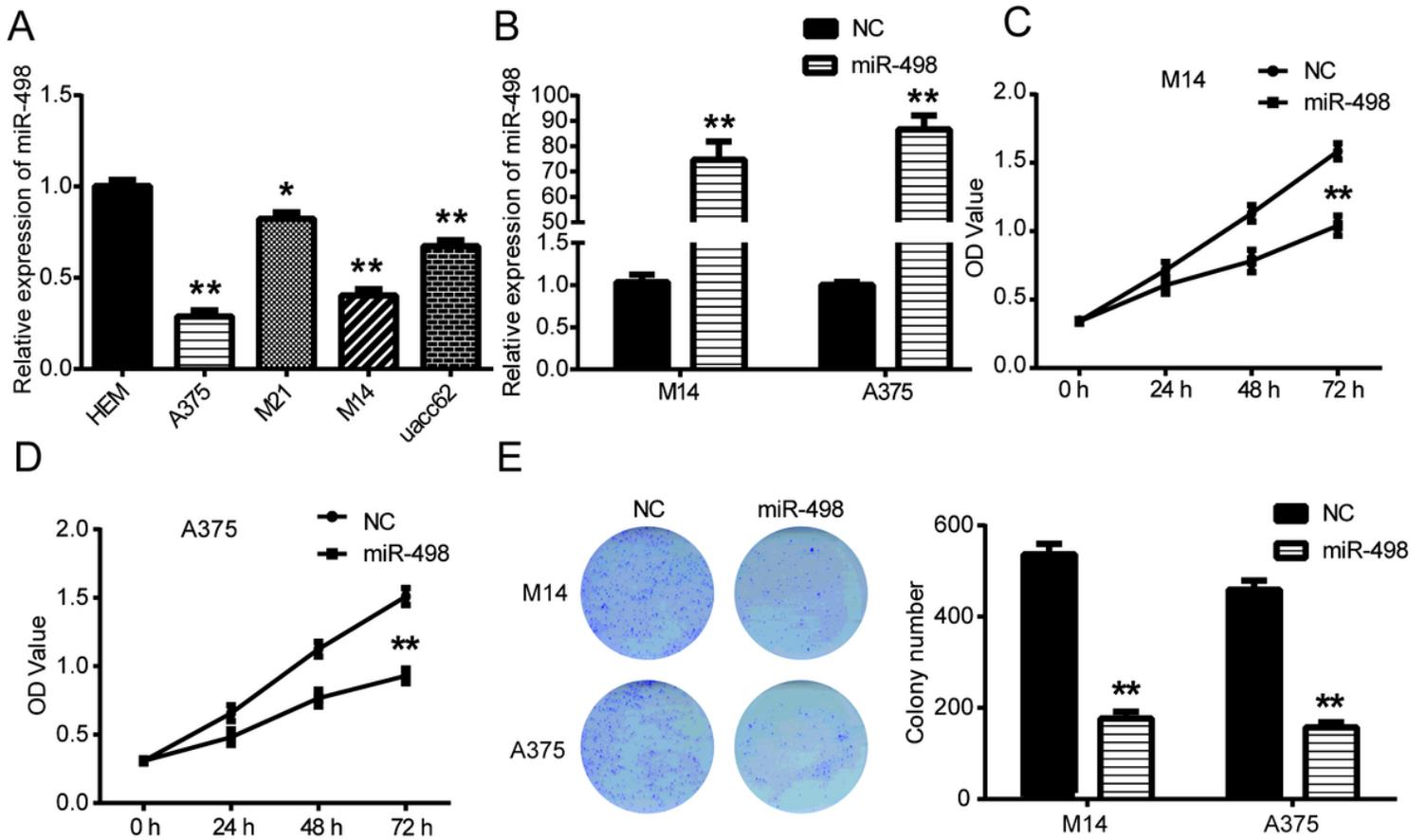


Figure 1

miR-498 inhibited MM cell proliferation. (A) qRT-PCR assay detected relative expression of miR-498 in four human MM cell lines. (B) Relative expression levels of miR-498 in M14 and A375 cells transfected with NC or miR-498 mimics were detected by qRT-PCR assay. (C-E) Cell counting kit-8 and clone formation assays measured cell proliferation in M14 and A375 cells. * represents $P < 0.05$, ** represents $P < 0.01$ compared with NC.

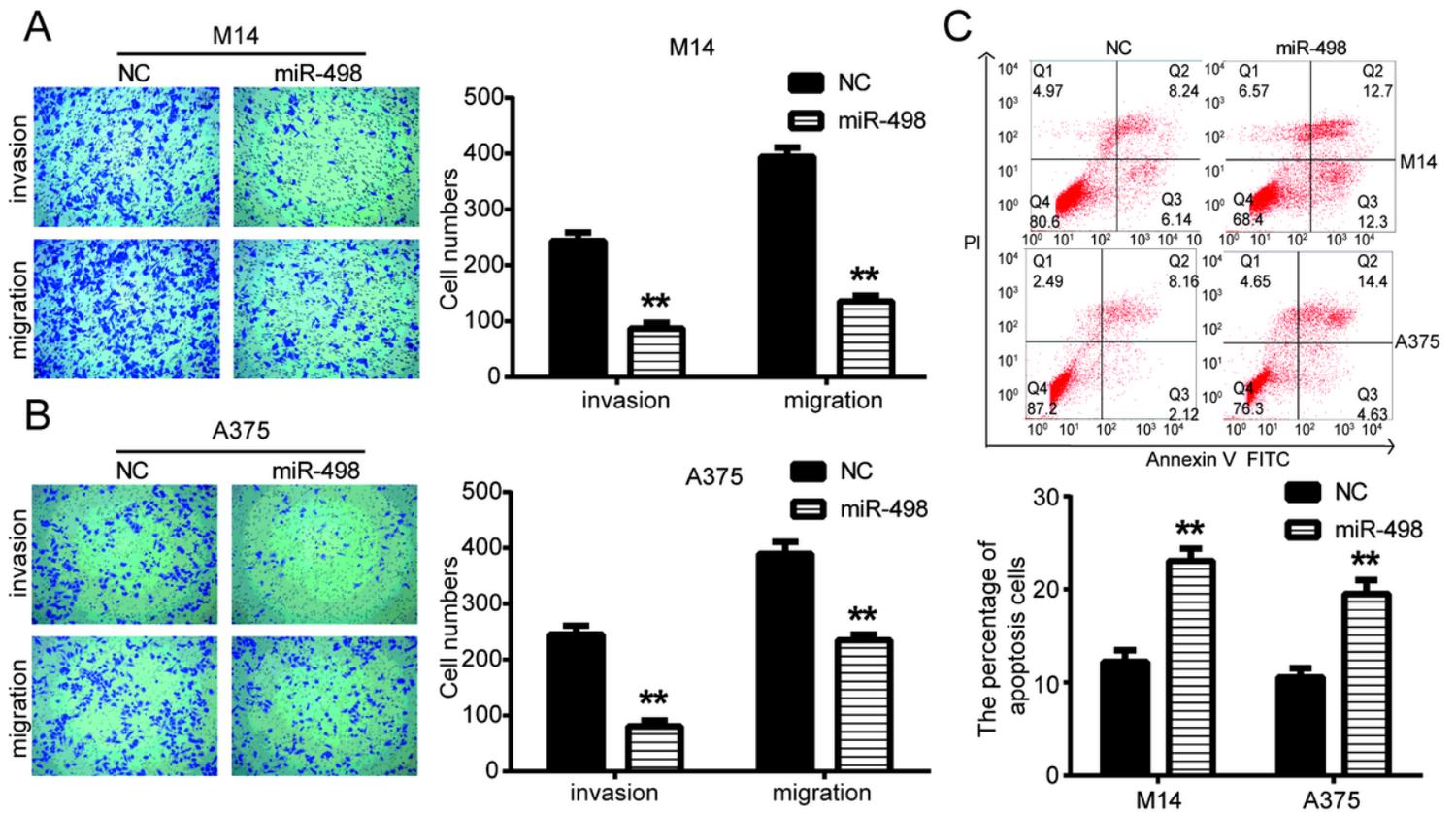


Figure 2

miR-498 inhibited MM cell invasion and migration and induces cell apoptosis. (A and B) Transwell assay detected MM cell invasion and migration. (C) Flow cytometry assay results showed that the percentage of apoptotic cells was significantly increased in M14 and A375 cells in miR-498 group compared to NC. ** represents $P < 0.01$ compared with NC.

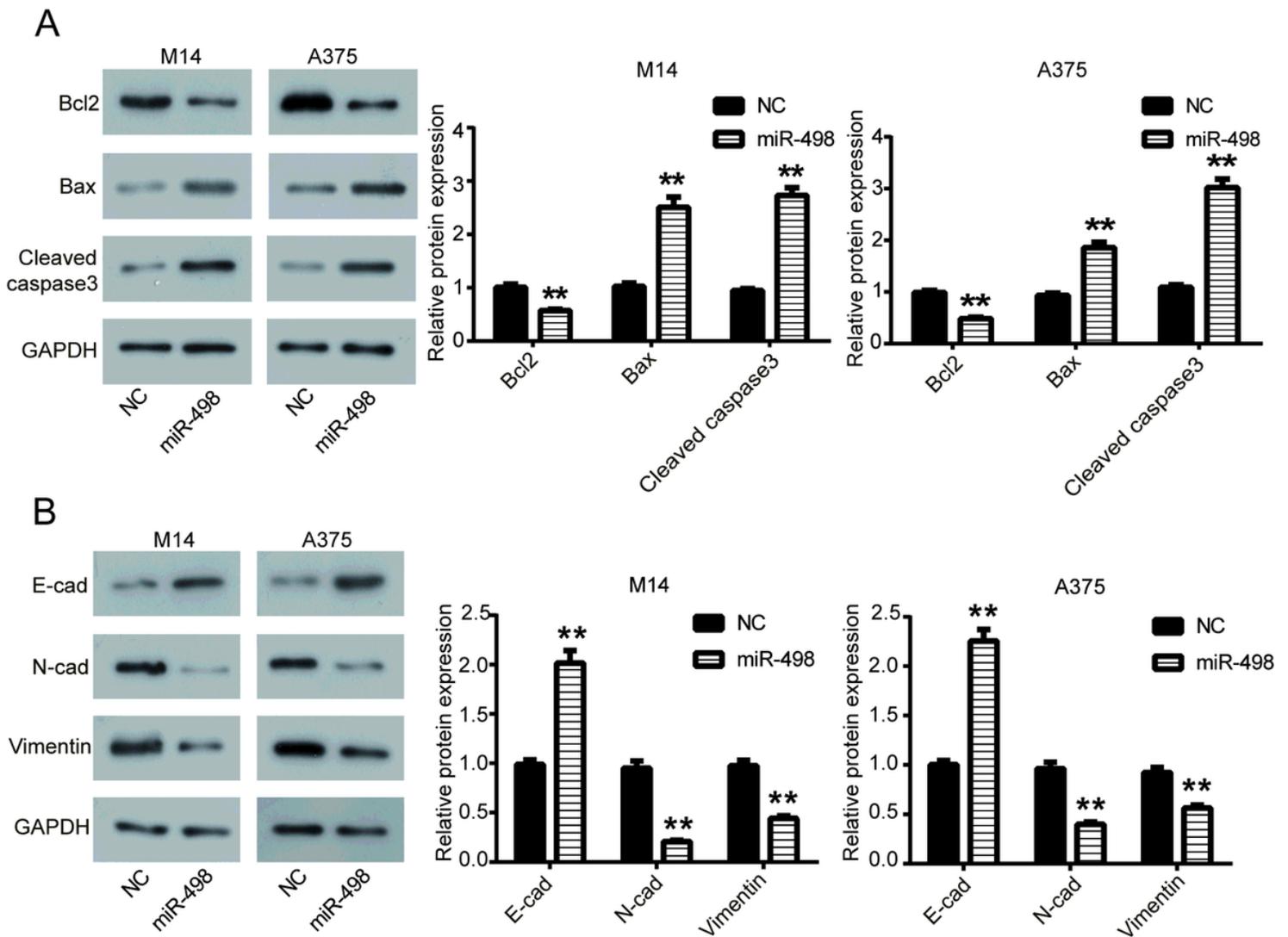


Figure 3

miR-498 regulated epithelial-mesenchymal transition (EMT) of MM cells. (A) Expression levels of Bcl-2, Bax and cleaved caspase3 in M14 and A375 cells were detected by western blot assay. (B) Western blot detected the expression levels of E-cad, N-cad and Vimentin in M14 and A375 cells. ** represents $P < 0.01$ compared with NC.

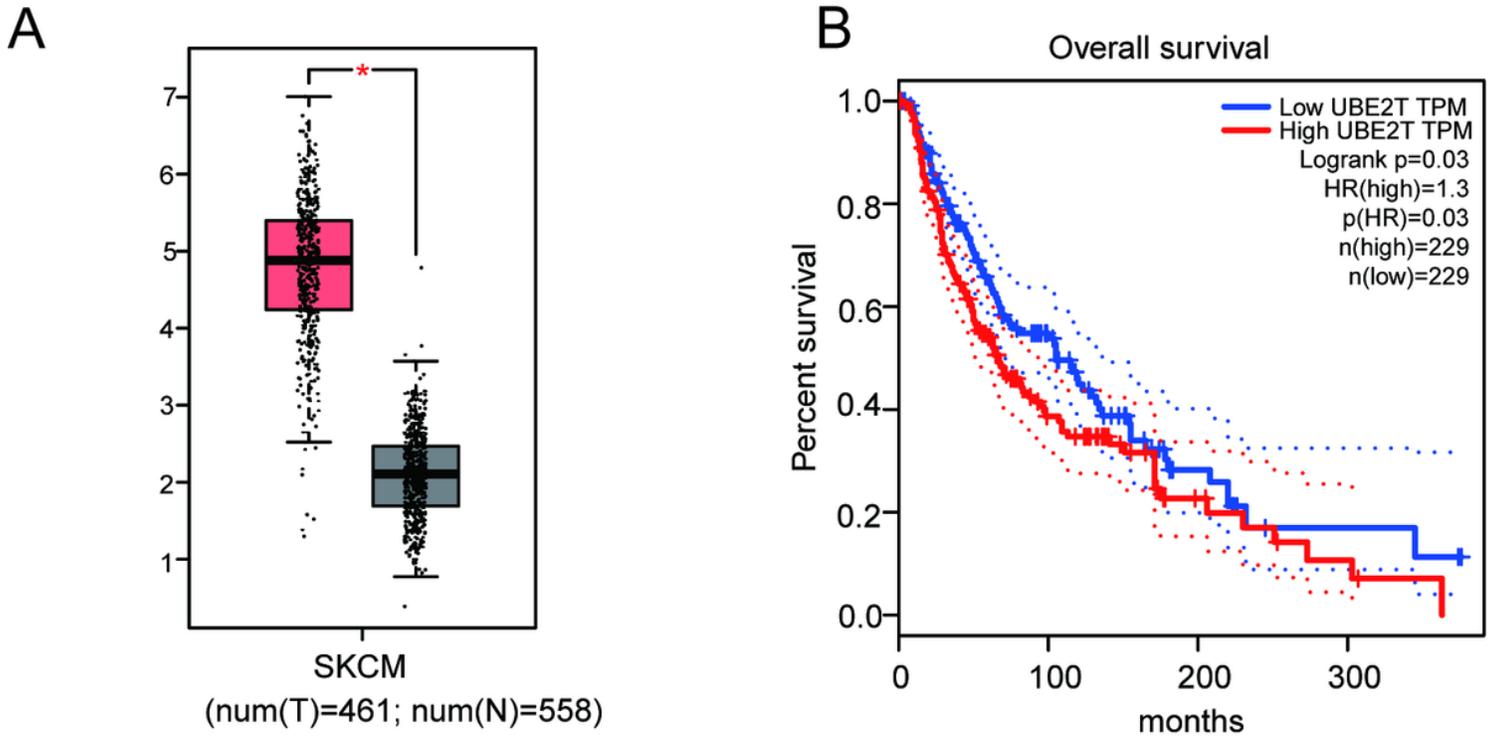


Figure 4

Higher level of UBE2T was associated with poor prognosis in MM cells. (A) The boxplot of mRNA expression of UBE2T in SKCM. The red and gray boxes represent MM and normal tissues, respectively. (B) The survival percentage of SKCM patients with high or low UBE2T expression.

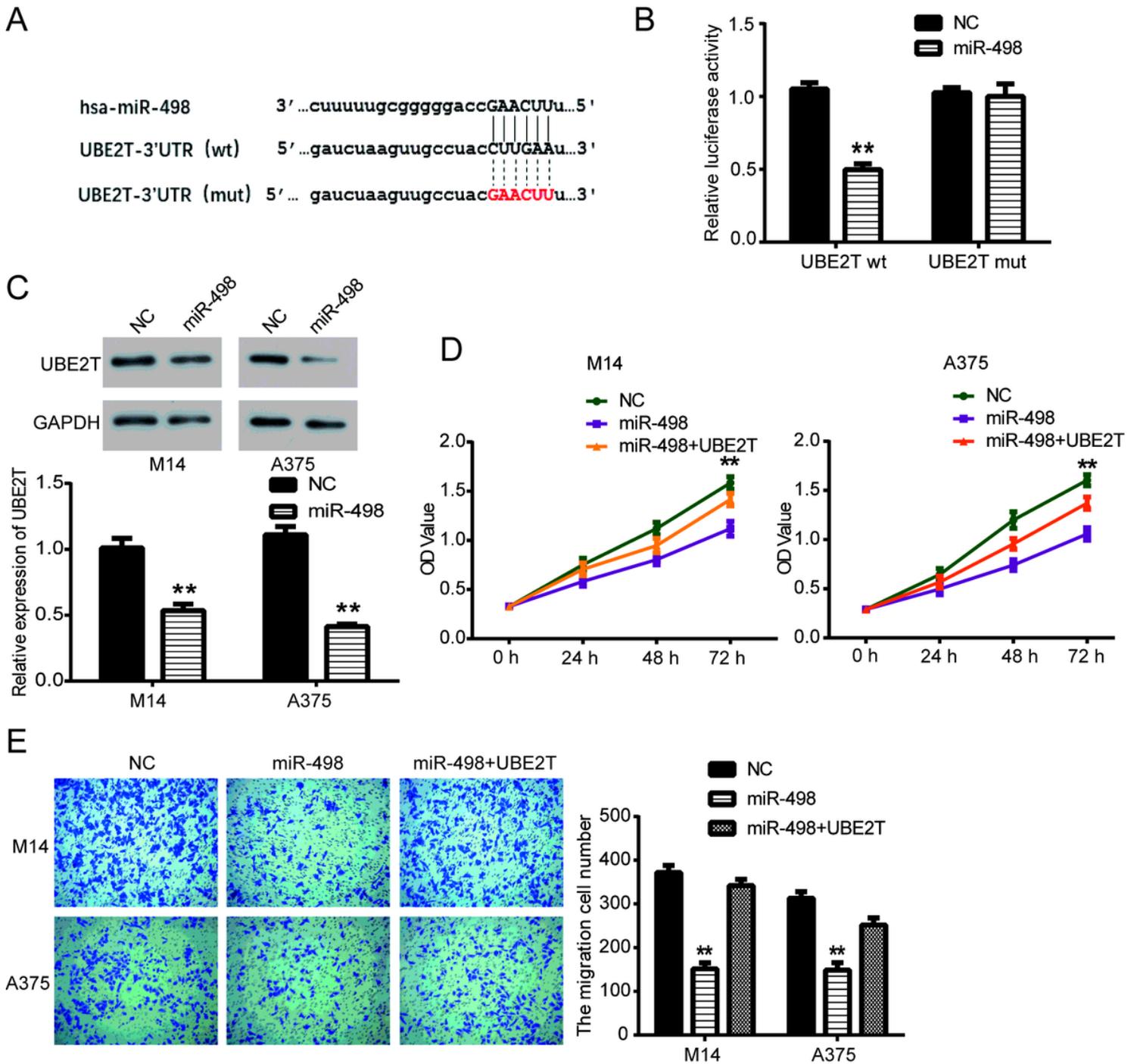


Figure 5

Overexpression of UBE2T reversed the inhibitory effects of miR-498 on MM cell progression. (A) miR-498 binding sites in the 3'-UTR of the UBE2T gene. (B) Results of luciferase-reporter assays performed. (C) Expression levels of UBE2T in M14 and A375 cells were detected by western blot assay. (D) Cell counting kit-8 assay detected cell proliferation in M14 and A375 cells. (E) Transwell assay detected MM cell migration. ** represents $P < 0.01$ compared with NC.